Etude de ressources végétales tropicales pour un usage anthelminthique en élevage de ruminants

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« L'inachevé…

…n'est rien !!! »

A Olga, Maéva et Aldo ….

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LISTE DES ABREVIATIONS

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ADF : Acid detergent fibre

AWM: Adult worm motility

CP: Crude protein

DM: Dry Matter

EHA: Egg Hatch Assay

FEC: Fecal Egg Count

HPLC : High Phase Liquid Chromatography

LDA: Larval Development Assay

LMI: Larval Migration inhibition

LS MEAN: Least Square Mean

LWG: Live Weight Gain

NDF: Neutral detergent fibre

OM: Organic Matter

OPG: nombre d'Oeufs Par Gramme de fèces

PBS : Phosphate Buffer Sample

PCV: Packed Cell Volume

PEG : Polyéthylène Glycol

SE: Standard Error

SEM: Standard Error of Mean

TC: Tanins condensés

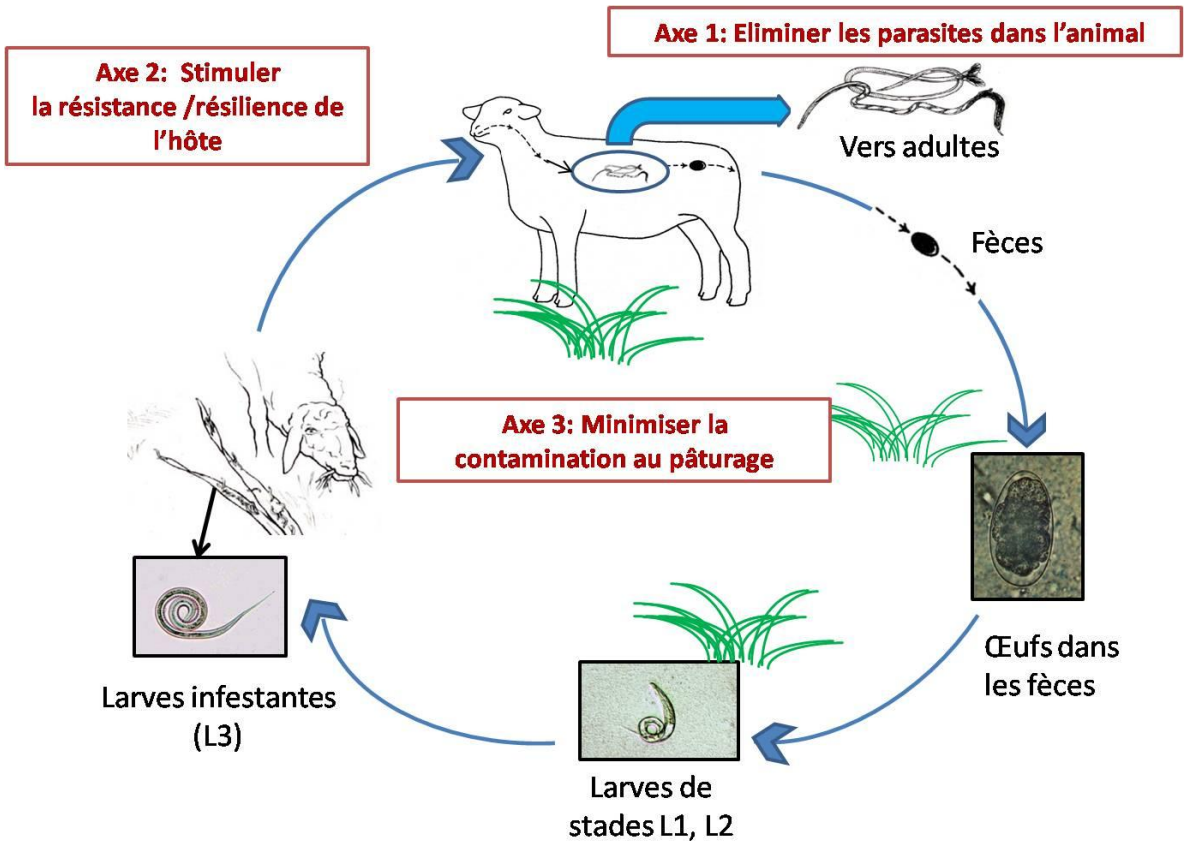
TLC: Thin Layer Chromatography

INTRODUCTION GENERALE

L'objectif de ce travail de thèse est d'étudier l'activité nématicide de certaines ressources végétales tropicales contre le parasite *Haemonchus contortus* ; et secondairement de caractériser la classe chimique responsable de l'activité puis d'en expliquer le mode d'action.

1. Contexte des travaux : résistance des strongles gastro-intestinaux aux anthelminthiques de synthèse, la lutte intégrée contre le parasitisme gastro-intestinal

Le parasitisme gastro-intestinal est un problème majeur de santé et de bien-être des ruminants d'élevage (Sykes, 1994). Il affecte la santé animale en causant un manque d'appétit, de la diarrhée, de l'anémie, et dans les cas les plus sévères, la mort. En plus d'affecter la santé et le bien-être, le parasitisme a un impact négatif sur la productivité globale des troupeaux (Aumont et al., 1997; Coop et al., 1982; Dakkak, 1995; Hoste et al., 2005): ralentissement de la croissance, augmentation du taux de mortalité, problèmes de reproduction. A titre d'exemple, cette pathologie peut engendrer une perte en gain de poids vif de -70% en quelques semaines (Coop et al., 1982). Depuis les années 1960, période de l'arrivée des premiers traitements chimiques contre le parasitisme gastro-intestinal (GI), la chimioprophylaxie a été le principal moyen de lutte. Cependant, depuis une quinzaine d'années, l'émergence de résistances des strongles aux anthelminthiques de synthèse est devenue un phénomène répandu dans le monde entier (Wolstenholme et al., 2004). De plus, l'inquiétude des consommateurs vis-à-vis des résidus dans les produits animaux ne cesse d'augmenter. Ainsi les limites de la lutte chimique et les pressions des consommateurs ont conduit la recherche à s'interroger sur des solutions alternatives pour lutter contre le parasitisme GI des ruminants.



<u>Figure 1</u> : Cycle des Trichostrongles gastro-intestinaux et les 3 axes de lutte

L'INRA URZ travaille à la construction d'une lutte intégrée contre les strongles gastro-intestinaux (SGI). Le travail de thèse suivant s'inscrit dans la programmation scientifique de l'unité INRA-URZ sur l'évaluation multicritères des ressources végétales (valeur alimentaire, santé, et environnementale) et la lutte intégrée contre le parasitisme gastro-intestinal des ruminants. La lutte intégrée combine plusieurs méthodes de lutte qui ciblent différents stades du cycle du parasite.

1.1. Moyens de lutte et cycle des strongles gastro-intestinaux

La lutte intégrée consiste à combiner différents moyens de maîtrise du parasitisme pour pouvoir mieux le traiter. Les différentes méthodes de lutte mises en jeu dans la lutte intégrée contre les SGI cibleront donc une ou plusieurs étapes du cycle du parasite et/ou de la réponse animale à l'agression parasitaire selon trois axes présentés en Figure 1.

Les différents moyens de lutte contre les parasites gastro-intestinaux agissent soit :

- Axe 1 : En éliminant la population vermineuse installée dans l'animal : C'est le cas de l'utilisation raisonnée des anthelminthiques, et de la phytothérapie.
- Axe 2 : En stimulant la résistance et/ou la résilience de l'hôte : C'est le cas de la sélection génétique, la supplémentation alimentaire, de la phytothérapie et de la vaccination.
- Axe 3 : En minimisant la contamination du pâturage : C'est le cas de la lutte biologique, de la gestion des pâturages, la phytothérapie et de l'utilisation raisonnée des anthelminthiques.

1.2. Méthodes alternatives à l'utilisation des anthelminthiques chimiques

Il existe plusieurs modes de gestion non ou partiellement chimique, du parasitisme par les SGI. Ces méthodes alternatives sont à des stades de développement plus ou moins avancés et, selon l'axe ciblé (Figure 1), les moyens utilisés seront de nature différente.

Les différents moyens de lutte seront explicités en insistant sur la phytothérapie, objet de ce travail de thèse.

- La lutte biologique contre les nématodes

La lutte biologique consiste en la limitation de la taille des populations d'une espèce en utilisant un autre organisme. Cette méthode vise à réduire la quantité, des œufs et des larves infestantes, sur les pâturages. Elle agit donc sur la phase de vie libre du parasite. Ainsi, la réduction de l'infestation des pâturages devrait aider les agriculteurs à maintenir ou à améliorer les niveaux de production, tout en réduisant la dépendance à l'égard des traitements anthelminthiques. Parmi les agents biologiques, les champignons nématophages (Larsen, 1999) et les vers de terre (Waghorn et al., 2002) ont montré un potentiel de réduction du nombre de parasites sur le pâturage. L'intérêt majeur de cette méthode est sa polyvalence d'actions contre la plupart des espèces de strongles gastro-intestinaux, mais il reste encore à démontrer l'efficacité observée en expérimentation, à l'échelle du troupeau.

- La sélection génétique d'animaux résistants

Des études (Gray, 1997; Mandonnet et al., 2001) ont montré qu'il est possible d'exploiter la variation génétique pour la sélection d'ovins et caprins résistants aux nématodes parasites. Le critère excrétion d'œufs (exprimé en OPG, œufs par grammes de fèces) reste le moyen le plus efficace pour sélectionner les moutons résistants (Gray, 1997). L'ajout d'autres critères (anticorps spécifiques de l'hôte, antigènes du parasite) et de marqueurs génétiques (QTL) de résistance, sont en cours d'étude. L'élaboration d'un programme de sélection doit non seulement prendre en compte le critère OPG, mais également les conséquences du parasitisme sur la productivité, ce qui amène in fine à combiner les concepts de résistance et de résilience. Cette méthode a donc pour avantages de conduire à une réduction de l'impact du parasitisme sur la production, réduire l'utilisation des produits chimiques et réduire la contamination des pâturages par les larves infestantes.

Elle permet aussi d'analyser les conséquences attendues sur la maîtrise du parasitisme, à moyen ou long terme.

- La supplémentation alimentaire des animaux parasités

Il existe une corrélation fortement positive entre les états de « malnutrition » et le parasitisme gastro-intestinal (Coop and Kyriazakis, 2001). En effet, le parasitisme GI entraine une baisse d'ingestion volontaire de la ration. La malabsorption des nutriments et la baisse de digestibilité des aliments alors occasionnées, entrainent une inefficacité à la fois dans l'utilisation des nutriments et dans la réponse immunitaire. Ces déséquilibres peuvent avoir un impact plus ou moins important en fonction de l'espèce, du stade physiologique de l'animal (*peri partum*, pré-sevrage) et de son statut génétique (Etter et al., 1999; Hoste et al., 2008). Le déficit protéique induit est le facteur majeur de l'affaiblissement de l'hôte. Cependant, une perte en énergie et minéraux est également observée. La supplémentation alimentaire des animaux, en protéines surtout, mais également en minéraux, en vitamines et en énergie permet d'augmenter à la fois la résistance et la résilience de l'hôte (Hoste et al., 2008; Torres-Acosta et al., 2004). Une supplémentation en cuivre chez des ovins et des caprins (Bang et al., 1990; Chartier et al., 2000; de Montellano et al., 2007) a révélé une réduction significative des charges parasitaires gastro-intestinales, ce résultat doit être précisé car il reste controversé.

- La vaccination contre les helminthes

Il existe à l'heure actuelle un vaccin commercialisé contre la dictyocaulose bovine, mais aucun vaccin n'est disponible contre les SGI des petits ruminants. Des travaux sont en cours notamment sur le parasite *H. contortus*, en utilisant les cystéine-protéases issues des cellules intestinales du parasite (LeJambre et al., 2008; Muleke et al., 2007). Cependant la recherche de vaccins anthelminthiques se heurte à la complexité du processus naturel d'élimination du parasite par la réponse immunitaire (Lacroux, 2006). En effet, cette réponse met en jeu entre autres : les antigènes du parasite (prise en compte des différents stades de développement et des différentes souches), l'induction d'une réponse immunitaire spécifique du phénotype de l'hôte et l'activation des mécanismes immunitaires appropriés (Meeusen et Piedrafita, 2003).

Alors que les nouvelles technologies ont permis de réaliser des progrès significatifs dans l'identification des antigènes du vaccin, la production à grande échelle de ces antigènes et de leur présentation avec les systèmes d'adjuvant approprié restent un problème majeur dans la recherche d'un vaccin. Ce sont donc l'identification des interactions moléculaires impliquées dans la réponse immunitaire innée contre les helminthiases et l'application de nouvelles technologies de génomique et de protéomique qui sont susceptibles de conduire à des avancées majeures dans ces domaines de recherche. Enfin, si un vaccin efficace est mis au point, la prochaine étape sera de prouver sa viabilité dans des conditions où les infestations sont mixtes.

- La gestion des pâturages

Ce moyen de lutte contre les SGI est basé sur une intervention directe sur la phase de vie libre du parasite. Il a pour but de diminuer les infestations des parcelles afin de réduire au maximum les possibilités de contact entre l'hôte et les larves infestantes (L3) et maintenir ainsi un niveau de productivité acceptable. Cette méthode est rapide à mettre en œuvre et peu coûteuse pour l'éleveur.

Cependant, si cette gestion des pâturages est réalisée avec succès dans les régions tropicales (Barger et al., 1994) où la durée de vie des L3 est courte dans le milieu extérieur, il n'en va pas de même dans les régions tempérées où la survie de ces larves est bien plus longue (Hoste et al., 1999). Elle se décline en trois méthodes : préventives, évasives et par dilution (Cabaret et al., 2002; Eysker et al., 2005; Mahieu, 1997; Yvoré et al., 1996).

- Méthodes préventives : Les animaux sains dès le départ sont placés sur des parcelles préalablement assainies.

- Méthodes évasives : Les animaux sont traités par un anthelminthique puis placés sur des parcelles également traitées.

Dans les deux cas, l'assainissement des parcelles peut se faire de la façon suivante : mise au repos des parcelles, chaulage, apport d'urée, retournement des prairies par labour, accélération de la mortalité des L3 par pratiques culturales.

- Méthodes par dilution : Il s'agit là de créer un système de pâturage mixte ou alterné (rotation) entre deux espèces animales, le plus souvent grands et petits ruminants. La spécificité de certains parasites pour une espèce animale donnée permet ainsi de briser les cycles parasitaires. Une autre méthode consiste à alterner le pâturage avec un seul type d'hôte, la différence de sensibilité entre jeunes et adultes peut être mise à profit afin de réduire la pression d'infestation rencontrée par les animaux jeunes qui sont les plus réceptifs. Le principe général consiste à ce que les jeunes animaux précèdent toujours les adultes sur des parcelles saines.

- Les traitements anthelminthiques raisonnés

Il s'agit là de traiter seulement les animaux incapables de résister au parasitisme, afin de conserver une population parasitaire sensible aux anthelminthiques encore efficaces.

La méthode Famacha© (Van Wyk and Bath, 2002) est une méthode d'évaluation clinique de l'état d'anémie des animaux les plus parasités par *Haemonchus*. Cette méthode appliquée en zone tropicale (Mahieu et al., 2007) sur des chèvres infestées par *Haemonchus contortus* a montré que son utilisation permettait de ménager un refuge efficace : moins de la moitié des chèvres du troupeau nécessite un traitement antiparasitaire pendant la période d'allaitement des chevreaux.

1.3. La phytothérapie

Les ressources végétales bioactives contiennent des substances actives contre certains pathogènes. Les plantes ou leurs extraits, sont utilisées depuis des siècles en médecine vétérinaire aussi bien en usage externe qu'en usage interne pour traiter toutes sortes de pathologies.

Les plantes élaborent en effet, une multitude de molécules organiques (glucides, acides, lipides et apparentés, substances peptidiques, saponosides, alcaloïdes, polyphénols, terpènes, stéroïdes, vitamines et éléments minéraux). Ces métabolites sont nécessaires à leur fonctionnement et à leur relation avec le milieu extérieur.

Parmi eux, les métabolites secondaires (MS) que constituent: les saponosides, alcaloïdes, polyphénols, terpènes, stéroïdes, acides aminés non protéiques, glucosides cyanogènes et autres hétérosides sont, comme leur dénomination l'indique, des composés qui ne sont pas *sensu stricto* indispensables aux fonctions principales de la plante.

Ces MS sont actuellement associés à la défense de la plante (Harborne, 1999, dans Athanasiadou and Kyriazakis, 2004) ; notamment: la défense contre les insectes herbivores et du pâturage, la défense contre les micro-organismes, y compris les bactéries, les champignons et les virus, la défense contre d'autres plantes en compétition pour les nutriments et la lumière, la protection contre l'effet néfaste des rayons UV (Fraenkel, 1969; Harborne, 2001; Wink, 1988). Ils peuvent également avoir un rôle nutritionnel, en particulier ceux contenant de l'azote, au cours de la germination des graines. La bio-activité des MS a été largement décrite dans la littérature (Acamovic et Brooker, 2005; Cheeke, 1995; D'Mello, 1992; Stegelmeier et al., 1999; Wang et al., 1994). Chez les MS de nombreuses structures ont été façonnées pour interagir avec de multiples cibles moléculaires et cellulaires, y compris les enzymes, les récepteurs d'hormones, de neurotransmetteurs et de récepteurs transmembranaires transporteurs, ce qui permet donc de montrer une réponse spécifique à la cible moléculaire.

Il n'y a donc pratiquement pas de cible cellulaire que les MS ne puissent moduler. Les substances bioactives des plantes peuvent donc avoir différentes cibles chez l'animal (Acamovic et Brooker, 2005). Dans de nombreux cas, les MS (par exemple les terpénoïdes des huiles essentielles) peuvent être plus efficaces que les composés purs issus des synthèses chimiques, car ils sont un mélange complexe de composés. Leur complexité leur permet d'interagir avec plusieurs cibles moléculaires et, par conséquent, il est plus difficile pour les micro-organismes ou des herbivores de développer une réponse efficace. Ainsi, les plantes produisent une grande variété de substances bioactives, dont plusieurs ont déjà été largement utilisées dans le domaine pharmacologique, la médecine et les industries agricoles, tandis que d'autres sont en cours de développement. Certains composés issus du métabolisme primaire peuvent également avoir des propriétés pharmacologiques (protéines, résines, lectines, hétérosides).

Les caractéristiques des métabolites actifs des végétaux, sont les suivantes (Bruneton, 1999) :

1.3.1. Composés du métabolisme primaire

- Les hétérosides

Les hétérosides sont caractérisés chimiquement par l'association d'un ou plusieurs oses avec une partie non sucrée appelée génine ou aglycone. L'action physiologique de plusieurs hétérosides est à l'origine de nombreuses applications thérapeutiques : hétérosides stéroïdiques cardiotoniques et saponosides hémolytiques (décrits plus bas), hétérosides anthracéniques purgatifs, hétérosides flavoniques à action vénotrope, hétérosides soufrés ou glucosinolates, aux propriétés révulsives voire anthelminthiques (Kermanshai et al., 2001), hétérosides cyanogénétiques qui libèrent par hydrolyse de l'acide cyanhydrique extrêmement toxique.

- Les gommes et mucilages

Les macromolécules osidiques forment des gommes et des mucilages. Ces derniers peuvent présenter des propriétés émollientes et laxatives, ou cicatrisantes.

- Les résines

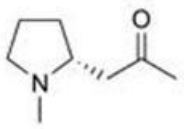
Les résines sont des substances amorphes, dures et visqueuses à température ambiante, et se ramollissent au chauffage. Chimiquement, elles sont constituées d'un mélange complexe de produits résultants du métabolisme des terpènes. On distingue les oléorésines, mélanges de résines et d'huiles essentielles, les baumes qui sont des oléorésines où dominent des dérivés des acides benzoïque et cinnamique, les glucorésines, unies à des sucres; les gommes-résines, mélanges de gommes et de résines. Certaines sont fongicides; ou encore fluidifiantes, voire suspectées comme anthelminthiques (Githiori et al., 2006) .

- Les protéines

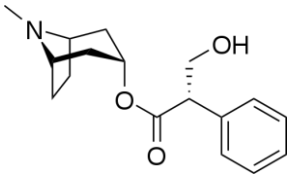
Parmi les protéines végétales, il existe des protéines ou des glycoprotéines, d'origine non induites, qui se fixent de façon réversible ou non à la membrane cellulaire, sans montrer d'activité enzymatique: ce sont les lectines. Elles sont pour la plupart localisées dans les graines, se forment autour de la maturation, puis disparaissent en cours de germination. Elles sont pour leur majorité, capable d'agglutiner les hématies, d'autres sont mitogènes, quelques unes savent différencier les cellules normales des cellules cancéreuses. Certaines plantes à lectines ont montré une efficacité nématicide (Gaofu et al., 2008; Makkar et al., 2007).

- Les enzymes

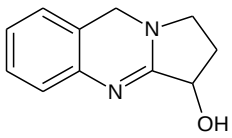
Certaines plantes contiennent des enzymes protéolytiques, qui peuvent agir sur les nématodes, c'est le cas de la bromélaïne, issue de l'ananas. Il s'agit d'une protéase sulfhydrylée, activée par la présence de composés réducteurs, comme la cystéine des nématodes (De Amorin et al., 1999; Makkar et al., 2007).



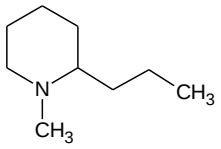
Hygrine A



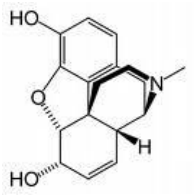
Hyoscyamine E



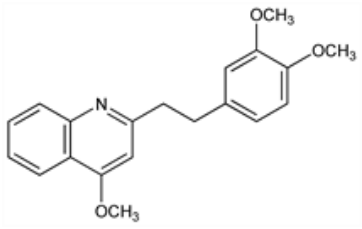
Vasicine I



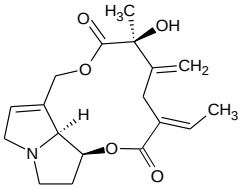
N-methyl coniine B



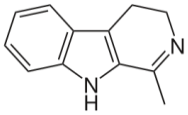
Morphine F



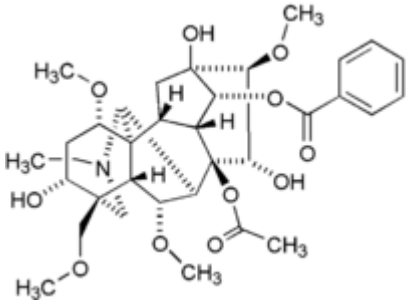
Cusparine J



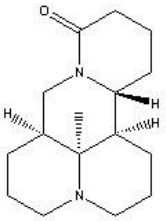
Seneciphylline C



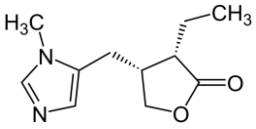
Harmane G



Aconitine K



Matrine D



Pilocarpine H

<u>Figure 2</u> : Exemples de structures alcaloïdiques illustrant les principaux systèmes hétérocycliques rencontrés

A: pyrrolidine ; B: pipéridine ; C: pyrrolizidine ; D : quinolizidine ; E : tropane ;

F : isoquinoléine ; G : indole ; H : imidazole ; I : quinazoline ; J : quinoléine ; K : terpénique

1.3.2. Composés du métabolisme secondaire

- Alcaloïdes

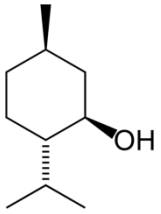
Ils constituent l'un des groupes les plus importants des substances naturelles d'intérêt thérapeutique. Ce sont des composés azotés complexes, plus ou moins basiques et doués, à faible dose, de propriétés pharmacologiques importantes. Les alcaloïdes sont issus du règne végétal mais aussi animal (éponges marines, salamandres, anoures…Bruneton, 1999). Dans les plantes, ils existent rarement à l'état libre; le plus souvent, ils figurent sous forme de combinaisons solubles. Ils sont dérivés d'acides aminés (Figure 2): de l'ornithine, la lysine, de l'acide nicotinique, de la phénylalanine et de la tyrosine, du tryptophane, de l'acide anthranilique, de l'histidine, du métabolisme terpénique (Bruneton, 1999). Les actions pharmacologiques des alcaloïdes sont très variées : certains agissent au niveau du système nerveux central (dépresseurs, stimulants), d'autres sur le système nerveux autonome (sympathomimétiques, sympatholytiques, parasympathomimé-tiques, anticholinergiques, ganglioplégiques).

D'autres sont des curarisants, des anesthésiques locaux, des antitumoraux et anticancéreuses, antiprotozoaires, antipaludiques. Certains sont suspectés pour leur activité nématicide (Eguale et al., 2007b; Githiori et al., 2006; Kusano et al., 2000; Zhao, 1999), mais sont aussi des facteurs antinutritionnels (Athanasiadou et Kyriazakis, 2004).

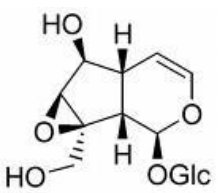
- Terpénoïdes et stéroïdes

Ces deux classes de métabolites secondaires sont issues des mêmes précurseurs. Il s'agit du plus vaste ensemble connu de MS. Parmi eux, les terpènes peuvent être issus à la fois du règne animal et du règne végétal. Les triterpènes, eux sont spécifiques du règne végétal. Quant aux stéroïdes végétaux, leur structure est spécifique de leur origine végétale (Bruneton, 1999). Les terpénoïdes se décomposent en (Figure 3): Monoterpènes (huiles essentielles, oléorésines, iridoïdes et pyréthrines) ; sesquiterpènes (huiles essentielles et lactone sesquiterpéniques); diterpènes ; triterpènes et stéroïdes (saponosides, hétérosides cardiotoniques, phytostérols et triterpènes modifiés) ; carotènes ; polyisoprènes (Bruneton, 1999).

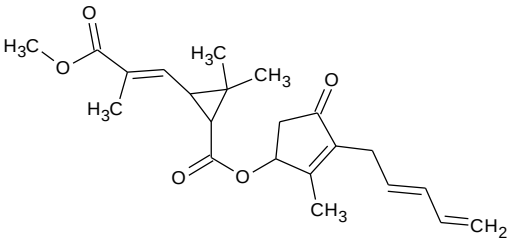
MONOTERPENES



Menthol
(Huiles essentielles)

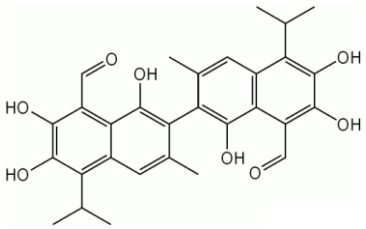


Catalpol
(Iridoïdes)

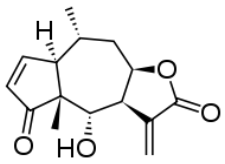


Pyréthrine II
(Pyréthrines)

SESQUITERPENES

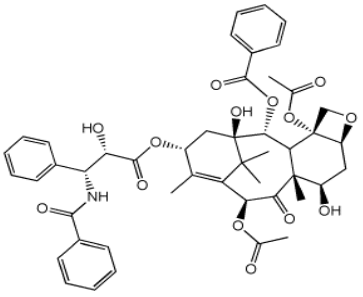


Gossypol



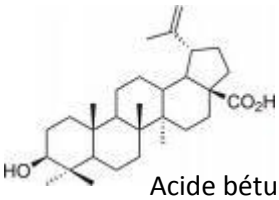
Hélénaline
(Lactones sesquiterpéniques)

DITERPENES

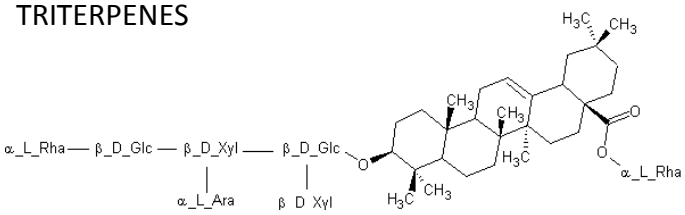


Taxol

TRITERPENES

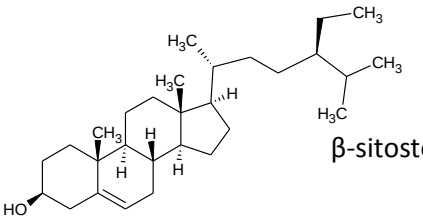


Acide bétulinique



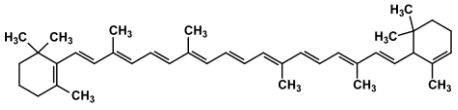
Mimonoside
(Saponosides)

STEROIDES



β-sitostérol

CAROTENES



β-carotène

<u>Figure 3</u> : Exemples de structures de terpénoïdes

- Les monoterpènes : issus du couplage de deux unités isoprèniques, ce sont les composants habituels des huiles essentielles. Les huiles essentielles ont des propriétés antiseptiques, spasmolytiques et sédatives, mais sont aussi irritantes, certaines sont bactéricides et peuvent avoir un impact sur le rumen (Acamovic et Brooker, 2005). Certaines huiles essentielles sont nématicides *in vitro* (Camurça-Vasconcelos et al., 2007; Githiori et al., 2006).

- Les oléorésines : il s'agit de mélanges d'huiles essentielles et de résines, elles peuvent être expectorantes.

- Les iridoïdes : il s'agit de glucosides pour la plupart, mais certains peuvent être alcaloïdiques, polycycliques, esters, éthers internes (Bruneton, 1999). Beaucoup sont impliqués dans les interactions plante-animal. L'action pharmacologique est plutôt anti-inflammatoire.

- Les pyréthrines : ce sont des esters cyclopropaniques à squelette chrysanthémane. Ils ont une action insecticide non toxique pour les mammifères, mais toxique pour les animaux à sang froid : poissons, batraciens, insectes. Ce sont des poisons nerveux (Bruneton, 1999).

- Les lactones sesquiterpéniques : appelées « principes amères », elles réagissent avec les groupes thiols et amines de divers enzymes, provoquant leur alkylation irréversible. Elles sont antibactériennes, antifongiques, anthelminthiques (Hoskin et al., 1999); et agissent sur la motilité de larves de nématodes (Molan et al., 2003a).

- Les diterpènes : ces molécules possèdent des propriétés antihypertensives, antirétrovirales, antitumorales, anti-inflammatoires et analgésiques, anti-oxydantes. Certains ont des activités co-cancérogènes (esters diterpéniques), ou paradoxalement, cytotoxiques (Bruneton, 1999).

- Les saponosides : Ce sont des hétérosides aux propriétés tensio-actives. La plupart sont hémolytiques et toxiques à l'égard des animaux à sang froid (poissons principalement). Mais ces propriétés ne sont pas communes à tous les saponosides.

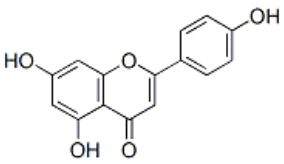
Selon la nature de leur génine, ils sont classés en deux groupes : stéroïdiques, et triterpéniques (Bruneton, 1999). Leur action hémolytique se fait par interaction avec des stérols de la membrane des érythrocytes. Beaucoup de saponosides ont des propriétés antimicrobiennes et antifongiques, mais aussi expectorantes, anti-inflammatoires, dépuratives, diurétiques, cicatrisantes, veinotoniques. Dans l'industrie pharmaceutique, les sapogénines stéroïdiques servent de matière première pour l'hémi-synthèse des dérivés stéroïdiques corticoïdes ou progestatifs. Ils peuvent également assurer la défense du végétal contre l'attaque microbienne ou fongique ; être anti tumoraux, cytotoxiques, analgésiques, antiviraux, immunomodulateurs, peuvent favoriser l'absorption intestinale du cholestérol (Bruneton, 1999).

Les saponosides représentent des facteurs antinutritionnels pour les ruminants (Athanasiadou et Kyriazakis, 2004), mais sont aussi antiprotozoaires (Makkar et al., 2007) et pré-sentis comme anthelminthiques (Athanasiadou and Kyriazakis, 2004; Githiori et al., 2006).

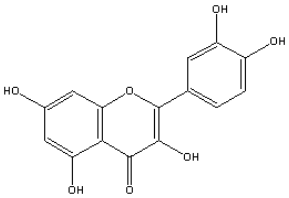
- Les hétérosides cardiotoniques : comme leur nom l'indique, ils permettent de traiter l'insuffisance cardiaque en augmentant la contractilité du myocarde (Bruneton, 1999).

- Phytostérols, stéroïdes et autres triterpènes : Des plantes à stéroïdes et à triterpènes ont montré une efficacité anthelminthique, notamment sur des nématodes à différents stades de développement (Githiori et al., 2006; Hoskin et al., 1999; Maciel et al., 2006; Qamar et al., 2005).

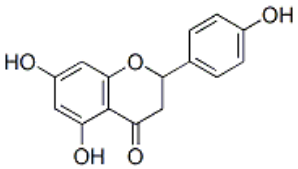
FLAVONOIDES



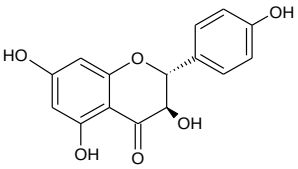
Apigenol
(Flavones)



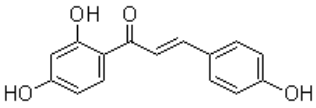
Quercétol
(Flavonols)



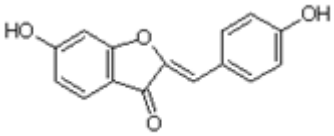
Naringétol
(Flavonones)



Dihydrokaempférol
(Dihydroflavonols)

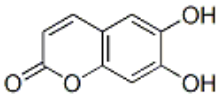


Isoliquiritigenin
(Chalcones)



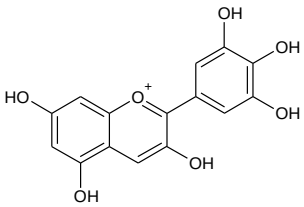
Hispidol
(Aurones)

COUMARINES



Esculétol

ANTHOCYANIDOLS



Delphinidol

<u>Figure 4 A</u> : Exemples de structures de composés phénoliques : flavonoïdes, Coumarines et anthocyanidols

- Composés phénoliques

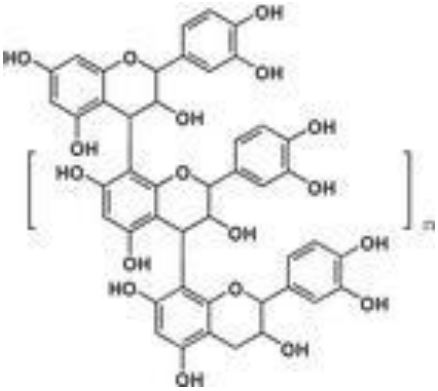
Les composés phénoliques forment un ensemble de substances caractérisées au niveau chimique par la présence d'au moins un noyau benzénique auquel est lié au moins un groupe hydroxyle, libre ou engagé dans une autre fonction (Figures 4A et 4B). Selon leur voie de génèse dans le végétal, on distingue les shikimates et les acétates (Bruneton, 1999). Concernant les shikimates, nous traiterons des coumarines, flavonoïdes, anthocyanosides et tanins. Pour ce qui est des acétates, nous traiterons des quinones.

- Les coumarines sont des molécules de structures très variées possédant des propriétés anticoagulantes, veinotoniques et vasculoprotectrices ou vasodilatatrices. Certaines, comme les furano-coumarines, sont antispasmodiques, photosensibilisantes et utiles dans le traitement du *psoriasis* (Bruneton, 1999). Certaines plantes contenant des coumarines ont des effets sur des nématodes (Eguale et al., 2007b; Githiori et al., 2006; Hoskin et al., 1999).

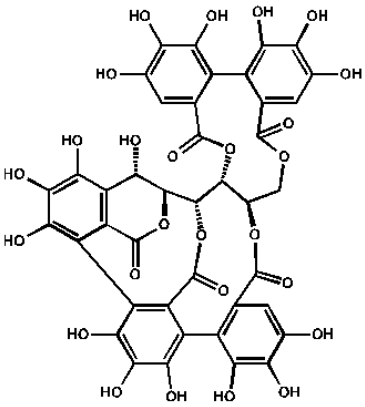
- Les flavonoïdes sont des composés phénoliques responsables de la coloration de nombreuses fleurs et de certains fruits mais aussi attracteurs polliniques des insectes. Ils possèdent des vertus anti-oxydantes, anti-radicalaires et anti-inflammatoires. Leur propriété vitaminique P qu'ils partagent avec les flavanes, leur permet, en diminuant leur perméabilité capillaire, de renforcer la résistance des vaisseaux et ainsi d'être des médicaments de l'insuffisance veineuse. Ils présentent également d'autres actions : diurétique, antispasmodique, anti-oedémateuse, hypocholestérolémiante, hypoglycémiante, hépatoprotectrice, hypocholestérolémiante, anti-inflammatoire et diurétique (Bruneton, 1999). Les flavonoïdes sont suspectés pour participer à une activité nématicide *in vitro* et *in vivo* sur *H. contortus* (Eguale et al., 2007b).

- Les anthocyanes (ou anthocyanidols) sont des pigments hydrosolubles responsables de la coloration rouge ou bleue des plantes. Dans la nature, ils se présentent sous forme d'hétérosides, les anthocyanosides. Ils sont considérés comme des facteurs vitaminiques P, protecteurs des capillaires sanguins et utiles dans certains troubles oculaires. Des plantes à anthocyanes ont été évaluées sur *H.contortus in vivo* (Al-Qarawi et al., 2001).

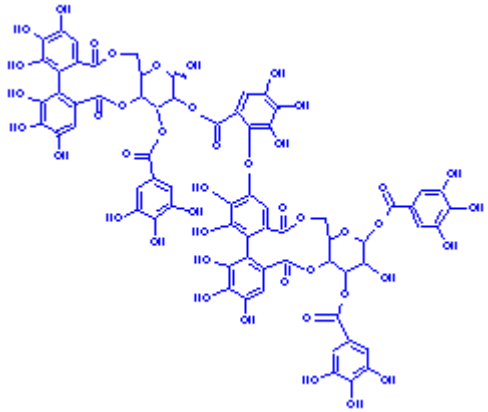
TANINS



Structure des proanthocyanidols
(ou tanins condensés)



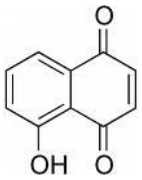
Castalagine



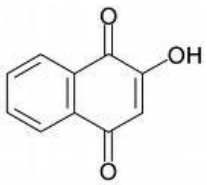
Oenothéine

Exemples de tanins galliques
(ou tanins hydrolysables)

QUINONES

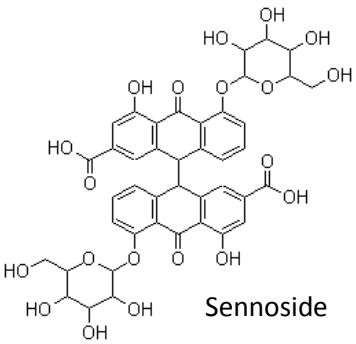


Juglone

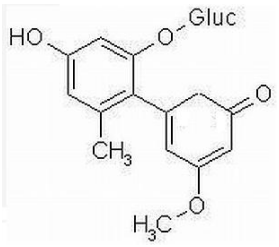


Lawsone

Exemples de naphtoquinones



Sennoside



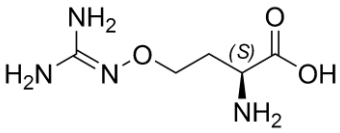
Aloïne A

Exemples d'anthraquinones

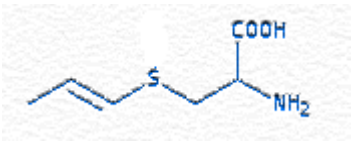
<u>Figure 4 B</u> : Exemples de structures de composés phénoliques : tanins et quinones

- Les tanins sont des composés polyphénoliques hydrosolubles de structure variée, caractérisés par de propriétés astringentes internes et externes. La plupart des propriétés biologiques des tanins sont liées à leur pouvoir de complexation avec les macromolécules, en particulier les protéines. Certains possèdent des propriétés antibactériennes, antifongiques et hypoglycémiantes. Ce sont d'excellents antidiarrhéiques et protecteurs veineux. Ils sont également hémostatiques, antioxydants, et peuvent aussi être des inhibiteurs enzymatiques (Bruneton, 1999). Il existe deux groupes de tanins : les condensés et les hydrolysables. Ils sont astringents, antioxydants, inhibiteurs d'enzymes. La majorité des propriétés biologiques des tanins sont dues à leur capacité de complexation avec les protéines (protéines fongiques ou virales, enzymes digestives, formation d'acides humiques en agriculture, action antinutritionnelle sur fourrages). Ce phénomène, selon les conditions (conditions non oxydantes, pH physiologique), peut être réversible (Bruneton, 1999). Les tanins hydrolysables sont des polyesters d'un sucre avec des molécules d'acide-phénol, on les appelle aussi tanins ellagiques, certains ont un effet antimutagène et sont toxiques vis-à-vis des ruminants (Paolini, 2004). Les tanins condensés ou proanthocyanidols, sont des polymères de flavan-3-ol, qui sont eux, issus du métabolisme des flavonoïdes. Les tanins condensés ont beaucoup été étudiés *in vitro* et *in vivo* pour leur effet nématicide, notamment chez les strongles gastro-intestinaux des ruminants (Acamovic and Brooker, 2005; Bahuaud et al., 2006; Barrau et al., 2005; Githiori et al., 2006; Hoste et al., 2006; Molan et al., 2003b; Paolini, 2004). Ils peuvent également présenter un effet antinutritionnel (Kumar et Singh, 1984; Nguyen et al., 2005; Silanikove et al., 1996).

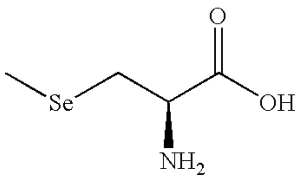
- Les quinones : Il s'agit de composés oxygénés correspondant à l'oxydation de dérivés aromatiques. Parmi ces composés, les naphtoquinones sont des antibactériens et des fongicides (Bruneton, 1999), ces propriétés étant dues à leur nucléophilie. Certaines plantes à quinones sont citées pour leur activité anthelminthique (Githiori et al., 2006).



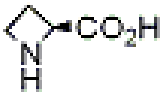
Canavanine



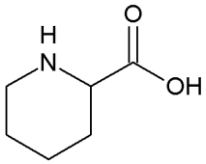
S-(1-propényl)-cystéine



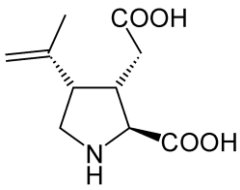
Se-méthlsélénocystéine



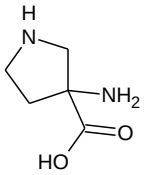
Acide azétidine carboxylique



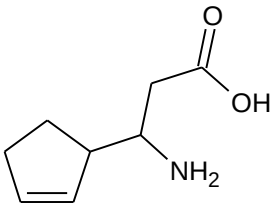
Acide pipécolique



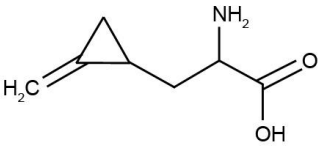
Acide kaïnique



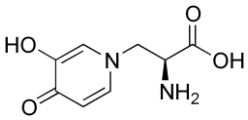
Cucurbitine



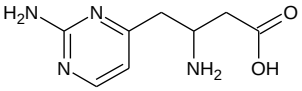
Cyclopentènylglycine



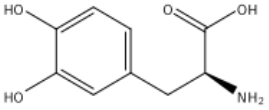
Hypoglycine A



Mimosine



Lathyrine



3,4-Dihydroxyphénylalanine

<u>Figure 5</u> : Exemples de structures d'acides aminés non protéiques

- Les acides aminés non protéiques

Il existe près de 300 acides aminés naturels chez les végétaux, mais une vingtaine seulement sont des constituants normaux des protéines. Donc, la grande majorité de ces composés (amino-acides, imino-acides, amides) peut être considérée comme faisant partie de la catégorie des métabolites secondaires (Bruneton, 1999). Beaucoup existent à l'état libre sauf chez les champignons où ils sont parfois engagés dans de petits peptides. Ils possèdent quelques variations structurales (Figure 5): acides aminés diacides ou dibasiques, acides aminés soufrés, homologues de la cystéine. Il y a également des acides aminés séléniés correspondant à la cystéine, à la méthionine ou à leurs homologues. Il existe aussi des imino acides: dérivés de la proline, mais aussi leurs homologues inférieurs (acide azétidine carboxylique) et supérieurs (acides pipécoliques). On a également décrit des acides aminés alicycliques (cyclopropaniques, cyclopenténiques), hétérocycliques (furaniques, dihydropyridiniques, imidazoliques, pyrazoliques, pyrimidiniques, etc.) et aromatiques (aryl-alanines). Certains acides aminés non protéiques s'accumulent dans les graines de quelques espèces puis disparaissent au cours de la germination. Ils peuvent, de ce fait, être considérés comme une forme de stockage de l'azote. Dans d'assez nombreux cas, ils participent à la survie de l'espèce en se révélant toxiques à l'encontre des prédateurs. Ils opèrent donc par une action anti métabolite, en interférant avec les acides aminés normaux au cours de la biosynthèse des protéines, donnant des protéines non fonctionnelles ou bloquant la biosynthèse protéique (Singh et al., 2003). Pour ce qui est de l'action anthelminthique, on peut signaler les propriétés taenicides de la cucurbitine (3-amino-3-carboxypyrrolidine) (Bruneton, 1999).

Compte tenu que peu de résistances sont observées avec les molécules naturelles (complexité moléculaire) par rapport aux molécules de synthèse dont il est fait un usage intensif; et que les plantes contiennent des métabolites pouvant avoir une efficacité anthelminthique (Githiori et al., 2006), la phytothérapie pourrait constituer une autre méthode de lutte contre les strongyloses gastro-intestinales. Cette méthode consisterait en la recherche de nouvelles molécules anthelminthiques dans les ressources végétales, soit pour en faire de nouveaux médicaments, soit pour la consommation directe des ressources végétales bioactives sous forme de fourrages (Coop et Kyriazakis, 2001).

II. PARTIE EXPERIMENTALE

Le contexte d'application de ces travaux étant la **Guadeloupe**, la **Martinique** et la **Guyane**; nous nous sommes intéressés à des ressources présentes dans ces régions. Toutefois au delà de la ressource elle-même, nous souhaitions avoir des éléments explicatifs sur son activité afin de pouvoir étendre les connaissances à d'autres ressources cibles.

Compte tenu de la forte biodiversité existante et la multitude des ressources potentiellement candidates, nous avons fait le choix de travailler à partir d'une typologie basée sur les principales classes chimiques connues pour des propriétés pharmacologiques. En plus de ce critère scientifique, le souci de transfert et d'appropriation des connaissances par les agriculteurs nous a conduits à nous intéresser à des ressources agricoles particulières.

Notre travail s'inscrit donc dans une perspective de valorisation de plantes tropicales afin de pouvoir nourrir et améliorer la santé des populations animales. D'autre part, en termes d'indicateurs économiques, nous souhaitions :

- Réduire le volume d'intrants sur les exploitations agricoles en utilisant des molécules naturelles produites sur place.

- Faciliter l'accès à des traitements médicamenteux à moindre coût pour les éleveurs.

Au travers de ce travail de thèse, nous nous attacherons **dans une première partie**, à recenser les ressources végétales disponibles dans la Caraïbe pour un usage vétérinaire. **Dans une deuxième partie**, certaines ressources seront évaluées pour leur activité nématicide sur *H.contortus*. Les classes phytochimiques responsables de l'activité seront identifiées. **Dans une troisième partie**, deux modèles de ressources végétales seront évalués *in vivo*, afin de mettre en évidence leur potentiel nématicide et nutritionnel.

1. Démarche du travail de thèse

1.1. Choix des ressources végétales

La Caraïbe dispose d'un véritable arsenal en matière de ressources à potentialités anthelminthiques. Il a donc fallu choisir des ressources d'intérêt pour un sujet de thèse et établir des critères de sélection.

Nous avons dans un premier temps réalisé un inventaire des ressources d'intérêt disponibles en nous basant sur la bibliographie et des enquêtes de terrain réalisées par nous-mêmes en Guadeloupe (article n°1). Sur la base des ressources ainsi identifiées pour leur usage vétérinaire, un screening *in vitro* a été réalisé sur 14 plantes, (les plus cités dans la bibliographie) pour tester leur potentiel anthelminthique (article n°1).

Le screening préliminaire des 14 plantes a été réalisé uniquement avec les tests de référence pour les anthelminthiques chimiques (Coles et al., 1992; Maingi et al., 1998). Les 14 plantes criblées sont: *Carica papaya L. (graine), Annona muricata (graine), Momordica charantia L. (feuille), Chenopodium ambrosioides L. (partie aérienne), Crescentia cujete L. (pulpe), Neurolaena lobata L. (feuille), Bidens pilosa L. (parties aérienne), Senna alata L. (feuille), Cucurbita moschata L. (graine), Musa x paradisiaca L. (feuille et tige), Tabernaemontana citrifolia L. (feuille, fruit et racine), Manihot esculenta Crantz(feuille), Lantana camara L. (partie aérienne), Rauvolfia viridis Willd. (feuille).*

Parmi ces 14 plantes, nos choix se sont orientés vers des ressources communes sur le territoire, à valeur fourragère potentielle en tant que tel ou comme coproduit. Par ailleurs l'état des connaissances scientifiques déjà acquises a été pris en considération.

L'effet nématicide de ces ressources végétales, à l'encontre du parasite *H. contortus*, a ensuite été évalué de manière plus approfondie en procédant à de nouveaux tests *in vitro,* (articles n°2, 3, 4 et 5), puis *in vivo* (articles n°6 et 7).

Les 4 plantes suivantes ont été choisies pour les tests *in vitro* plus approfondis: *Cucurbita moschata L. (graines), Tabernaemontana citrifolia L. (feuille, fruit et racine), Musa x paradisiaca L. (feuille et tige), Manihot esculenta Crantz (feuille).* Le choix s'est porté sur ces ressources végétales, d'une part, compte tenu de leur efficacité sur les tests préliminaires et d'autre part, parce qu'elles contiennent des familles de composés diversifiés. Les deux dernières plantes ont été choisies pour les tests *in vivo*, compte tenu de leur possibilité de valorisation également en nutrition animale. De plus, il s'agit de ressources végétales d'intérêt économique. En effet :

- ✓ D'une part, la banane (*Musa x paradisiaca L.*) représente la première richesse agricole des îles de Guadeloupe et Martinique. En effet, dans ces îles, il est produit 300 000 tonnes de bananes par an. Cultivé pour son fruit, le bananier devient donc un déchet agricole après chaque récolte.
- ✓ D'autre part, le manioc (*Manihot esculenta Crantz*) introduit en Guadeloupe à l'époque précolombienne, tient une place importante dans l'alimentation humaine. Cultivé pour ses tubercules, ses feuilles constituent également des déchets agricoles importants.

De telles productions représentent donc une biomasse disponible importante qu'il est nécessaire de valoriser, permettant ainsi un recyclage des déchets agricoles.

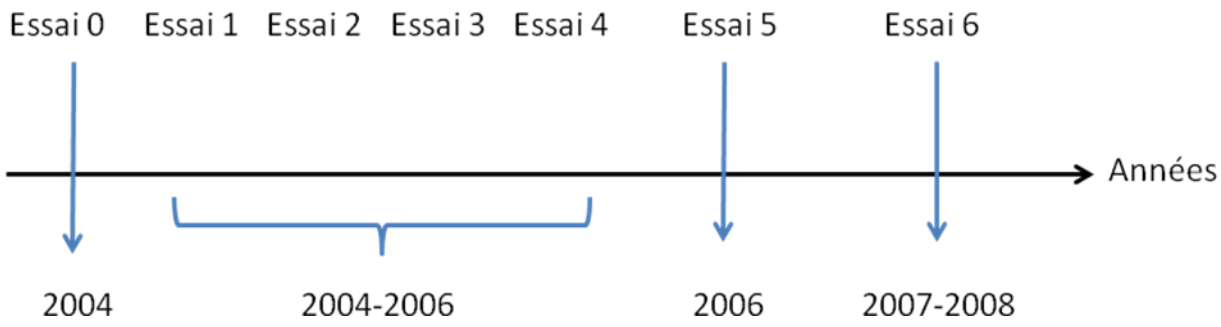


Figure 6 : Chronologie des essais réalisés durant la thèse

1.2. Planification des expérimentations

Les différentes expérimentations (essais) réalisées lors de la thèse sont les suivantes (Figure 6) :

- Essai 0: Screening EHA/LDA sur 14 ressources végétales (article 1)
- Essai 1 : Test *in vitro* sur *Cucurbita moschata L. ,* « Giromon » (article 2)
- Essai 2 : Test *in vitro* sur *Tabernaemontana citrifolia L.*, « Bwalèt » (article 3)
- Essai 3 : Test *in vitro* sur *Musa x paradisiaca L. ,* « Bananier » (article 4)
- Essai 4 : Test *in vitro* sur *Manihot esculenta Crantz. ,* « Manioc » (article 5)
- Essai 5 : Test *in vivo* sur *Musa x paradisiaca L.*(article 6)
- Essai 6 : Test *in vivo* sur *Manihot esculenta Crantz* (article 7)

1.3. Principales méthodes pour la caractérisation chimique et biochimique des ressources végétales

1.3.1. Analyses physico chimiques classiques

- **Matière sèche, matière organique**

La matière sèche des aliments et des fèces a été déterminée par séchage à l'étuve jusqu' à poids constant à 60 ° C pendant 48 h (AOAC, 1997). La teneur en cendres a été déterminée par chauffage des échantillons à 550 ° C pendant 4 h selon la méthode de l'AOAC (1997).

- **Matière azotée totale**

Des échantillons secs ont été prélevés et broyés (sur grille de 1 mm) pour les analyses chimiques : la matière azotée totale (MAT) a été calculée après détermination de la quantité d'azote (N*6.25), par dosage chromatographique selon la méthode de combustion micro-Dumas (auto-analyseur élémentaire des matières azotées totales, NA2100 Protein ; CE instruments).

- **Dosage des fibres des parois cellulaires végétales**

La méthode de Van Soest et al. (1991) a été utilisée afin de déterminer le NDF et l'ADF (méthode d'hydrolyses séquentielles des fibres des parois végétales) sur une base sans cendres, à l'aide d'un incubateur Ankom 2000 Fiber Analyser.

1.3.2. Principales méthodes phytochimiques

- **Dosage des glycosides cyanogènes (HCN)**

Le dosage de l'HCN dans les feuilles et tubercules de manioc a été réalisé selon la méthode Hogg et Ahlgren (1942). La plante (0,15g) est coupée en petites particules plus 3 à 4 gouttes de Toluène, sont mélangés dans un tube à essai. Une bande de papier filtre (10 à 12 cm de long sur 0,5 cm de large) humide saturée d'une solution de picrate de soude (12.5g de Na2CO3 et 2.4g d'acide picrique dans 500mL d'eau distillée) est suspendue 0,5 cm au dessus de la mixture. Le papier filtre saturé est maintenu par un fil de maillechort fixé au bouchon fermant le tube. L'ensemble est maintenu à température ambiante (23 °C) durant 24h. Le picrate de sodium se trouvant sur le papier filtre est réduit en un composé rougeâtre en proportion de la quantité d'HCN dégagé. La coloration produite est dissoute en mettant le papier dans 10 mL d'eau distillée et l'intensité de la coloration est mesurée au spectrophotomètre (DO 525 nm) par rapport à une gamme étalon : Dissoudre 0,241g de KCN dans 100 mL d'H_2O, on obtient une solution renfermant 0,1mg d'HCN/ mL (HCN = 41.46 % de KCN). On mélange dans un tube hermétiquement fermé : 5 mL de la solution de picrate et 5 mL de solution HCN à 0,1mg d'HCN/ mL, (Soit une solution à 0,05 mg/mL). Le tube est mis à chauffer 5 min dans l'eau bouillante. Cette solution est ensuite répartie dans différents tubes à essais et diluée avec 10 mL d'H_2O de façon à établir la gamme suivante :

N° tube	1	2	3	4	5	6	7	8
solution picrate de soude + KCN (0,05mg HCN/mL) (mL)	0,00	0,1	0,2	0,4	0,6	0,8	1,0	1,6
HCN/tube (mg)	0,00	0,005	0,01	0,02	0,03	0,4	0,5	0,08
H2O (mL)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

- **Dosage des polyphénols totaux**

Les polyphénols totaux sont dosés par la méthode de Folin-Cioclateu (Singleton et al., 1998). 50 mg de plante sèche pulvérisée sont macérés dans 5 ml de méthanol durant 30 min puis lavés 2 fois avec 2,5 ml de méthanol.

Le solvant est évaporé et le résidu est repris dans 5 ml d'eau distillée. La solution aqueuse est lavée avec 3 fois 5 ml d'acétate d'éthyle. L'ensemble des phases organiques est déshydraté avec du sulfate de magnésium anhydre. Le solvant est évaporé et le résidu repris dans 1ml d'éthanol.

Une gamme étalon (d'au moins 5 points de concentration allant de 0.5 à 0.0156 mg/L) est préparée à partir d'une solution mère aqueuse d'acide gallique de concentration massique 0.5mg/L. Chaque solution (point de gamme ou échantillon) est introduite dans des tubes a essais à raison de 100µl, suivi de l'addition de 500 µl de réactif de Folin -Ciocalteu dilué au 1/10ème dans de l'eau distillée. Après 2 minutes, 2 ml de carbonate de sodium (Na_2CO_3) à 20% (m/v) sont rajoutés. Le mélange est mis à incuber à l'obscurité pendant 30 minutes à 22°C. L'absorbance de chaque solution est lue à une longueur d'onde de 760 nm contre un blanc (500 µl de réactif de Folin-Ciocalteu plus 2 ml de Na_2CO_3).

La teneur en composés phénoliques totaux de l'extrait de plante est calculée à partir de la courbe d'étalonnage et exprimée en milligrammes pour 100 grammes de la matière sèche équivalent en acide gallique.

- **Dosage des tanins condensés**

La concentration en tanins condensés dans les extraits de plantes (essais 5 et 6) a été déterminée par la méthode vanilline-HCl (Nakamura et al., 2003), en utilisant comme standards les tanins condensés (TC) extraits de chaque plante sur Sephadex LH-20 (Giner-Chavez et al., 1997), et les tanins condensés du quebracho (*Schinopsis* sp.). A 1 ml de solution de TC (0-300µg/ml en méthanol) ou de solution d'extrait de plante (10mg/ml en méthanol) sont ajouté soit 2,5ml de méthanol (témoin) ou 2,5ml de solution de vanilline 1% en méthanol (échantillon); plus 2,5 ml de HCl 9 mol/l en méthanol.

Chaque échantillon est répété 3 à 5 fois. Les échantillons sont incubés durant 20 min à 30°C et l'absorbance est mesurée à 500 nm. Les différentes absorbances A0, Ab, Ac, As pour les échantillons sont calculées de la façon suivante :

A0= absorbance à 500 nm du blanc « méthanol » à 0 mg/ml de TC (1ml méthanol+2,5 ml méthanol+2,5 ml HCL)
Ab= absorbance à 500 nm du blanc « vanilline» à 0 mg/ml de TC (1ml méthanol+2,5 ml solution vanilline 1% +2,5 ml HCL)
Ac= échantillon méthanol (1ml échantillon+2,5 ml méthanol+2,5 ml HCL)
As = échantillon vanilline (1ml échantillon+2,5 ml solution vanilline 1% +2,5 ml HCL)

L'absorbance A est finalement calculée pour chaque échantillon :

$$A = (As - Ab) - (Ac - A0)$$

Deux courbes de calibration sont réalisées (l'une avec TC quebracho et l'autre avec TC manioc) en calculant A pour les différentes concentrations de TC (0-300µg/ml en méthanol), permettant ainsi d'évaluer la quantité de TC (flavan-3-ol) dans chaque échantillon.

- **Chromatographie sur couche mince (CCM)**

L'analyse qualitative par CCM a été réalisée sur plaque de silice (Al coated Kieselgel 60 F_{254} (Merck) plates). Après migration sur 15 cm, les plaques sont observées sous lumière UV aux longueurs d'ondes 254 nm et 365 nm pour la détection des composés fluorescents (flavonoïdes, phénoliques). Les différents systèmes de migration et les révélations utilisées selon les essais sont les suivants (Wagner, 1996):

- Giromon, *Cucurbita moschata L.* (essai 1)

Système de migration: n-butanol/ acide acétique /eau (6:2:2, v/v/v).

Révélations: Ninhydrine pour la détection de composés acides aminés. H_2SO_4 (20% dans methanol, à conserver à 4°C), pour la détection des terpénoïdes.

- Bwalèt, *Tabernaemontana citrifolia L.* (essai 2)

Système de migration: Acétate d'éthyle/ acide acétique/ acide formique/ eau (100:11:11:26, v/v/v/v).

Révélations: Réactif de Dragendorff pour la détection des alcaloïdes.

- Bananier, *Musa x paradisiaca L.*et manioc, *Manihot esculenta Crantz*(essais 3 et 4)

Système de migration: Chloroforme/ acide acétique/méthanol/eau (64:32:12:8, v/v/v/v).

Révélations:

Essais 3 et 4 : Vanilline-sulfurique pour la révélation des terpénoïdes (tri-terpènes and saponines) et phénoliques : pulvérisation avec 10 ml de solution de vanilline à 1% dans l'éthanol, suivi immédiatement de 10 ml de solution d'acide sulfurique à10% dans l'éthanol.

Essai 3: Naphtorésorcinol phosphorique pour la détection d'hétérosides : (1) solution éthanolique de naphtorésorcinol à 0,2% (m/v) ; (2) acide orthophosphorique à 85%. Mélange de 22 volumes de (1) avec 1 volume de (2). Après vaporisation de la solution sur la plaque ccm, chauffer à 110°C durant 10 min pour révéler les sucres.

- **Chromatographie liquide haute pression, HPLC (essai 4)**

La chromatographie liquide haute pression (HPLC) a été utilisée pour la séparation des tanins condensés (TC) des extraits de feuilles de manioc. L'analyse estréalisée avec un chromatographe liquide Varian® Prostar pump 210/18, équipé d'une valve d'injection rheodyne de 20µL, ainsi qu'un détecteur à barrettes de diodes (DAD, Varian® Prostar 335) et du logiciel Galaxy 1.9 pour l'intégration. Les extraits sont séparés sur colonne Microsorb 100-5 C18, S250 x 4.6, à la température de 23°C. L'analyse HPLC est effectuée selon une modification de la méthode Marin-Martinez et al. (2008). Les TC sont élués durant 15 min avec un débit de 1ml/min selon un système isocratique ayant pour phase mobile le mélange : acide acétique/ acétonitrile/eau (2:28:70, v/v/v).

La phase mobile a été dégazée grâce au dégazeur Degassit Power Supply (Varian® Prostar 210 to 240 V, 10ml/min).

Le quebracho étant un extrait de plante riche en TC ayant un effet sur les larves du parasite *H. contortus* (Athanasiadou et al., 2001), il est utilisé en plus des TC extraits directement des feuilles de manioc (sur Sephadex LH 20; Giner-Chavez et al., 1997) pour une estimation qualitative des TC communs aux deux plantes. Les composés séparés sont identifiés à la longueur d'onde 280 nm (sachant que la zone d'absorbance des TC est 280-300 nm), et comparés en se basant sur les temps de rétention et les spectres UV.

- **Screening phytochimique (essais 3 et 4)**

Les tests phytochimiques sont réalisés en suivant les méthodologies classiques (Dohou et al., 2003; Togola, 2002). Ces tests sont basés sur l'ajout de réactifs spécifiques au matériel végétal, soit directement après broyage, soit sur l'infusé. L'ajout de réactifs provoque des changements de colorations, ou bien les formations de précipités témoignant de la présence des composés recherchés. Les composés chimiques suivants ont été recherchés: alcaloïdes, coumarines, saponosides, tanins, triterpènes et stérols, quinones libres, anthraquinones, anthraquinone hétérosides, flavonoïdes, flavonols, flavanes, and anthocyanines.

- Pour évaluer la présence d'**alcaloïdes,** 5g de plante séchée pulvérisée sont repris avec 30 ml d'acide chlorhydrique 1N, laissés macérer durant 30 min, puis filtrés. Les alcaloïdes sont recherchés sur des prises d'essai de 1ml de filtrat, réparties dans des tubes à hémolyse, au moyen de 5 gouttes de réactifs de Mayer et Dragendorff. L'importance du précipité permet une appréciation grossière de la teneur en alcaloïdes de la plante.

- La présence de **coumarines** est évaluée de la façon suivante : Faire macérer Durant 24 h, 1g de poudre de plante dans 20 ml d'éther éthylique.

Evaporer à sec 5 ml du filtrat et ajouter 2 ml d'eau chaude, partager la solution entre deux tubes à essai. Au contenu de l'un des tubes, rajouter 0,5 ml de soude à 25% et procéder à l'observation sous UV à 365 nm. Une fluorescence intense (violette ou jaune) indique la présence de coumarines.

- La présence des **saponosides** est déterminée quantitativement par le calcul de l'indice de mousse, degré de dilution d'un décocté aqueux donnant une mousse persistante dans des conditions déterminées. Deux grammes de matériel végétal sec broyé à tester sont utilisés pour préparer une décoction avec 100 ml d'eau. On porte à ébullition pendant 30 min. Après refroidissement et filtration, on réajuste le volume à 100 ml. À partir de cette solution mère, on prépare 10 tubes (1,3 cm de diamètre interne) avec 1, 2, … 10 ml, le volume final étant réajusté à 10 ml avec de l'eau distillée. Chacun des tubes est agité avec énergie en position horizontale pendant 15 secondes. Après un repos de 15 min en position verticale, on relève la hauteur de la mousse persistante en cm.

  Si elle est proche de 1 cm dans le Nième tube, alors l'indice de mousse est calculé par la formule suivante : I = hauteur de mousse (en cm) dans le Nième tube x 5 / 0,0N. La présence de saponines dans la plante est confirmée avec un indice supérieur à 100.

- Pour évaluer la présence de **tanins** 1,5 g de matériel végétal sec sont ajoutés à dans 10 ml de MeOH 80 %. Après 15 minutes d'agitation, l'extrait est filtré et récupéré dans un tube. L'ajout de FeCl3 1 % permet de détecter la présence ou non de tanins. La couleur vire au bleu noir en présence de tanins galliques et au brun verdâtre en présence de tanins catéchiques.

- La présence de **triterpènes et stérols** est évaluée par la réaction de Lieberman Burchard. 1g de matériel végétal pulvérisé est mis à macérer dans 20 ml d'éther éthylique durant 24h. L'extrait est filtré et 10 ml de filtrat sont évaporés à sec. Le

résidu est dissout dans 1 ml d'anhydride acétique puis 1 ml de chloroforme. La solution est répartie dans 2 tubes à essai. A l'aide d'une pipette, 1 ml d'H2SO4 concentré est déposé au fond du tube sans agiter. La formation d'un anneau rouge brun à la zone de contact des deux liquides ainsi qu'une coloration violette du surnageant révèlent la présence de stérols et triterpènes.

- La présence d'**anthraquinones libres** est mise en évidence par la réaction de Borntragger. A 1 g de poudre ajouter 10 ml de chloroforme, chauffer au bain marie pendant 3 mn et filtrer. A 1 ml de ce filtrat ajouter 1 ml d´ammoniaque 1N. Une coloration rouge indique la présence d´anthracéniques libres. Afin d'évaluer la présence d'**hétérosides d'anthraquinones**, à la poudre précédemment épuisée par le chloroforme sont ajoutés 10 ml d´eau distillée et 1 ml de HCl. Le mélange est chauffé au bain- marie pendant 15 mn puis filtré. 5 ml de ce filtrat, sont lavés avec 5 ml de chloroforme. A la phase organique, 1 ml de NH4OH dilué sont rajoutés. L´apparition d´une coloration rouge indique la présence d´anthraquinones sous la forme *O*-hétérosides.

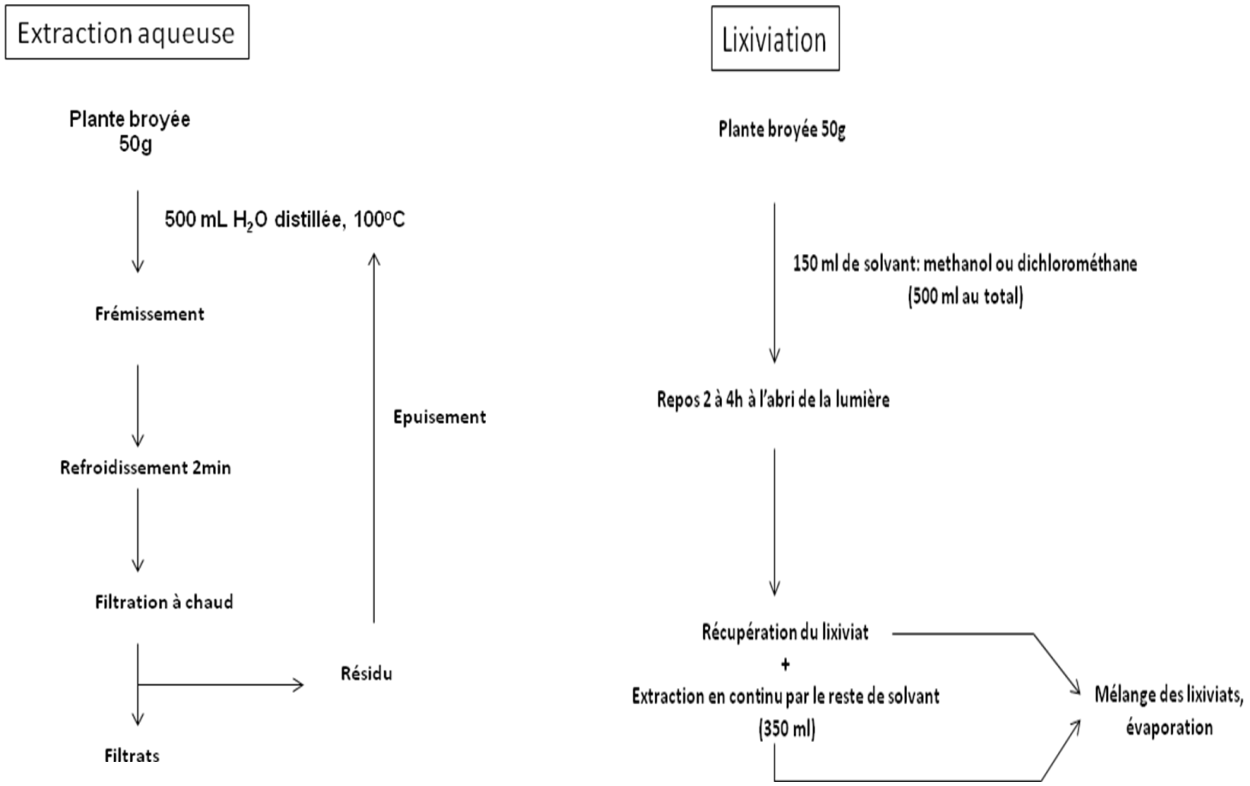
- La présence de **quinones** est mise en évidence de la façon suivante : Un gramme de matériel végétal sec pulvérisé est placé dans un tube avec 15 à 30 ml d'éther de pétrole. Après agitation et un repos de 24 h, l'extrait est filtré et concentré au rotavapor. La présence de quinones libres est confirmée par l'ajout de quelques gouttes de NaOH 1/10, lorsque la phase aqueuse vire au jaune, rouge ou violet.

- En ce qui concerne les **flavonoïdes**, leur présence est évaluée par la réaction à la cyanidine. La solution à analyser est un infusé aqueux à 5 % (5 g de poudre dans 100 ml d´eau distillée bouillante, infusés pendant 15 mn, puis filtrés).

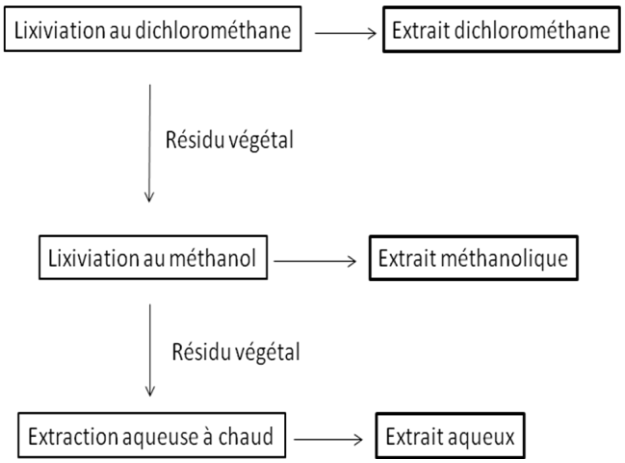
A 5 ml de l´infusé ajouter 5 ml d´alcool chlorhydrique, 1 ml d´alcool iso amylique et quelques copeaux de magnésium, l´apparition d´une coloration:

- rose orangée indique la présence de **flavones**
-rose violacée indique celle des **flavanones**
-rouge indique la présence de **flavanols et flavanonols**

La même réaction sans copeaux de magnésium mais chauffée au bain marie pendant 15 min permet de détecter la présence de **Leucoanthocyanes** en cas d´apparition de coloration rouge cerise ou violacée.



Concernant les feuilles de *Manihot esculenta*, une extraction séquentielle a été opérée, de la façon suivante :



<u>Figure 7</u> : Les modes d'extraction utilisés : extraction aqueuse à chaud et lixiviation

1.4. Choix et présentation des tests anthelminthiques

1.4.1. Tests *in vitro*

1.4.1.1. Préparation des échantillons pour les tests *in vitro*

Les échantillons de plantes ont tous été collectés en Guadeloupe. Préalablement aux tests *in vitro*, ils ont subi un séchage et des extractions.

- **Séchage :**

Afin de ne pas altérer les susbtances potentiellement actives, les ressources végétales ont subi un séchage doux à 55°C en étuve ventilée et ce, jusqu'à obtenir un poids constant. Dans un souci de meilleure conservation, les échantillons ont été par la suite lyophilisés.

- **Extractions :**

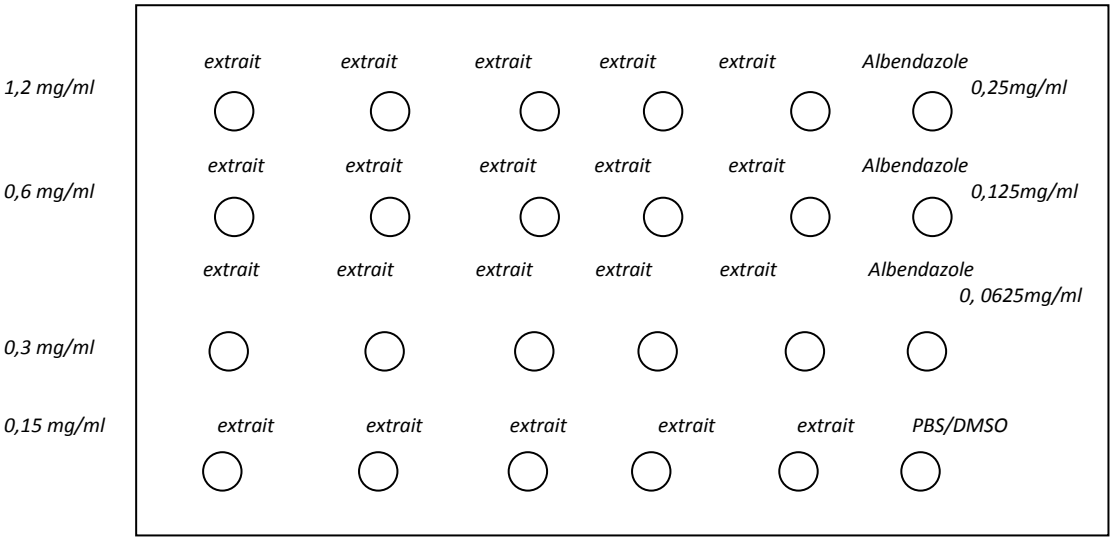
Deux types d'extractions sont réalisés à partir de la ressource végétale séchée. Il s'agit soit de l'extraction aqueuse à chaud, soit de l'extraction par lixiviation au méthanol ou au dichlorométhane (Figure 7). Le principe consiste à choisir des solvants de polarités différentes (Balansard, 1991), de manière à extraire la plus grande variabilité possible de molécules: des moyennement polaires (dichlorométhane) aux plus polaires (eau) en passant par les molécules polaires (méthanol). Les métabolites sont donc, en général, extraits séparément avec chacun des solvants de la manière suivante avant lyophilisation (Figure 7).

1.4.1.2. Expérimentations *in vitro* (essais 0, 1, 2, 3 et 4)

Les différentes doses testées *in vitro* ont été déterminées et établies à l'aide d'une part d'une étude bibliographique concernant les tests d'extraits de plantes et d'autre part, de tests préalables au laboratoire.

- **Test d'éclosion** (Egg hatch assay, EHA; Hubert et Kerboeuf, 1984)

L'objectif est de tester *in vitro* l'activité anthelminthique d'extraits de plantes sur l'éclosion des œufs du parasite *H. contortus.* Les œufs sont issus des féces d'un animal infesté expérimentalement, et sont extraits selon la méthode (Hubert and Kerboeuf, 1984). La suspension d'œufs de concentration 400 œufs/ ml est distribuée dans des boîtes de 24 puits à raison de 0,5ml par puits. Ensuite, 0,5ml des solutions d'extraits de plantes (diluées dans du tampon PBS) de concentrations croissantes (300, 600, 1200, et 2400µg.ml^{-1}) sont ajoutées à ces puits. Il y a une boîte par type d'extrait (eau, méthanol et dichlorométhane) et chaque concentration est testée cinq fois. Des témoins négatifs (PBS ou DMSO à 6%) et un témoin positif: l'albendazole aux différentes concentrations : 0,125%, 0.25%, 0,5% sont également distribués dans les boîtes à raison de 0,5ml par puits (Figure 8). Les œufs sont ensuite incubés durant 48h à température ambiante à 25°C. Une solution de Lugol's iodine est alors ajoutée à l'issue de ce délai aux afin de stopper l'éclosion. Le comptage des larves écloses dans chaque puits se fait au microscope inversé. Le pourcentage d'œufs éclos est déterminé comme étant le rapport entre le nombre de larves L1 formées et le nombre d'œufs déposé par puits.



<u>Figure 8</u>: Distribution et concentrations finales dans une boîte de 24 puits pour les tests EHA et LDA

- **Test d'inhibition du développement larvaire** (Larval development assay, LDA; Hubert et Kerboeuf, 1992)

L'objectif est de tester *in vitro* l'activité anthelminthique d'extraits de plantes sur le développement larvaire du parasite *H. contortus.* Les larves L1-L2 sont obtenues à partir des œufs d'un mouton infesté par *Haemonchus contortus* (méthode de récupération des oeufs identique au test précédent), ces œufs sont mis à incuber dans les boites après distribution d'environ 200 par puits a température ambiante pendant 48h (temps nécessaire à l'éclosion). Suite à cette incubation, les larves formées sont placées dans un milieu de culture rajouté à chaque puits. Les extraits de plante dilués dans du tampon PBS à différentes concentrations sont de la même manière ajoutés à leur tour dans les puits. Il y a une boîte par type d'extrait (eau, méthanol et dichlorométhane) et chaque concentration est testée cinq fois. Des témoins négatifs (PBS ou DMSO à 6%) et un témoin positif Albendazole sont également distribués dans chaque boîte de test. Pour toutes les solutions, le volume réparti est de 0,5ml par puits (Figure 8). Une incubation de huit jours est nécessaire pour le développement des larves L1 en larves L 3. Le développement larvaire est alors stoppé par l'ajout d'une solution de Lugol's iodine. Le comptage des larves se fait ensuiteau microscope inversé en séparant les larves au stade L3 des larves aux stades L1 et L2. Le pourcentage des larves L 3 est calculé comme étant le rapport entre le nombre de larves L3 et le nombre de larves au total, observées dans chaque puits.

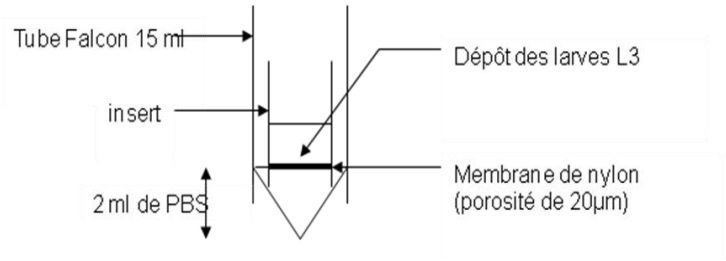
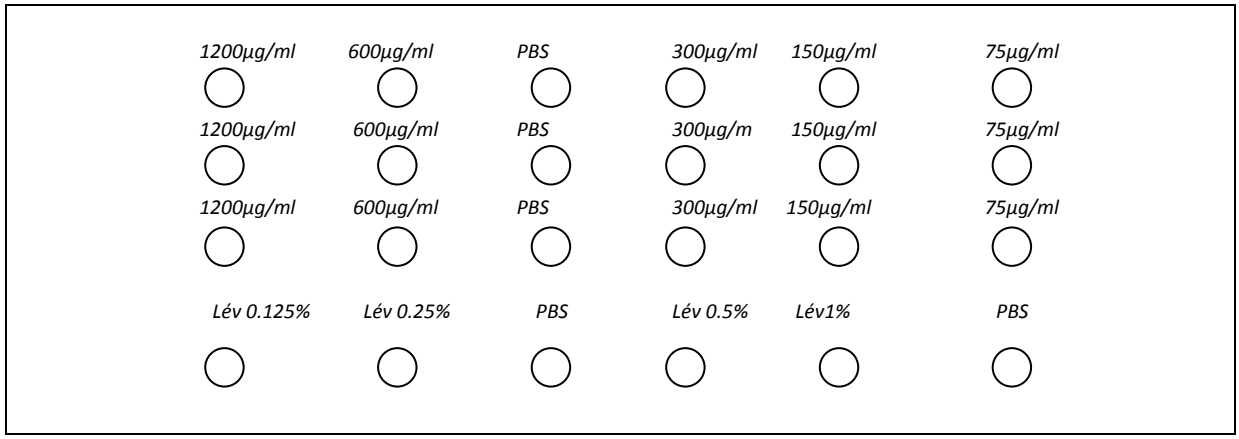


Figure 9 : Schéma du dispositif pour le test LMI

- **Test d'inhibition de la migration des larves L3** infestantes *d'Haemonchus contortus* (Larval migration inhibition, LMI; Rabel et al., 1994)

L'objectif est de tester *in vitro* l'effet d'extrait de plantes sur la capacité de migration des larves L3 d'*H. contortus*. Les larves au stade infestant (L3) sont obtenues par coproculture des fèces d'un mouton « donneur » infesté expérimentalement par *Haemonchus contortus*. Ils atteignent le stade L3 au bout de dix jours. Un dispositif appelé « appareil de Baerman » permet de recueillir ces larves par sédimentation. Une suspension larvaire d'un volume correspondant à 1000 larves L3 est déposée dans une série de tubes. Les solutions des différents extraits de plantes mises en contact avec les larves L3 ont pour concentrations : 300, 600 et 1200 $\mu g.ml^{-1}$. Des témoins sont également mis en contact avec les larves : le contrôle anthelminthique positif lévamisole aux différentes concentrations : 0,125%, 0,25% et 0,5 % (g/v); le tampon PBS comme contrôle négatif, et une solution de DMSO à 3% (v/v). Chaque dose et chaque contrôle (DMSO, PBS, lévamisole) est testé 5 fois. Tous ces tubes sont mis à incuber pendant 3h à 25°C. Suite à cette incubation, les larves sont rincées au tampon PBS par trois centrifugations successives. Après le dernier rinçage, le culot de L3 restant est placé dans des inserts munis d'une membrane de nylon ayant une porosité de 20µm. Ces inserts sont eux-mêmes positionnés dans des tubes contenant du tampon PBS jusqu'au niveau de la membrane (Figure 9). La porosité de 20µm permet de sélectionner les larves L3 qui ont encore la capacité de migrer de manière active. Les inserts sont retirés au bout de 3h d'incubation à 25°C. Afin d'évaluer le nombre de larves ayant migré, un comptage est effectué au microscope optique sur 25 % du volume total de la suspension larvaire. Cette valeur lue rapportée au volume total permet d'obtenir le nombre de larves L3 ayant migré.



<u>Figure 10:</u> Distribution et concentrations finales dans une boîte de 24 puits pour le test AWM

- **Test d'inhibition de la motilité des vers adultes** *Haemonchus contortus (*Adult worm motility, AWM; Hounzangbe-Adote et al., 2005)

L'objectif est de tester *in vitro* l'effet d'extraits de plantes sur la motilité des vers adultes du parasite *H.contortus.* Les vers adultes sont récupérés dans la caillette d'un mouton abattu quatre semaines après avoir été infesté expérimentalement (par voie orale) par des larves *d'Haemonchus contortus*. La caillette est incisée et placée dans du sérum physiologique à 37°C afin de récupérer les vers. Ces vers sont répartis (à raison de trois par puits) dans des boîtes de culture de 24 puits contenant du sérum physiologique à 37°C. Les vers sont incubés une heure à l'étuve à 5% de CO2 et à 37°C. Après avoir retiré le sérum physiologique de tous les puits, les différentes doses d'extraits de plantes (75, 150, 300, 600 et 1200 µg.ml^{-1}), le témoin positif lévamisole (de concentrations : 0,125%, 0,25%, 0,5% et 1%) et les témoins négatifs, tous dilués dans une solution PBS-pénicilline-streptomycine, sont répartis dans les puits (Figure 10). Trois répétitions sont effectuées pour chaque type d'extrait (méthanol dichlorométhane et eau) ainsi que pour chaque concentration. Le milieu est renouvelé au bout de 24h. L'observation de la motilité des vers adultes est effectuée sous une loupe binoculaire à différents intervalles de temps d'incubation : 6h, 24h et 48h. La motilité des vers adultes est calculée pour chaque observation comme étant le rapport entre le nombre de vers mobiles et le nombre total de vers dans le puits.

1.4.2. Tests *in vivo*

1.4.2.1. Préparation des échantillons pour les tests *in vivo*

Les deux plantes testées *in vivo* ont subi des prétraitements afin, d'une part, de faciliter leur préhension par les animaux et d'autre part d'éliminer certaines substances toxiques.

- *Musa x paradisiaca L.*(Bananier, essai 5)

Afin d'optimiser la préhension des feuilles et stipes de bananier frais par les animaux, ils ont préalablement été broyés grossièrement à l'aide d'un broyeur à herbe et le mélange homogénéisé avant distribution.

- *Manihot esculenta Crantz*(Manioc, essai 6)

Dans un souci d'optimiser la distribution et la préhension, les branches de manioc ont subi un effeuillage manuel. Afin d'évacuer un maximum de glycosides cyanogéniques avant distribution (Guillermo Gómez, 1985), les feuilles et tubercules de manioc ont été préalablement séchés dans un séchoir à herbe durant une journée avant d'être distribués aux animaux.

1.4.2.2. Expérimentations *in vivo*

Dans ces expériences (essais 5 et 6), il s'agit de comparer les traitements par les plantes avec ou sans infestation par le parasite *Haemonchus contortus*. Les essais ont été menés en hors sol afin de travailler en conditions contrôlées. L'effet adulticide (OPG et bilan à l'abattage), ovicide (coproculture) et larvicide (coproculture, OPG) de la plante est également évalué, ainsi que l'effet sur la fécondité des vers femelles (bilan à l'abattage). Dans les deux cas (essais 5 et 6), l'expérimentation a été réalisée avec des agneaux mâles, préalablement traités contre les helminthes. Dans le cas de l'essai 6, l'effet de tanins condensés est évalué en utilisant le polyéthylène glycol (PEG), inhibiteur des tanins condensés (Kabasa et al., 2000).

D'autre part, des mesures parasitologiques et zootechniques ont été réalisées lors de ces deux expérimentations *in vivo*.

- Mesures parasitologiques :

  - **L'excrétion d'œufs (OPG)**: Il s'agit du nombre d'œufs de strongle excrétés par gramme de fèces (OPG). Il est obtenu par la technique de coproscopie (flottaison des œufs en solution de NaCl saturée) et lecture sous cellule Mac Master (Henriksen, 1976). Cette mesure permet de suivre l'excrétion des œufs de strongles pendant la période de ponte, qui débute environ 21 jours après infestation.

  - **Coproculture** : par le dispositif de Baerman, pour la mise en évidence de l'effet ovicide (Roberts et O'Sullivan, 1952). Six échantillons de fèces d'environ 4g chacun sont mis en culture. L'identification des stades larvaires a été réalisée par les clés de diagnose décrites par Gruner et Raynaud (1980). L'OPG est déterminé en parallèle afin de calculer le développement larvaire effectif.

  - **Eosinophilie**: Cette mesure permet d'évaluer l'importance de la réponse immunitaire mise en place par l'hôte lors de l'infestation parasitaire. Plus l'agression est importante, plus ce nombre augmente. Le nombre d'éosinophiles circulants est mesuré selon la methode de Dawkins (1989), et les cellules sont comptées sur lames de Malassez.

  - **Hématocrite** ou Packed Cell Volume (PCV) : *H.contortus* étant un parasite hématophage, il provoque une anémie plus ou moins importante chez l'hôte. La mesure de l'hématocrite permet de suivre l'infestation mais également de surveiller l'évolution de l'anémie chez l'hôte. L'hématocrite est le pourcentage représenté par le volume d'hématies par rapport au volume total de sang. Dans les présents travaux, il est estimé en utilisant la méthode de capillarité microhématocrite.

- **Bilan parasitaire à l'abattage** : Sept semaines après l'infestation, les agneaux sont abattus pour le dénombrement des vers mâles et femelles dans la caillette, selon la méthode décrite par Gaba et al., (2006).

  La caillette est retirée de l'animal de moins de 15 min après l'abattage et immédiatement traitée. Le contenu de la caillette est récupéré dans un plateau, puis placé dans un flacon (lavage 1).

  L'intérieur de la caillette est rincé soigneusement à l'aide d'un jet d'eau et l'eau de lavage est également récupérée dans le même flacon. Après lavage, la muqueuse de la caillette est conservée dans un deuxième flacon puis immergée dans du serum physiologique à 37°C et maintenue à cette température durant 4-5 h afin de récupérer les larves inhibées ou non, et les autres vers jeunes et adultes (lavage 2). Les lavages 1 et 2 sont réunis et mis à décanter avant d'être ramenés jusqu'à 1 ou 2 L de volume. Ces volumes peuvent varier en fonction de la quantité de vers récupérés. Le volume final est tamisé sur un tamis de porosité 125 mm suivi d'un tamis de porosité 32 mm. On obtient alors un échantillon, correspondant aux contenus, des lavages 1 et 2, respectivement pour chaque mouton.

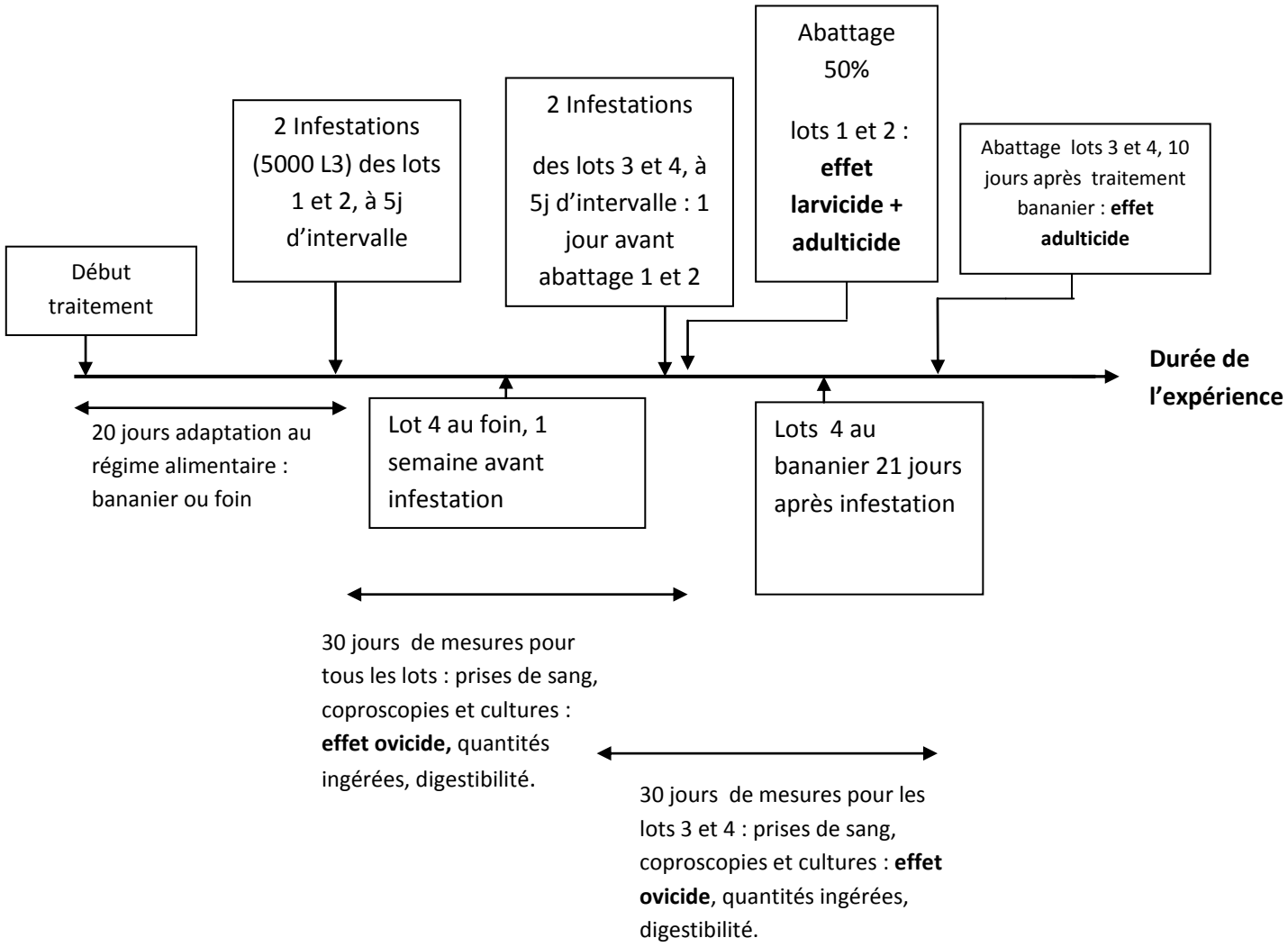
  Les vers déposés sur les tamis sont récupérés à l'aide d'un jet d'eau et ramenés, après décantation, à un volume final minimum. Les échantillons sont conservés par ajout d'une solution de conservateur (35% d'éthanol, 2,5% de formol, de l'eau distillée qsp 1L) à raison de 5% (v/v). Les vers sont ensuite comptés sous une loupe binoculaire (grossissement 150), en séparant les mâles des femelles. Les vers femelles sont mis en conservateur (5%, v/v) dans l'attente de mesure de la taille et du comptage des œufs *in utero*. La mesure de la taille des vers femelles et le comptage des œufs *in utero* est effectué selon la méthode Kloosterman et al. (1978), en utilisant l'hypochlorite de potassium (eau de javel) à 0,4% comme agent de lyse. Chaque vers est désintégré individuellement, à raison de 100µL de solution d'hypochlorite de potassium par vers. Le comptage des œufs est réalisé au microscope optique.

- Mesures zootechniques :

  - **Quantités ingérées** journalières : Il s'agit de la différence entre les quantités d'aliment proposées et refusées. Cette mesure est importante car le parasitisme gastro-intestinal peut avoir un impact négatif sur ce paramètre en fonction du degré et du stade de l'infestation. La composition chimique de l'aliment peut également avoir un impact sur son ingestion par l'animal.

  - **Croissance :** Pesée des animaux tous les 15 jours. Ce paramètre doit être mesuré car non seulement, le parasitisme gastro-intestinal peut faire diminuer la croissance des animaux, mais certaines rations ont également un effet positif sur la croissance. La croissance est aussi liée à la quantité d'aliment digestible ingérée et à son équilibre nutritionnel.

  - **Digestibilité** : c'est le bilan global de l'utilisation digestive de l'aliment, la proportion d'aliment ingéré non excrété via les fèces par l'animal. La digestibilité des aliments diffère selon leur composition chimique. De plus, le parasitisme gastro-intestinal provoque une diminution de la digestibilité de la matière azotée, de la matière sèche, des glucides et de l'énergie.



<u>Figure 11</u> : Schéma de l'expérience *in vivo* bananier, essai 5

2. Plans expérimentaux

2.1. Essai 5 : Test *in vivo* avec les feuilles et stipes de bananier

4 lots d'animaux ont été mis en place :

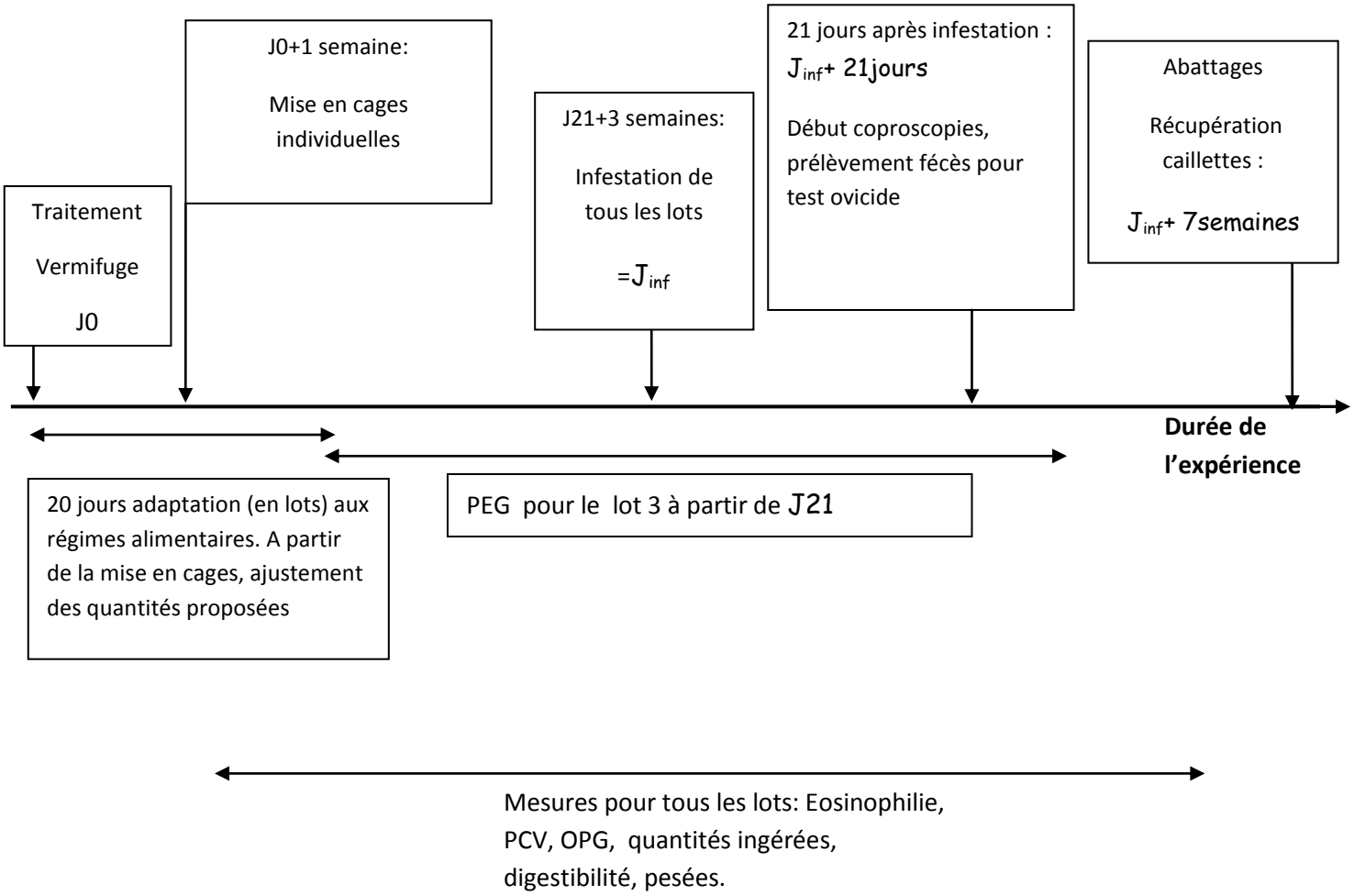
- Lot 1 : Foin, infesté

- Lot 2 : Bananier, infesté

- Lot 3 : Foin, non infesté

- Lot 4 : Bananier, non infesté.

Le plan expérimental de l'essai 5 est illustré par la Figure 11, ci-contre.



<u>Figure 12</u> : Schéma de l'expérience *in vivo* manioc, essai 6

2.2. Essai 6 : Test *in vivo* avec les feuilles de manioc (*Manihot esculenta Crantz*)

Pour cette expérimentation sur feuilles de manioc, les animaux sont répartis en 3 lots :

- Lot 1 : luzerne, foin
- Lot 2 : feuilles de manioc, foin
- Lot 3 : feuilles de manioc + Polyéthylène Glycol (PEG), foin

Le plan expérimental de l'essai 6 est illustré par la Figure 12, ci-contre.

III. RESULTATS

Article n°1

Inventory of vegetal resources potentially available for veterinary medicine in Guadeloupe and Martinique Islands: a focus on their anthelmintic properties.

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Soumis à Livestock Science

Abstract

This article presents a review of literature and ethnobotanical studies, which is based on the utilisation of plants in veterinary medicine in the French West Indies. Also a comprehensive survey was conducted which involved direct interviews of people experienced in the field of ethnoveterinary medicine in the French West Indies. A total of 194 plants belonging to 74 families were identified as possessing medicinal properties and of potential application in veterinary medicine. The highest number of species belong to the family of the *Asteraceae* (7.73%), followed by the *Euphorbiaceae* and the *Fabaceae* (4.64% each), and then, the *Cucurbitaceae* and the *Rutaceae* (4.12% each). When considering plants with anthelmintic properties, the most important family was also *Asteraceae* (14%).

Keywords: Ethnoveterinary medicine; Medicinal plants; Animal health; French West Indies; Review; Anthelmintic; Screening

1. Introduction

Since the last two decades, two new concepts have emerged in agricultural science. These are sustainable agricultural production system and high food quality to preserve human health. These new concepts have resulted in a shift from inorganic to organic agriculture. One direct consequence of this shift is the increasing interest of agronomists and veterinary researchers for medicinal plants with potential use in veterinary medicine, a field which is already advanced in human health.

In organic agriculture, the use of chemical drug is very limited (CEC, 1999) whereas animal diseases continue to exist irrespective of the technical management of the farmers. The situation becomes even more urgent in intensisve agriculture with the ever growing development of drug resistance in pathogen agents coupled with the deficiencies of industrial drugs to provide new molecules to treat some diseases. For example, for gastro-intestinal infections with parasitic nematodes in small ruminants, the development and diffusion of AH resistance is nowadays a worldwide phenomenon (Kaplan, 2004). Hence the need of alternative approach like ethnoveterinary medicine becomes indispensable.

Several methods are commonly used for testing nematicidal activity of both chemical drugs and plant extracts. Amongst them, *in vitro* assays revealed to be relevant and cheaper than *in vivo* methods. The Egg Hatch Assay (EHA), (Hubert and Kerboeuf, 1984), Larval Development Assay (LDA), (Hubert and Kerboeuf, 1992) are currently used for the detection of anthelmintic resistance in gastrointestinal nematodes (Coles et al., 1992). In the present study, these two *in vitro* assays were performed on 14 plants selected according to anthelmintic criteria from an ethnoveterinary survey in Guadeloupe Island.

The objectives of this paper are: 1) To gather informations on plants potentially useful in veterinary medicine in Guadeloupe and Martinique (French West Indies) and further in the other Caribbean Islands; 2) to review previous works and studies about the scientific data validating the anthelmintic efficacy of those plants 3) to try to identify strategies for future research, with particular interest on anthelmintic plants.

2. Materials and methods

2.1 Method of study

The data were, for most part, sourced from comprehensive literature search. Ethnoveterinary articles around the world were compiled, from which Guadeloupe and Martinique plants were picked out and classified using the Flore of (Fournet, 2002). The data were rigorously explored one by one according to the Flore, to ascertain the plants belonging to the Caribbean vegetal patrimony. In the same way data from websites (www.vetwork.org) were also examined.

Additional data were obtained from interviews carried out in the field with Guadeloupean breeders, finding out about their own experiences with animal's disease and the remedies used (traditional or chemical). For traditional use, each individual was asked to list all the names of plants that were used for each disease they had encountered and the method of preparation. They were also asked to show, on the field, the plant species used, which were identified with their latin name. Veterinarians of Guadeloupe were also contacted to list the major diseases frequently encountered. During this study, a special interest was accorded to worm treatment.

The medicinal uses of the plants have been classified in groups following the veterinary criteria: digestive, skin, locomotive, reproductive, respiratory, nervous, cardiovascular, urinary, ocular and metabolic systems. Subsequently, the collected data were analysed taking into account experimental demonstration (*in vitro* and *in vivo*) of plants activities.

2.2 Screening of plants against Haemonchus contortus

After analysis of data from literature and local surveys, 14 plants (*Carica papaya L., Annona muricata L., Momordica charantia L., Chenopodium ambrosioides L., Crescentia cujete L., Neurolaena lobata L., Bidens pilosa L., Senna alata L., Cucurbita moschata L., Musa x paradisiaca L., Tabernaemontana citrifolia L., Manihot esculenta Crantz, Lantana camara L., Rauvolfia viridis Willd.)* were screened for assessment of their nematicidal activity against *H. Contortus* performing the Egg Hatch Assay (EHA), (Hubert and Kerboeuf, 1984) and the Larval Development Assay (LDA), (Hubert and Kerboeuf, 1992). Three extracts were prepared: aqueous, methanolic and dichloromethane, for each plant.

2.2.1. The EHA

The *in vitro* anthelmintic activity of the three extracts of the 14 plants on the egg hatching of *H. contortus* was carried out according to a modification of the method used for testing anthelmintic resistance (Assis et al., 2003). Eggs were extracted from a donor sheep according to the method described by Hubert and Kerboeuf, 1984. The donor sheep had previously been per os infested with an aqueous suspension of pure 10 000 *Haemonchus contortus* L3 larvae.

2.2.2. The LDA

The objective of this assay was to test the *in vitro* efficiency of the extracts to inhibit the larval development of *H. contortus* from the L1-L2 stage to the infective L3 stage. The assay used was a modification (Assis et al., 2003) of the technique described by Hubert and Kerboeuf (1992). Eggs were also extracted from a donor sheep previously per os infested with an aqueous suspension of pure 10 000 *Haemonchus contortus* L3 larvae.

2.3. Statistical analysis

Data from bibliographic and surveys were gathered for descriptive analysis using the SAS software. Data from antiparasitic screening were analysed using the general linear model of the Minitab Release software.

3. Results

The results are presented in tables 1, 2 and 3 (p. 80 to 96). The plants were grouped according to their ethnoveterinary indications: treatment of internal table1) or external disorders (table2) and anthelmintic use (table 3) and following the systematic order of plant families. The preparation modes and medicinal uses were also described.

3.1. *Surveys in fields*

On the whole breeders interviewed in Guadeloupe, 10 were using traditional remedies for treating their animals. Among them, 4 were using sea water for external parasites and 6 were using plants. So, for the continuation of the study, the majority of the data were collected from literature and websites.

3.2. Data obtained and botanical families

At least 194 plants, belonging to 74 families used in folk veterinary medicine throughout the world, are usable or used in Guadeloupe and Martinique for veterinary purposes. Among these plants, the different botanical families encountered were: *Asteraceae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Poaceae, Rutaceae, Lamiaceae, Caesalpiniaceae, Verbenaceae, Zingiberaceae, Rubiaceae, Meliaceae, Apocynaceae, Araceae, Armaranthaceae, Anacardiaceae, Annonaceae, Asclepiadaceae, Brassicaceae, Malvaceae, Sapotaceae, Mimosaceae, Myrtaceae, Arecaceae, Apiaceae* and *Solanaceae*. Most species with ethnoveterinary indications belong to the family of *Asteraceae* (7.7%), and then Euphorbiaceae and Fabaceae (4.6% each one), Cucurbitaceae and Rutaceae (4.1% each one). *Asteraceae* (14%) was also the most important family used for deworming plants.

3.3. Internal uses

3.3.1. Pathologies and plant families

80 diseases have been classified into 10 main pathological indications taking into account the symptoms: digestive system (bloating, gastro-intestinal diseases, intestinal parasitism, rumination), skin system (coat irritation, wounds, oedema); locomotive system (musculary bruise, joint deseases), reproductive system (fertility, sterility, parturition, abortion), nervous system (convulsions, aggressiveness), cardiovascular system (haemorragy, blood pressure troubles...), blood system (blood parasitism), metabolic system (agalacty, astheny, poisoning, anorexy, fever, colds, septicaemia), urinary system, respiratory system.

There were 131 plants available for treating all these diseases. Among those, 43 plants were used for digestive system, 10 for skin system, 4 for locomotive system, 27 for the reproductive system, 3 for nervous system, 2 for cardiovascular system, 27 for blood system, 65 for metabolic system, 5 for urinary system and 8 for respiratory system. *Euphorbiaceae* was the plant family that were employed to cure most diseases (52%) followed by *Apiaceae*, *Alliaceae*, *Asteraceae, Fabaceae and Poaceae* (respectively 40% and 36% for the last four plant families). *Euphorbiaceae* plant family was particularly used for metabolic system (representing 36% of the diseases treated by this botanical family) and for digestive and reproductive systems (representing each one 18% of the diseases treated). *Apiaceae* were particularly used for reproductive, metabolic and urinary systems (representing each one 22% of the disesases treated).

Alliaceae was particularly used for digestive system (40% of the diseases treated by this family). *Asteraceae* plant family was particularly used for digestive system (33% of the diseases treated by this family). *Fabaceae* were particularly used for metabolic systems (representing 56% of the diseases treated by this plant family) and *Poaceae* was particularly used for digestive system (representing 44% of the diseases treated).

3.3.2. Preparation modes

Raw plants and mixtures were the most used preparation modes for internal treatments (28% for each other).

The mixture consists of mixing a decoction or maceration of the plant with other plants and/or other ingredients. This preparation is then administered to the animal *per os*.

3.3.3. Plant parts

For internal use, the commonest part of plant used is leaves (33%), followed by fruits (18%) and then the aerial parts (10%). Most frequently, these parts of plants were given directly raw or after a decoction.

3.4. External uses

3.4.1. Pathologies and plant families

The diseases have been classified into 6 pathological systems taking into account their external expression: skin system (wounds, burn, and external parasitism), locomotive system (joint diseases, bruise), reproductive system (reproductive, delivery troubles), blood system (blood parasitism), metabolic system (septicaemia, colds) and ocular system (eye troubles).

73 plants are available to treat these diseases and among theses plants: 58 plants are available for skin system, 3 for locomotive system, 3 for reproductive system, 6 for blood system, 17 for metabolic system and 5 for ocular system.

The families with the largest application field are *Asteraceae* (36%) and *Solanaceae* (36%). *Asteraceae* were particulary used for the skin system (representing 57% of the diseases treated by this plant family). *Solanaceae* were particulary used for the skin and ocular systems (33% of the diseases treated). It was also observed that *Meliaceae* were often used (32%), and at 80% for the treatment of external parasites.

3.4.2. Preparation modes

The most widespread preparation mode for external use was the mixing of plants with other plants and/or ingredients (28%). Otherwise, plants were used raw either ground or directly applied on skin (25%). Decoction and maceration were also well-used (respectively 10% and 11%) methods.

3.4.3. Plant parts

Leaf was the most used part of plant (54%). People often ground leaves and apply this preparation directly on animal skin. Leaves can also be directly placed in the target area without any preparation.

3.5. Anthelmintic uses

The results are presented in table 3. The plants were grouped according to the systematic order of botanical families. The preparation modes and pathological indications (according to organic systems) were also described.

3.5.1. Pathologies and plant families

74 plants were identified corresponding to 11 categories of worm infections. *Asteraceae* and *Meliaceae* were the most widely used families. No botanical family was exclusive to treat a specific worm disease. *Cucurbitaceae* were cited 3 times against *Haemonchus contortu*s and *Asteraceae* 3 times against *Ascaris*.

3.5.2. Preparation modes

The most widely used modes of preparation for anthelmintic uses are: essential oils (23%), decoctions (17%), raw plant (8%), solvents (alcoholic extractions) (6%) and followed by mixed preparations, macerations (in water) and infusions.

3.5.3. Plant parts

Concerning the parts of plant used for anthelmintic treatment, leaves and seeds are the most frequently quoted (respectively 14% and 18%). And then the use of whole plant (12%) followed by fruits, roots and bark. Aerial parts (stem and leaves), stem and latex are in the last position (4%).

3.5.4. Data on experimental validation

Analysis of literature showed that among the plants for anthelmintic use, 39 plants have been tested in order to confirm or to invalidate their potential use, by experimental tests on various helminth species by way of *in vitro* or *in vivo* assays (Table 3).

Efficacy tests on tapeworms *(*Akthar et al., 2000) were carried out on *Callistemon viminalis, Anacardium occidentale, Piper betle* (greater efficacy *in vitro* than piperazine phosphate)*, Randia dumetorum* (varying degree of anthelmintic activity)*, Cyperus rotundus* (good anthelmintic activity)*, Ageratum conyzoides* (very potent anthelmintic activity)*, Lagenaria siceraria* (EPG reduction > 80%)*, Eupatorium triplinerve* (good efficacy against *Taenia solium*)*, Artabotrys odoratissimus* and *Hedychium coronarium* (better efficacy *in vitro* than piperazine phosphate). *Ananas sativus,* fruit bark of *Punica granatum* and *Melia azedarach* had a good activity against tapeworms.

As far as hookworms were concerned (Akthar et al. 2000)*,* assays were performed on *Callistemon viminalis* (same activity as hexylresorcinol)*, Calotropis gigantea* (remarkable vermicidal activity)*, Hedychium coronarium* (better activity than piperazine phosphate), *Artemisia vulgaris* (Duval, 1997; SBSUA, 1994) and *Piper betle* (better activity than hexylresorcinol).

I*n vitro* tests confirmed the efficacy on lungworms for: *Leucaena leucocephala* (Vilasenor et al. 1997), *Momordica charantia, Callistemon viminalis, Neolamarckia cadamba, Datura metel, Citrus medica* and *Zingiber officinale* (Akthar et al. 2000). The *in vitro* activity of *Allium sativum* against *Ascaris* was moderate (Akthar et al. 2000; SBSUA, 1994). Seeds of *Mangifera indica* and flowers of *Tanacetum vulgare* were efficient as well as aerial parts of *Chenopodium ambrosioides; Melia azedarach* was efficace against *Ascaridia galli; Carica papaya* has an 80 to 100% efficacy *in vivo* and effective *in vitro* (Akthar et al. 2000; SBSUA, 1994; Duval, 1997; Satrija et al., 2001)*,* bark of *Punica granatum* had a moderate activity (Akthar et al. 2000). *Artabotrys odoratissimus* had better efficacy *in vitro* than piperazine phosphate; *Eupatorium triplinerve* has been shown to possess good efficacy (Akthar et al. 2000). *Ocimum gratissimum* showed 55.8% *in vivo* efficacy (Njoku et al., 1998).

Concerning nematodes, assays were made on nematodiasis in calves (Pradhan et al. 1992) for *Punica granatum, Cucurbita maxima* (respectively 78.2% and 40.6% efficacy). *Azadiracta indica* reduced bovine nematode EPG (Pietrosemoli et al., 1999). *Annona squamosa (*no efficacy*,* Vieira et al., 1999*), Ananas sativus* (Hordegen et al., 2003), *Carica papaya* (90% efficacy; Fajimi and Taiwo, 2005), *Manihot esculenta Crantz* (Vieira et al. (1999); *Amaranthus spinosus*, *Khaya senegalensis* (Fajimi and Taiwo, 2005) were tested on *Trichostrongyloids. Annona squamosa* (51.96% *in vivo* activity), *Carica papaya* (90% efficacy), *Khaya senegalensis* and *Calotropis gigantea* had efficacy on *Oesophagostomum* (Vieira et al., 1999; Akthar et al. 2000; Fajimi and Taiwo, 2005).

In vitro tests against the parasite *Haemonchus contortus,* were performed with *Momordica charantia* (Vieira et al. 1999; Sharma et al., 1971); *Musa x paradisiaca, Calotropis gigantea, Cucurbita pepo, Mangifera indica* (Sharma et al., 1971); *Carica papaya* (Hounzangbe-Adote et al., 2005*)*, *Spigelia anthelmia* (Assis et al., 2003) *Khaya senegalensis* (Fajimi and Taiwo, 2005), *Ocimum gratissimum* (Njoku and Asuzu, 1998), *Azadiracta indica, Ananas sativus* (Hordegen et al., 2006), *Punica granatum* (dose dependent activity on eggs; Akthar et al. 2000).
In vivo assays on *Haemonchus contortus* were also carried out (Pamo et al., 2006; Sokerya et al. 2001, 2003; Vieira et al., 1999; Hordegen et al., 2003; Kahiya et al., 2003; Akthar et al. 2000; Houzangbe-Adote, 2001, 2005) with: *Olea europaea* (low activity), *Dodonea viscosa* (non significant effect), *Musa sp.* (EPG 0%), *Carica papaya (*EPG 32.2%), *Allium sativum* (EPG 3.2%), *Canavalia brasiliensis* (EPG 33%), *Momordica charantia* (EPG 36% and 17.6% reduction of adult worms), *Annona squamosa* (EPG 40% and 30.4% reduction of adult worms)*, Chenopodium ambrosioides* (EPG 33.6% and 9.6% reduction of adult worms), *Hymenaea courbaril* (EPG 25.8%), *Azadiracta indica, Ananas sativus, Acacia nilotica* (10% efficacy), *Calotropis gigantean, Cucurbita pepo* and *Manihot esculenta Crantz* (EPG reduction).

For the *Oxyuridae* the activities of *Chenopodium ambrosioides, Tanacetum vulgare, Carica papaya* (Houzangbe-Adote et al., 2001) and *Melia azedarach* have been tested.
Carica papaya was also tested against *Trichocephale* and gastro-intestinal parasites of poultry (Mpoame et al., 2000; Singh and Nagaich, 1999).

3.6. Anthelmintic screening

3.6.1. EHA

Compared to the negative control PBS, nematicidal effect on egg hatching of *H. contortus* ($P < 0.05$) was observed for 9 plants (table 4): *Carica papaya L., Annona muricata L., Momordica charantia L., Chenopodium ambrosioides L., Crescentia cujete L., Neurolaena lobata L., Bidens pilosa L., Senna alata L., Cucurbita moschata L.*

3.6.2. LDA

Compared to the negative control PBS and depending on the extracts, effect on larval development of *H. Contortus* ($P < 0.05$) was observed for each one of the 14 plants screened (table 5). Significant effect was observed compared to the positive control albendazole ($P > 0.05$) for: *Carica papaya L.*

aqueous extract*, Annona muricataL.* aqueous and methanolic extracts*, Lantana camara L.* aqueous and methanolic extracts*, Senna alata L.* aqueous and methanolic extracts and *Cucurbita moschata L.* aqueous, methanolic and dichloromethane extracts.

4. Discussion

4.1. Plants indicated against main veterinary pathologies.

From this investigation, it was observed that the French West Indies possesses a large arsenal of plants potentially useful for veterinary therapeutics. *Euphorbiaceae* was the plant family that was most widely quoted to treat diseases. The latex of this plant family contains alkaloid compounds and has large therapeutic indications. It was also observed that plants of the *Meliaceae* family were often used and only for the treatment of external parasites. *Asteraceae* and *Solanaceae* have been described for external and internal uses. The use of essential oils was frequently mentioned. The *Asteraceae* family could contain essential oils, terpenic lactones, anti-inflammatory azulens, alkaloids, polyins (polyacetylens), and inulin (from roots), (Bruneton, 1999). In this family, many plants are refered as antispasmodic, tonic, antiparasitic (antimalarian), insecticide (containing pyrethrine).

Solanaceae contain tropanic/steroidic alkaloids, some of them being toxic (Bruneton, 1999). The favoured use of essential oils could be due to the fact that they are efficient on a wide spectrum of diseases, because of the presence of several chemical compounds, each of them having a specific target and whose activities are balanced overall.

These observations indicate that plants from these botanical families could have a greater potential for veterinary medicine.

Considering the frequency of animal illness in Guadeloupe (Aumont et al., 1997), there are potentially available resources to treat major diseases (internal and external parasites, physiologic troubles, external traumatisms) except the main pandemics. Most of the plants are generally non cultivated resources. Unlike the human traditional medicine, where people typically cultivate their own medicinal field (usually common plants), veterinary medicine lags far behind in this respect particularly in the French West Indies.

From this investigation, veterinary medicinal fields would be essentially composed with none of the classically cultivated resources.

This constraint could be a limiting factor for the development of veterinary medicinal field. Even so, in this inventory still exists some traditionally cultivated resources (cassava, banana, some legumes…) with perspective of possible development given their potential as sources of energy as well as medicinal values.

There are few *in vitro* and *in vivo* studies to confirm the efficacy of these plants. Moreover the ethnoveterinary approach is still in its infantry stage and is not as developed as practices in human medicine. For example, the researchers of the TRAMIL association (program developed to record and preserve Caribbean medicinal plant lore) stipulate that a plant resource has to be studied when its frequency of use exceed 20%.

Ethnoveterinary medicine is yet far to attain this level and approach of study. To progress in the use of plant in veterinary medicine, the first step could be the development of statistical analysis of the traditional use of plants as in human medicine. This could be a project worth to develop within the Caribbean area where the flore is homogeneous.

4.2 Vegetal ressources with anthelmintic properties

Helminthioses are major pathologies of ruminant farm animals (Aumont et al, 1997). In Guadeloupe they represent 41% of goat's diseases, 15% for cats, and about 5% for pigs. Cattle suffer from pulmonary strongylosis (15%). It is nowadays admitted that the use of chemotherapy against helminths is not a sustainable method of control because of the constant development of resistance to the chemical molecules, this phenomenon being particularly prominent for gastrointestinal nematodes (Kaplan et al, 2004). Today resistance have been reported for most of the available molecules. There is an urgent need to find new molecules and/or new methods of control to support the development of small ruminant production.

The present study indicates that in the French West Indies, there are potential vegetal resources (containing alkaloids, polyphenols, essential oils and/or non proteic amino acids) which could be employed to treat helminthiases. However, very little scientific information exists to validate the use of these plants and the exact nature of the targeted helminths. The screening carried out in the present study confirmed the anthelmintic effect of the 14 plants used in ethnoveterinary, and clarified it as a nematicidal use against *H. contortus*.

As in human medicine, resources rich in alkaloids (e.g; *Tabernaemontana citrifolia L., Rauvolfia viridis Willd.)* are cited as possible veterinary anthelmintic treatment. Although the alkaloid family is widely studied in human medicine, far fewer studies are reported in veterinary research.

Plant resources containing condensed tannins (e.g; *Leucaena leucocephala (Lam.) de Wit.)* or foliages rich in proteins (e.g. *Manihot esculenta Crantz, Musa x paradisiaca L.*) are often cited for their anthelmintic (AH) activity (Morton, 1981). Forages containing condensed tannins (CT) have the potential to complement the control of anthelmintic-resistant gastrointestinal (GI) parasites. Two non exclusive hypotheses have been proposed to explain the AH effect of CT against GI nematodes, either directly, or indirectly, through the stimulation of the local host mucosal response. However, most current data supports the hypothesis of direct effect, mediated through condensed tannins - nematode interactions, which affect the physiological functions of different stages of the gastrointestinal parasites (Athanasiadou et al., 2000; 2001; Brunet et al., 2007; Brunet and Hoste, 2006; Brunet et al., 2008; Hoste et al., 2006; Molan et al., 1999; 2002; Molan et al., 2000). The direct mechanism of action of the CT plant resources is also strongly supported by *in vitro* trials. CT have been shown to decrease faecal egg counts in sheep and goats and may decrease hatching rate and larval development in faeces. CT from *Leucaena leucocephala* (Ademola and Idowu, 2006) were effective at reducing the migration of exsheathed larvae, and the development of nematode eggs to the third larval stage was found to be reduced in the presence of CT (Alonso-Diaz et al., 2008a; Alonso-Diaz et al., 2008b; Kahn and A.Diaz-Hernandez, 2000). Eggs per gram counted in goats fed with *Manihot esculenta Crantz* leaves declined during the experiment from 4000-5000 in the first 30 days to about 1500 after 70 days (Sokerya and Preston, 2003b; Sokerya and Rodriguez, 2001).

Furthermore, based on production trials with ruminants, there might be confusion between the impact of the level of condensed tannins and the one of the level of proteins (Hoste et al., 2005). Condensed tannins play a significant role in animal nutrition, causing either adverse or beneficial effects, depending on their concentration in plants, on nutrient utilisation, health and production (Waghorn and McNabb, 2003). The major benefit of tannins in feed is the protection of plant proteins from ruminal degradation, making them available for digestion and absorption in the abomasum and the small intestine (Nguyen et al., 2005). It is also known that a better protein nutrition decrease the pathological consequences due to nematode infections (Coop and Kyriazakis, 1999). Protein supplementation appears to be effective in enhancing specific immune responses against intestinal parasite infection. Therefore, condensed tannins may counteract parasites by one or more mechanisms, and this might differ depending on the nature of condensed tannins from different forage species (Nguyen et al., 2005, Brunet and Hoste, 2006; Brunet et al, 2007).

Plant resources with non proteic amino acids, mainly seeds of *Cucurbita moschata*, (Morton, 1981), are also cited for their anthelmintic activity. One of the functions attributed to these non proteic amino acids is defense against predators (Bruneton, 1999).
Nevertheless, the availability of this kind of resources (seed) in a large quantity could be a limitation to develop its use in farming. It seems to be more appropriate for an industrial approach.
Foliages rich in essential oils (generally volatile aromatic compounds) (*Chenopodium ambrosioides, Tanacetum vulgare L.*); were also associated with anthelmintic properties (Morton, 1981).
In human pharmacology some of these molecules were cited for their activity on the nervous system and for their anthelmintic activity (Bruneton, 1999). This could also be applicable in veterinary medicine. Other secondary metabolites of plants, for instance, terpenoids present in some legumes, are known to reduce the mobility and the migration ability of ovine nematode larvae (Molan et al., 2003).

5. Future Strategies and conclusion

Today in the French West Indies, it is increasingly difficult to find farmers having used or having knowledge on ethnoveterinary phytotherapy. In the Caribbean, with the decline of agriculture (competition with urbanism and tourism) there is an obvious increasing risk that the traditional knowledge will disappear before it is documented for the future generations. Consequently, it seems urgent to develop a Caribbean network to organise surveys on veterinary phytotherapy practices on similar bases to TRAMIL which aims to present a selection of medicinal plants based on precise scientific criteria, including field ethnopharmacological surveys conducted by multidisciplinary teams in each member country. Their work has given impressive good results in human medicine and it could be adopted for veterinary medicine. This type of network could be developed for the ethnoveterinary approach and complemented with laboratory approaches to validate traditional knowledge useful for the development of sustainable agriculture.

Concerning the laboratory approaches, it is necessary to develop *in vivo* trials. *In vitro* trials are useful for screening, but *in vivo* methods are necessary for validation. Moreover, conclusions of *in vitro* methods should be cautiously analysed since *in vivo* is a more complex system than *in vitro*, because some inactive compounds *in vitro* could be active *in vivo*.

The strategy of validation of vegetal resources seems to be an open question. Traditional use can be a limiting factor for large appropriation of phytotherapy but industrial method has also limits. Probably the solution is a holistic approach to animal health integrating: nutrition, breeding practices and use of traditional medicine.

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Table 1. Inventory of main tropical vegetal resources potentially useful for internal use in veterinary medicine[1]

Botanical family	Scientific name	Pathological indications	Preparation mode	References
Acanthaceae	*Ruellia tuberosa L.*	REP	leaves, roots D,M	(Lans and Brown, 1998a); (Lans et al., 2001)
Alliaceae	*Allium sativum L.*	Me, Di, N, C	Fruit I	(Akthar et al., 2000; BAIF, ; Lans and Brown, 1998a; Lans et al., 2001; Minja, 1994; Uncini Manganelli et al., 2001)
-	*Allium cepa L.*	Di	Corm M	(Uncini Manganelli et al., 2001)
Amaranthaceae	*Achyranthes aspera L.*	REP, Di, B	leaves, roots R,M	(Ali, 1999; Lans and Brown, 1998a; b; Lans et al., 2001; Rangnekar, 1994)
-	*Gomphrena serrata L.G.decumbens jacq*	REP	whole plant R	Ali 1999
-	*Amaranthus spinosus L.*	REP, Me	whole plant, leaves	(Byavu et al., 2000; Lans and Brown, 1998a; b; Lans et al., 2000; 2001)
Anacardiaceae	*Anacardium occidentale L.Masika & Afolayan 2003*	Di	Bark D	
-	*Mangifera indica L.*	Me	Bark M	Personal survey
-	*Spondias mombin L Masika & Afolayan 2003*	REP, S, Me, B	aerial part, bark R,M	Personal survey (Lans and Brown, 1998a; b; Lans et al., 2001)
Anthericaceae	*Chlorophytum comosumThunb. Jacques*	Me	Fruit I	(Tabuti et al., 2003)
Apiaceae	*Petroselimum crispum*	L, REP	aerial part D	(Blanco et al., 1999)
-	*Foeniculum vulgare Mill*	Me, Di, U	fruits, flowers R,M	(Rangnekar, 1994; Uncini Manganelli et al., 2001)
-	*Petroselium sativum Hoffm.*	U, REP	aerial parts R	(Uncini Manganelli et al., 2001)
-	*Eryngium foetidum L.*	Me, RESP	leaves	Lans, 2001
Apocynaceae	*Carissa carandas L.*	B	aerial parts R	Ali, 1999
-	*Rauvolfia viridis L.*	Me	aerial part D	Personal survey

[1]Veterinary criteria systems: REP: reproductive ; Me: metabolic, Di: digestive , N: neurologic, C: cardiovascular, B: blood, S: skin, RESP: respiratory , U: urinary

Preparation modes: D: decoction; M:mix with other ingredients; J: juice; R: raw; I: infusion; MA: maceration

Botanical family	Scientific name	Pathological indications	Preparation mode	References
raceae	*Pistia stratiotes L.*	B	Whole plant M	Tabuti et al., 2003
Araliaceae	*Schefflera attenuata Sw frodin*	Di	Roots M	Personal survey
Arecaceae	*Cocos nucifera L.*	Me	Fruit J	Lans and Brown 1998a ; b; Personal survey
-	*Phoenix acaulis Buch*	REP	Roots D	.
Aristolochiaceae	*Aristolochia elegans Masters*	B	Leaves I	Tabuti et al. 2003
Asclepiadaceae	*Calotropis gigantea May.*	Me, Di	leaves R, latex M	(Abbas et al., 2002; Ali, 1999)
Asphodelaceae	*Tylophora indica Burm. f.*	Me	Leaves M	(Reddy et al., 1998)
_	*Aloe ferox Mill.*	B	aerial parts MA	(Masika and Afolayan, 2003)
Asteraceae	*Aloe vera L.*	Di, REP, Me	leaves R,MA	Lans and Brown 1998a ; b; Lans et al., 2001
-	*Tanacetum vulgare L.*	C	aerial part D,MA in wine	Blanco et al., 1999
-	*Cichorium intybus L.*	REP, Me	Roots M	Ali, 1999
-	*Artemisia absinthium L.*	Di	aerial part I	Uncini Manganelli et al. 2001
-	*Tridax procumbens L.*	Me, S	whole plant, leaves M	Byayu et al., 2000; Reddy et al.,1998
-	*Neurolaena lobata L.L. Cass.*	Me	Leaves D	Personal survey
				Lans and Brown 1998a ; b; Lans et al. 2001
-	*Conyza bonariensis L. Cronq.*	Di	Leaves J	(Minja, 1994)
-	*Tithonia diversifolia A. Gray*	Di	Leaves I	Minja, 1994
Basellaceae	*Basella alba L.*	REP, B, Di	Leaves D, MA	Byavu et al., 2000; Minja, 1994
Bignoniaceae	*Crescentia cujete L.*	REP, Me	fruit R	BAIF 1999
Bombacaceae	*Ceiba pentandraL. Gaertn.*	REP, B	bark, leaves I, MA	BAIF 1999; (Bâ, 1996)
-	*Pachira insignis L.*	REP, Me	leaves	Personal survey
-				
Brassicaceae	*Lepidium sativum L.*	Di,S	leaves, seeds	Abbas et al., 2002
Bromeliaceae	*Ananas sativus Schult f.*	B	Fruit I	Tabuti et al., 2003
-	*Bromelia pinguin L.*	Me	Leaves M	Personal survey

Botanical family	Scientific name	Pathological indications	Preparation mode	References
Caesalpiniaceae	*Tamarindus indica L.*	Di, U	Fruit M	Reddy et al., 1998
-	*Haematoxylon campechianum L*	Me	Bark M	Personal survey
-	*Senna fistula L.*	B	Fruit M	Personal survey
Cannabaceae	*Cannabis sativa L.*	B	Leaves M	Tabuti et al., 2003; Byayu et al., 2000
Caryophyllaceae	*Stellaria mediaLCirillo*	Me	leaves R	Uncini Manganelli et al., 2001
Chrysobalanaceae	*Chrysobalanus icaco*	B	.	Lans et al., 2001
Commelinaceae	*Commelina benghalensis L.*	Di	Bark MA	Reddy et al., 1998
Convolvulaceae	*Ipomea cairicaL.*	Me	whole plant R	Bâ, 1996
Crassulaceae	*Bryophyllum pinnatum Lam.*	Me	leaves, whole plant J	Lans and Brown 1998a ; b; Lans et al., 2001
Cucurbitaceae	*Momordica charantia L.*	Me,Di	leaves, stems D, M	Lans and Brown 1998a; b; Lans et al., 2001
-	*Trichosanthes cucumerina L.var anguinaL*	S	fruits R	Ali, 1999
-	*Momordica balsamia L.*	Di, REP	growth, bark M	(Alawa et al., 2002)
Cucurbitaceae	*Luffa acutanglaL. Roxb.*	Di	fruit	BAIF 1999
-	*Luffa aegyptiaca Mill.*	Di	unripe fruit	BAIF 1999
Cupressaceae	*Cupressus sempervirens L.*	Di,S	aerial parts R	Uncini Manganelli et al., 2001
Dracaenaceae	*Sansevieria sp.*	Me	Roots D	Minja, 1994
Euphorbiaceae	*Ricinus communis L.*	Di, B, RESP, Me, S, REP	Seeds, leaves and fruits, roots MA, R, O	Ali, 1999; (Mbarubukeye, 1992); Byayu et al., 2000; Lans et al., 2001; Reddy et al.,1998; Minja, 1994 ; Personal survey
-	*Chamaesyce hirtaL. Millsp*	Me	whole plant R	Alawa et al., 2002; Reddy et al., 1998
-	*Mercurialis annua L.*	Me, REP	Whole plant D	Uncini Manganelli et al., 2001
-	*Jatropha curcas L.*	Me	Leaves D	Byavu et al., 2000
-	*Acalypha indica L.*	Di	Leaves M	Reddy et al., 1998
Fabaceae	*Cajanus cajan L. Huth*	Me	leaves R	Personal survey
-	*Sesbania bispinosa Jacq.Wight*	Di	leaves R	Ali, 1999
-	*Arachis hypogaea L.*	Me, Di	seeds R	Alawa et al., 2002
-	*Sesbania sesban L. Merr.*	Me	.	Masika and Afolayan, 2003

Botanical family	Scientific name	Pathological indications	Preparation mode	References
-	*Rhynchosia minima L.*	U	Leaves D	Byayu et al., 2000
-	*Mucuna sp.*	Me, REP	.	Hounzangbé-Adote 2003
-	*Neonotonia wightii Wight and Am.*	Me	Leaves D	Minja, 1994
Flacourtiaceae	*Oncoba spinosa Forssk.*	B	Fruit and roots I	Tabuti et al., 2003
Lamiaceae	*Mentha pulegium L.*	Me	Leaves D	Uncini Manganelli et al., 2001
-	*Rosmarinus officinalis L.*	Di	aerial parts MA	Uncini Manganelli et al., 2001
-	*Leucas martiniencis jacq. R. Br.*	B	Leaves M	Byayu et al., 2000
-	*Ocimum sanctum L.*	Me	Leaves	Akthar et al., 2000; Tabuti et al., 2003
-	*Ocimum gratissimum L.*	RESP	.	Hounzangbé-Adote 2003
Lauraceae	*Cassytha filiformis L.*	Di, Me, REP	Whole plant D	Minja, 1994
Laureaceae	*Laurus nobilis L.*	Di, Me, REP	Fruit D	Blanco et al., 1999; Ucini Manganelli et al., 2001
Malvaceae	*Sida rhombifolia L.*	Di	Leaves M	Ali, 1999
-	*Gossypium barbadense L*	Me	Seeds M	Alawa et al., 2002
Meliaceae	*Melia azedarach L.*	Me	Leaves M	Ali, 1999
-	*Azadiracta indica L.*	Me	flowers R	Ali, 1999
-	*Khaya senegalensisDesr.A.Juss.*	Di, Me, REP	Bark MA	Alawa et al., 2002
Menispermaceae	*Cissampelos pareiraL. Var arbiculata*	Me, REP, Di, L	Roots, leaves R, M	Minja, 1994
Mimosaceae	*Acacia nilotica L;*	Di	Fruit MA	(Bâ, 1996)
	Leucaena leucocephala L	Me	R whole plant	BAIF, 1999
-	*Dichrostachys cinereaL. Wight & Arn.*	B	Leaves M	Byayu et al., 2000
-	*Mimosa pudica L.*	REP	Roots M	Lans and Brown, 1998 a ; b; Lans et al., 2001
Moraceae	*Ficus carica L.*	Me	leaves R	Uncini Manganelli et al., 2001
-	*Morus nigra L.*	Me, L	leaves R	Uncini Manganelli et al., 2001
Musaceae	*Musa x paradisiaca L.*	B, Me	leaves, fruit R	Tabuti et al., 2003; Byayu et al., 2000
Myrtaceae	*Syzygium cumini L.*	S	Stem and barkM	Ali, 1999

Botanical family	Scientific name	Pathological indications	Preparation mode	References
-	*Psidium guajava L. Masika & Afolayan 2003*	Di, N, Me	leaves, buds D, I	Lans et al. 2000; Lans and Brown 1998 a ; b; Lans et al., 2001; Minja, 1994
-	*Pimenta racemosa Mill J. W. Moore*	RESP, Me	.	Lans and Brown, 1998a ; b; Lans et al., 2001
Nyctaginaceae	*Boerhavia diffusa L.*	B, Me	Aerial parts I, M	Ali 1999; Tabuti, Dhillion, et al. 2003
Oleaceae	*Olea europaea L.*	Me, Di, B	Leaves, bark D	Ucini Manganelli et al., 2001; Masika and Afolayan, 2003
Onagraceae	*Ludwigia octovalvisJacq. Raven*	REP, Me	whole plant R	Ali, 1999
Pedaliaceae	*Sesamum indicum L*	S	Seeds O	Ali, 1999
Phytolaccaceae	*Petiveria alliacea L.*	REP	Whole plant M	BAIF, 1999; Lans and Brown, 1998a; b; Lans et al., 2001
Poaceae	*Paspalum conjugatum P. Bergius*	N	M	BAIF, 1999
-	*Oryza sativa L.*	Di, Me, S	R, M	Ucini Manganelli et al., 2001; Lans and Brown 1998a ; b; Lans et al., 2001
-	*Zea mays L. Sp.*	Di	.	Hounzangbé-Adoté 2003
-	*Cymbopogon schoenanthus 1Masika & Afolayan 2003*	Di	Whole plant, roots D	Abbas et al., 2002
-	*Sorghum bicolor L. Moench*	Di, B	Whole plant M	Byayu et al., 2000
	Bambusa vulgaris Schrad Masika & Afolayan 2003	Me	leaves R	Lans and Brown 1998 a ; b; Lans et al., 2001; Personal survey
-				
	Rumex acetosa L.	Me	fruit R	Uncini Manganelli et al., 2001
Polygonaceae				
-	*Rumex crispus L.*	Di	Leaves D	Uncini Manganelli et al., 2001
Polygonaceae	*Coccoloba uvifera*	B	Bark M	Personal survey
Rubiaceae	*Pentas sp.*	Di, REP, B, Me	Leaves, roots I	Minja, 1994
-	*Morinda citrifolia L.*	B	Fruit M	Personal survey
Rutaceae	*Citrus medica L.*	REP	Fruits M	Ali, 1999
-	*Citrus limonL.*	B	Fruit M	Tabuti,et al., 2003
-	*Citrus aurantifolia Christm.&Panzer Swingle*	RESP	Fruit J	Lans and Brown 1998a ; b; Lans et al., 2001

Botanical family	Scientific name	Pathological indications	Preparation mode	References
Rutaceae	*Citrus aurantium L.*	RESP, Me	Fruit J	Lans and Brown 1998a ; b
-	*Coffea robusta linden ex De wildeman*	RESP	seeds R	Akthar et al. 2000; Tabuti et al., 2003
-	*Coffea arabica L.*	RESP	seeds R	Akthar et al. 2000; Lans and Brown 1998a ; b; Tabuti et al., 2003
-	*Cleome gynandra L.*	Di	Bark M	Reddy et al., 1998
Sapindaceae	*Dodonea viscosa L. Jacq.*	B, N, Me, L	Leaves I, M	Minja, 1994
Sapotaceae	*Mimusops elengi L.*	S	Fruit M	Ali, 1999
Smilacaceae	*Smilax guianensis Vitman*	Me	Roots M	Personal survey
Solanaceae	*Nicotiana tabacum L*	B, Me	Leaves M	Ucini Manganelli et al., 2001; Byayu et al., 2000
-	*Datura metel L.*	Di	fruits R	Ali, 1999
-	*Capsicum annuum L.*	Di	Fruits M	Ali, 1999; BAIF, 1999
-	*Solanum capsicoides All. Masika & Afolayan 2003*	Me	Fruit M	Byayu et al., 2000
Tiliaceae	*Triumfetta sp.*	Di	roots R	Minja, 1994
Urticaceae	*Laportea aestuans L Chew*	U	Leaves I	Lans and Brown 1998a ; b; Lans et al., 2001
Verbenaceae	*Lantana camara L .*	Me	Leaves MA	Tabuti et al., 2003
-	*Vitex negundo L.*	Me	R leaves	.
-	*Lantana trifolia L.*	B, Me	LeavesM	Mbarubukeye, 1992; Byayu et al., 2000
-	*Stachytarpheta jamaicensisL. Vahl. Masika & Afolayan 2003*	Me	Leaves I	Lans et al., 2000; Lans and Brown 1998a ; b; Lans et al., 2001
Vitaceae	*Cissus quadrangularis L. Masika & Afolayan 2003*	Di, S, Me	Roots, shoot MA, M	Byavu et al., 2000
Zingiberaceae	*Curcuma longa L.*	Me	Roots I	Lans and Brown 1998a ; b; Lans et al., 2001

Veterinary criteria systems: REP: reproductive ; Me: metabolic, Di: digestive , N: neurologic, C: cardiovascular, B: blood, S: skin, RESP: respiratory , U: urinary. Preparation modes: D: decoction; M:mix with other ingredients; J: juice; R: raw; I: infusion; MA: maceration; O: oil

Table 2. Inventory of main tropical vegetal resources potentially useful for external use in veterinary medecine[2]

Botanical family	Scientific name	Pathological indications	Preparation mode	References
Acanthaceae	*Justicia adhatoda L*	S	Leaves R	Ali (1999)
Alliaceae	*Allium cepa L.*	S	Leaves R	(Jamir et al., 1999)
Amaranthaceae	*Amaranthus spinosus L.*	Me	Roots R M, leaves R and roots M	Byavu et al. (2000)
Anacardiaceae	*Mangifera indica L.*	S, B	Leaves	(Rangnekar, 1994)
Annonaceae	*Annona squamosa L.*	S	Leaves, seeds R	BAIF (1999); Rangnekar (1994)
Apocynaceae	*Nerium oleander L.*	S	Dried flowers	Uncini Manganelli et al. (2001)
Araceae	*Caladium bicolor Ait. Vent*	S	D	BAIF (1999)
Arecaceae	*Elaeis guineensis L.*	S	No precision	Hounzangbé-Adoté 2003
Asclepiadaceae	*Calotropis gigantea May.*	S, L, Me	Roots and bark D, latex and leaves R	Ali (1999); Abbas et al. (2002); Rangnekar (1994); Reddy et al. (1998) ; (Al-Qarawi et al., 2001)
-	*Asclepia curassavica L.*	S	Flowers and leaves R	Lans and Brown (1998a ; b); Lans et al. (2001)
Asphodelaceae	*Aloe vera L.*	S	Pulp R, M	Lans and Brown (1998a; b); Lans et al. (2001); Personal survey
Asteraceae	*Achillea millefolium L.*	S, Me	Leaves M	Uncini Manganelli et al. (2001)
-	*Bidens pilosa L.L.*	S	Leaves R	Byavu et al. (2000)
-	*Tridax procumbens L.*	Me	Whole plant D M	Byavu et al. (2000)
-	*Eclipta prostata L.*	S	Leaves R	Lans et al. (2000); Lans et al. (2001)
-	*Neurolaena lobata L.L.*	S	Leaves	Personal survey
-	*Conyza bonariensis L.Cronq.*	S, Me	Leaves R	Minja (1994)
Bignoniaceae	*Crescentia cujete L.L.*	S	Pulp	Lans et al. (2000); Lans et al. (2001); Personal survey
Bixaceae	*Bixa orellana L.*	S	Seeds R	Lans et al. (2000); Lans et al. (2001)
Boraginaceae	*Cordia curassavicaJacq. R&S.*	S	Leaves M	Lans et al. (2000); Lans et al. (2001)
Brassicaceae	*Lepidium sativum L.*	Me	Latex	Abbas et al. (2002)
Cactaceae	*Nopalea cochenillifera L.*	S	Leaves R	Lans and Brown (1998a; b); Lans et

[2] Veterinary criteria systems: REP: reproductive ; Me: metabolic, L: locomotive , B: blood, S: skin, Oc: ocular. Preparation modes: D: decoction; M:mix with other ingredients; J: juice; R: raw; I: infusion; MA: maceration; O: oil

Botanical family	Scientific name	Pathological indications	Preparation mode	References
	Salm-Dyc			al. (2001)
Caesalpiniaceae	*Senna alata L.L.*	S	Leaves	Abbas et al. (2002); Hounzangbé-Adoté 2003
-	*Senna tora L. Roxb*	S	Heated leaves	Abbas et al. (2002); BAIF (1999)
-	*Cassia occidentalis L.*	REP, Me	I ,D ,M	Abbas et al. (2002); Byavu et al. (2000); Lans and Brown (1998a; b); Lans et al. (2001)
Chrysobalanaceae	*Chrysobalanus icaco L.subsp*	S	No precision	Lans et al. (2001)
Clusiaceae	*Mammea americana L.*	S	Bark; dried seeds M	BAIF (1999); Lans et al. (2000); Lans et al. (2001)
Crassulaceae	*Bryophyllum pinnatum Lam.*	S, Me	Heated leaves	Lans and Brown (1998a ; b); Lans et al. (2001)
Cucurbitaceae	*Momordica charantia L.*	S, Me	Fruit M, leaves D	Ali (1999); Personal survey
-	*Cucurbita citrullus L.*	S	Leaves J	Ali (1999)
Cupressaceae	*Cupressus sempervirens L.*	S, L	Bark D M	Uncini Manganelli et al. (2001)
Euphorbiaceae	*Euphorbia turicalli L.*	B, Me	Latex, roots	Tabuti et al. (2003); Mbarubukeye (1994) Minja (1994)
-	*Ricinus communis L.*	S	Seeds O	Reddy et al. (1998)
-	*Manihot esculenta CrantzCrantz.*	Me	Leaves and roots MA M	Byavu et al. (2000)
-	*Croton astroites Dryand*	S	D	Personal survey
Fabaceae	*Abrus precatorius L.*	Oc	Seeds	Tabuti et al. (2003)
-	*Cajanus cajan L. Huth*	S	Leaves	Lans et al. (2001)
-	*Arachis hypogaea L.*	S	Plant ash	Alawa et al. (2002)
-	*Indigofera sp.*	S	Whole plant R, roots ethanolic extraction	Minja (1994); Personal survey
Lamiaceae	*Ocimum gratissimum L.*	Oc, S	Leaves and seeds	Minja (1994)
Lauraceae	*Cassytha filiformis L.*	Oc, Me	Whole plant J	Minja (1994)
-	*Ocotea eggersiana Mez*	S	D	Personal survey
Malvaceae	*Hibiscus cannabinus L.*	S	Leaves R	Byavu et al. (2000)
-	*Sida rhombifolia L.*	S	Whole plant M	Personal survey
Meliaceae	*Melia azedarach L.*	B, S	D, alcoholic extract	Masika and Afolayan (2003); Akthar et al. (2000)

Botanical family	Scientific name	Pathological indications	Preparation mode	References
-	*Azadiracta indica L.*	S	Bark I, M	Tabuti et al. (2003); Lans and Brown (1998a; b); Lans et al. (2001); Reddy et al. (1998)
-	*Khaya senegalensis (Desr.) A. Juss.*	S	bark D, M	Alawa et al. (2002)
-	*Cedrela odorata L.*	S	Leaves	Lans and Brown (1998a; b); Lans et al. (2001)
Menispermaceae	*Cissampelos pareiraL. Var arbiculata*	Me	Plant I, M	Minja (1994)
Mimosaceae	*Acacia nilotica L.*	B, S, Me	Stem and bark M , plant tannins M	(Adewunmi et al., 2001); Bâ (1996)
Musaceae	*Musa x paradisiaca L.L.*	S	Fruit peelings M, stem latex	Byavu et al. (2000); Lans et al. (2000); Lans and Brown (1998a; b); Lans et al. (2001)
Myrtaceae	*Eugenia uniflora L*	B	Ethyl acetate fraction	Adewunmi et al. (2001)
-	*Syzygium cumini L.*	S	Stem and bark D M	Ali (1999)
Myrtaceae	*Psidium guajava L.*	REP	D	Minja (1994)
Nyctaginaceae	*Boerhavia diffusa L.*	S	Roots R	Ali (1999)
Oleaceae	*Jasminum fluminense Vell.*	Me	Leaves, roots and bark	Minja (1994)
Phytolaccaceae	*Petiveria alliacea L.*	S	Leaves	Lans and Brown (1998a; b) ; Lans et al. (2001); Personal survey
Plantaginaceae	*Plantago major L.P.virginica sensu Duss,non L.*	S	Leaves R M	Uncini Manganelli et al. (2001)
Poaceae	*Zea mays L. Sp.*	S	Fruits and rye flour	Uncini Manganelli et al. (2001)
-	*Sorghum bicolor L. Moench*	S, B	Seeds R	Byavu et al. (2000)
-	*Bambusa vulgaris Schrad*	S	Leaves R, M	Lans et al. (2000); Lans et al. (2001)
Rutaceae	*citrus limonL.Burm.f.*	S	Fruit J M	Reddy et al. (1998)
Sapindaceae	*Dodonea viscosa*	S, L	Leaves R, M ; shoots M	Abbas et al. (2002); Reddy et al. (1998); Minja (1994)
Sapotaceae	*Manilkara zapota L. P. van Royen*	S	Dried seeds add to coconut O	Lans et al. (2000); Lans et al. (2001)
-	*Pouteria sapota Jacq. H. Moore & Stear*	S	Seeds	Lans et al. (2001)
Scrophulariaceae	*Verbascum thapsus L.*	REP	Heated leaves M	Uncini Manganelli, et al. (2001)
-	*Scoparia dulcis L.*	S	leaves R, stems and leaves MA	Lans et al. (2000); Lans et al. (2001)

Botanical family	Scientific name	Pathological indications	Preparation mode	References
Solanaceae	*Nicotiana tabacum L*	S, Oc	Dried leaves MA, leaves and green fruit MA M; green leaves J M, MA.	Uncini Manganelli et al. (2001); Byavu et al. (2000)
-	*Datura metel L.*	S	Whole plant	Reddy et al. (1998)
-	*Capsicum frutescens L.*	Me, Oc	Leaves J M	BAIF (1999); Byavu et al. (2000)
Zingiberaceae	*Curcuma longa L.*	S	Roots heated M	Lans and Brown (1998a ; b); Lans et al. (2001)
	Renealmia alpina Rottb. Mass	S	Leaves	Lans and Brown (1998a; b)

Veterinary criteria systems: REP: reproductive ; Me: metabolic, L: locomotive , B: blood, S: skin, Oc: ocular.

Preparation modes: D: decoction; M:mix with other ingredients; J: juice; R: raw; I: infusion; MA: maceration; O: oil

Table 3. Inventory of main vegetal resources potentially useful for anthelmintic use in veterinary medicine[3]

Family	Scientific name	Medicinal application or disease treated/ study	Preparation mode	References
Acanthaceae	*Ruellia tuberosa L.*	Ruminants AH	Roots or leaves D	Lans and Brown (1998a ; b); Lans et al. (2001)
Alliaceae	*Allium cepa L*	AH	Leaves J	Jamir et al. (1999)
-	*Allium sativum L.*	*Ascaris lumbricoides* moderate activity *in vitro*, anthelminthic	Fruit MA	Akthar et al. (2000) ; Uncini Manganelli et al. (2001) ; Blanco et al. (1999); SBSUA 2004; Minja (1994)
Anacardiaceae	*Anacardium occidentale L*	*Taenia*, earthworm	Eo	Akthar et al. (2001)
-	*Mangifera indica*	*Ascaris lumbricoides*, worms , *H. contortus* in vitro on adults no effective	Seeds D M; *H. contortus*: aqueous extract	Akthar et al. (2001); Reddy et al. (1998); Sharma et al. (1971)
Annonaceae	*Annona squamosa L.*	Strongyloids, *H. contortus, Trichostrongylus colubriformis, Strongyloides papillosus in vivo* low activity in goats, *Oesophagostomum in vivo* 51.96% activity in goats	No precision	(Githiori et al., 2003); (Vieira et al., 1999)
-	*Artabotrys odoratissimus R.Br*	*taenia, ascaris*	Leaves Eo	Akthar et al. (2000)
-	*Polyathia longifolia Sonnerat Thwait*	AH	Fruit and leaves D	Jamir, Sharma, et al. 1999
Apiaceae	*Daucus carota L.*	Worms	Seeds Eo	Rangnekar 1999; Duval 1997; SBSUA 2004
Apocynaceae	*Rauvolfia viridis Willd. exR.&S.*	Bovines and ovins worms	Leaves and stems D	Personal survey
-	*Tabernaemontana citrifolia L. L.*	Worms	Leaves D M	Personal survey
Araceae	*Amorphophallus paenifolius D.H.Nicolson*	AH	Stems D	Jamir, Sharma, et al. 1999
Arecaceae	*Areca catechu L.*	AH	Seeds	Lans 2001; Duval 1997
-	*Cocos nucifera L.*	Dogs' AH	Fruit jelly	Lans 2001
-	*Rostonea oleracea*	Ovines AH	Bark	Personal survey

[3] Veterinary criteria systems: AH: anthelmintic

Preparation modes: D: decoction; M:mix with other ingredients; J: juice; R: raw; I: infusion; MA: maceration; O: oil; Eo: essential oil

Family	Scientific name	Medicinal application or disease treated/ study	Preparation mode	References
	Jacq.O.F.Cook			
Asclepiadaceae	*Calotropis gigantea May.*	*Oesohagostomum columbianum, Bunostomum trigonocephalum* of sheep, *H. contortus in vivo, in vitro* on *larvae*, lowers mobility of mature *H cont*, proteolitic enzyme calotropain	Latex	Akthar et al. (2000); Sharma et al. (1971)(Al-Qarawi et al., 2001)
Asphodelaceae	*Aloe ferox Mill.*	AH	I	Masika and Afolayan (2003)
-	*Aloe vera L.*	AH	Leaves jelly alone or M	Personal survey
Asteraceae	*Achillea millefolium L.*	Worms	No precision	(Duval, 1997)
-	*Ageratum conyzoides L.*	Tapeworms, very efficient	Eo	Akthar et al. (2000)
-	*Artemisia absinthium L.*	Bovines worms, AH	Aerial parts and flowers M	Uncini Manganelli et al. (2001); Duval (1997); SBSUA 2004
-	*Artemisia vulgaris*	*Haemonchus contortus* eggs, *Bunostomum, protostrongylus* effective	Whole plant R	Duval (1997); SBSUA 2004
-	*Calendula officinalis L*	Worms	.No precision	Duval (1997)
-	*Chrysanthemum cinerariifolium L.*	Horse strongylose, poultry *ascaris*	Whole plant R	Duval (1997)
-	*Eupatorium triplinerve Vahl.*	*Taenia, ascaris*	Flowers Eo	Akthar et al. (2000)
-	*Neurolaena lobata L.*	Bovines and ovins AH	Leaves D	Personal survey
-	*Tanacetum vulgare*	*Ascaris*, oxyures, anthelminthic, nematodes	*Ascaris*: flowers I; AH, nematodes: aerial parts O	Duval (1997); SBSUA 2004 Personal survey
Bombacaceae	*Ceiba pentandraL. Gaertn.*	Ovine AH	Bark	Personal survey
Brassicaceae	*Brassica juncea L.*	Cattle worms	Seeds	SBSUA 2004
-	*Sinapsis arvensis L*	Worms, AH	Whole seeds or O	Duval (1997); SBSUA 2004
Bromeliaceae	*Ananas sativus Schult f.*	Bromelain: in vitro *H. contortus*, strongyloids, *trichostrongylids in vivo, H. contortus in vivo*	aqueous or alcoholic extract of whole plant. *In vivo*: leaves 70% EtOH extract or young fruit EtOH extract	Akthar et al. (2000); Githiori et al. (2004); (Satrija et al., 2001); (Hordegen et al., 2003)
Caesalpiniaceae	*Senna alata L.L.*	Dog AH	Leaves D	Lans et al.(2000); Lans et al. (2001)
-	*Hymenaea Courbaril L.*	*In vivo* assays on*: H. contortus, T.colubriformis, O. columbianum, S. papillosus.*	.	Vieira et al. (1999)

Family	Scientific name	Medicinal application or disease treated/ study	Preparation mode	References
Cannabaceae	*Cannabis sativa L.*	Worms	No precision	Duval (1997)
Caricaceae	*Carica papaya L.*	*Ascaridia galli in vitro , oxyures, trichocephales, ascaris lumbricoides, Ascaris suum in vivo 80-100% activity,* gastro intestinal strongyles *in vivo, in vitro Haemonchus contortus, Oeasophagostomum, Trichostrongylus*	Leaves, fruits, latex. Aqueous extract or whole seeds. Roots MA. Ovines, poultry strongyles: dried seeds	Akthar et al. (2000); Lans et al. (2000); Lans et al. (2001); SBSUA 2004; Satrija et al. (2001); (Hounzangbe-Adote et al., 2005; Houzangbe-Adote et al., 2001); (Mpoame and Essomba, 2000);Fajimi and Taiwo (2005); Personal survey
Chenopodiaceae	*Chenopodium ambrosioides L.*	*Ascaris, oxyures, H. contortus in vivo, in vitro*: eggs. Medium activities	O	Blanco et al. (1999); BAIF (1999); Lans et al. (2000); Lans et al. (2001); Reddy et al. (1998); (Villasenor et al., 1997); (Tamboura et al., 1998); SBSUA 2004 ; (Ketzis et al., 2006)
Cucurbitaceae	*Cucurbita pepo L.*	*Haemonchus contortus in vivo* in goat, *in vitro* effective on adults; nematodiasis in calves i*n vivo*	whole seeds, *in vitro* : aqueous extract	Rangnekar (1994); (Pradhan et al., 1992); Sharma et al. (1971); Akthar et al. (2000)
-	*Lagenaria siceraria*	Worms, cestodes	Seeds powder, methanol, aqueous extracts	Akthar et al. (2000)
-	*Momordica charantia L.*	*Ascaris galli, in vitro*. Lowers mobility of mature *H.contortus, in vitro* on adultes effective, *in vivo* non efficacy on gastro intestinal nematodes	*In vitro* on *Haemonchus contortus*:aqueous extract of seeds	Akthar et al. (2000); Sharma et al. (1971); Vieira et al. (1999); Sharma et al. (1971)
Cyperaceae	*Cyperus rotundus L.*	*Taenia*, good activity on earthworms and tapeworms	Eo	Akthar et al. (2000)
Euphorbiaceae	*Euphorbia tirucalli L.*	Dog verminose	No precision	Abbas et al. (2002)
-	*Manihot esculenta CrantzCrantz*	Goat nematodes , *in vivo H.contortus*	Fresh leaves	(Sokerya and Preston, 2003a; Sokerya and Rodriguez, 2001) (Nguyen et al., 2003) ; Pamo et al.(2006)
-	*Ricinus communis L.*	Rabbits AH. *Dracunculus medinensis* infestation	R leaves. *Dracunculus medinensis*: roots.	Personal survey Minja (1994)
Fabaceae	*Cajanus cajan L. Huth*	AH	Leaves mixed with other ingredients or aerial parts D	Lans et al. (2000); Lans et al. (2001)
	Canavalia brasiliensis L.	*In vivo* assays on*: H. contortus, T.colubriformis, O. columbianum, S. papillosus*	Seed J	Vieira et al. (1999)

Family	Scientific name	Medicinal application or disease treated/ study	Preparation mode	References
Lamiaceae	*Ocimum gratissimum*	*H. contortus in vitro* low activity: 15% paralysis, chicken *A.Galli* in vivo very efficient 55.8%	Leaves D	(Njoku and Asuzu, 1998)
Loganiaceae	*Spigelia anthelmia L.*	*H. contortus* eggs, *larvae* good activity for ethyle acetate and methanol extracts	Ethyle acetate, methanol, chloroforme, hexan extracts	(Assis et al., 2003)
Malvaceae	*Gossypium barbadense L*	Dog AH very efficient	Leaves D	Lans et al. (2000); Lans et al. (2001)
Meliaceae	*Azadiracta indica L.*	Worms, strongyloids *in vivo* no activity, *in vitro* 35-53%activity, *Angiostrongylus cantonensis* and *Trichinella spiralis* no activity on both nematodes, gastro-intestinal nematodes of bovines *in vivo* reduce EPG	R leaves. Dogs, ruminants: leaves I. Strongyloids: seeds 70% ethanol extract in vivo and in vitro	Lans et al. (2000); Lans and Brown (1998a ; b); Lans et al. (2001); Githiori et al. (2003); SBSUA 2004; Hordegen et al. (2003); (Maki et al., 1997); (Pietrosemoli et al., 1999)
-	*Khaya senegalensis (Desr) A. Juss.*	AH , moderate effects on *Haemonchus, Cooperia, oesophagostomum Trichostrongylus*	Bark D with other plants and ingredients	Alawa et al. (2002)(Fajimi and Taiwo, 2005)
-	*Melia azedarach L.*	Goat intestinal nematodes, Haemonchus contortus in vitro test 90% mortality for fresh leaves and very little toxic effect for dry leaves, ascaris, oxyures, ankylostomes, Taenia canina, paramphistomum cervi, strongyloids in vivo, low activity	*H.contortus*: aqueous or alcoholic extract, strongyloids: seeds 70% Ethanol extract *in vivo*. Nematodes: Ethanol extract *in vitro*	Akthar et al. (2000); Githiori et al. (2003); (SBSUA, 2004); Hordegen et al. (2003); Githiori et al. (2003); (Sangwan and Sangwan, 1998)
Mimosaceae	*Acacia nilotica L*	Haemonchus contortus in goats in vivo non significative efficacy 10%	Dried leaves	(Kahiya et al., 2003)
-	*Leucaena leucocephala L.*	Ascaris suum of pigs in vitro	Seeds chloroform extract	Vilasenor et al. (1997)
Musaceae	*Musa x paradisiaca L.*	Haemonchus contortus : lowers mobility of mature worms; in vivo effect on EPG	Roots aqueous extract, leaves R	Sharma et al. (1971) Morton (1981) (Sokerya and Rodriguez, 2001); Vieira et al. (1999); Personal survey
Myrtaceae	*Callistemon viminalis*	*taenia, ascaris*. More efficient than piperazine phosphate *in vitro* on earthworms and tapeworms, same activity as hexylresorcinol on hookworms	Eo	Akthar et al. (2000)
-	*Psidium guajava L.*	AH	Leaves	Minja (1994)
Oleaceae	*Olea europaea L.*	*H. contortus in vivo* low activity	No precision	Githiori et al. (2003)

Family	Scientific name	Medicinal application or disease treated/ study	Preparation mode	References
Phytolaccaceae	*Petiveria alliacea L.*	ruminants AH	Roots	Lans and Brown (1998a ; b); Lans et al. (2001)
Piperaceae	*Piper betle L.*	tapeworms, hookworms , earthworms: best activity than piperazine phosphate on tapeworms, better than hexylresorcinol on hookworms.	Eo	Akthar et al. (2000)
Poaceae	*Cymbopogon citratus L.*	moderate activity on earthworms	Eo	Akthar et al. (2000)
Punicaceae	*Punica granatum L.*	*Ascaris lumbricoides*: moderate activity of bark, *H. contortus* alcoholic extract: dose dependant inhibition of the transformation egg/larva. Efficacy on sheep intestinal nematodes EPG :fruit bark: 85%; aqueous extract:80%. Anticestodes activity: fruit bark:76%; aqueous extract 77%; glycosides fraction 225mg/kg, 6+-2%; alcaloides à 225mg/kg, 95+-12%. Nematicidal activity in calf.	Fruit and bark alcoholic or aqueous extract	Akthar et al. (2000); Pradhan et al. (1992)
Rubiaceae	*Neolamarckia cadambaRoxb. Bosser*	*in vitro* efficacy on *Ascaridia galli*	Bark	Akthar et al. (2000)
-	*Randia dumetorum Lam*	variable activity on worms,tapeworms	Seeds	Akthar et al. (2000)
Rutaceae	*Citrus aurantium L.*	worms	Fruit J M	Personal survey
-	*Citrus medica*	*A. lumbricoides* moderate activity	Bark	Akthar et al. (2000)
Sapindaceae	*Dodonea viscosa*	verminose*, H. contortus* low activity in vivo	No precision	Mbarubukeye (1992); Githiori et al. (2003)
Solanaceae	*Datura metel L.*	*Ascaridia galli*	Whole plant	Akthar et al. (2000)
-	*Nicotiana tabacum L.*	worms, endoparasites, Verminose	Leaves MA M	Rangnekar (1994); Duval (1997); Uncini Manganelli et al. (2001); Byavu et al. (2000)
Verbenaceae	*Lantana camara L.*	worms, good AH	Eo	Akthar et al. (2000)
-	*Stachytarpheta jamaicensisL. Vahl.*	ruminants AH	Leaves I	Lans et al. (2001)
Zingiberaceae	*Hedychium coronarium*	worms, best activity than piperazine phosphate on earthworms, tapeworms but less than hexylresorcinol on hookworms and nodule worms	Roots Eo	Akthar et al. (2000)
-	*Zingiber officinale Roscoe*	*A. lumbricoides*	Roots	Akthar et al. (2000)

Veterinary criteria systems: AH: anthelmintic. Preparation modes: D: decoction; M:mix with other ingredients; J: juice; R: raw; I: infusion; MA: maceration; O: oil; Eo: essential oil

Table 4. Egg Hatch Assay results

Plant part	Treatment	Efficacy %	P/alb	P/PBS
	Albendazole	79.38	-	***
	PBS	10.99	***	-
C. papaya seed	A	49.87	***	***
	M	19.84	***	*
	D	3.88	***	0.18
A. muricata seed	A	53.41	***	***
	M	54.83	***	***
	D	7.66	***	0.99
R. viridis leaf	A	14.29	***	0.94
	M	15.74	***	0.92
	D	7.89	***	0.98
M. charantia leaf	A	34.37	***	***
	M	4.63	***	0.88
	D	34.28	***	***
C. ambrosioides aerial part	A	22.07	***	0.103
	M	28.84	***	*
	D	30.27	***	***
C. cujete pulp	A	35.29	***	***
	M	15.63	***	0.996
	D	34.39	***	***
N. lobata leaf	A	35.38	***	***
	M	17.19	***	0.98
	D	0.965	***	0.56
B. pilosa aerial part	A	32.61	***	***
	M	18.25	***	0.96
	D	22.09	***	0.5
L. camara aerial part	A	20.07	***	0.63
	M	19.48	***	0.87
	D	14.79	***	0.998
S. alata leaf	A	7.81	***	0.96
	M	27.42	***	***
	D	8.8	***	0.99
M. paradisiaca leaf	A	6.14	***	1
	M	7.03	***	0.9971
	D	7.839	***	0.9557
T. citrifolia leaf	A	2	***	1
	M	1.6	***	1
	D	6.5	***	0.96
C. moschata seed	A	7	***	0.48
	M	19.6	***	***
	D	1.9	***	1
M.esculenta leaf	A	12.35	***	0.81
	M	7.78	***	0.97
	D	13.77	***	0.48

P/alb: P value for comparison between treatment and albendazole; P/PBS: P value for comparison between treatment and PBS; *P<0.05; **P<0.01; ***P<0.0001

Table 5. Larval Development Assay results

Plant parts	Treatment	Efficacy %	P/alb	P/PBS
	Albendazole	89.63	-	***
	PBS	28.55	***	-
C. papaya seed	A	82.89	0.98	***
	M	62.31	**	***
	D	54.81	***	***
A. muricata seed	A	78.04	0.87	***
	M	81.11	0.99	***
	D	60.72	*	**
R. viridis leaf	A	69.8	*	***
	M	53.63	***	***
	D	47.78	***	**
M. charantia leaf	A	70.25	0.2523	***
	M	51.74	**	0.15
	D	17.63	***	0.85
C. ambrosioides aerial part	A	43.87	***	0.58
	M	62.26	0.09	**
	D	63.33	0.095	**
C. cujete pulp	A	51.91	***	*
	M	45.63	***	0.45
	D	48.74	***	0.25
N. lobata leaf	A	53.95	***	**
	M	57.94	**	**
	D	40.51	***	0.75
B. pilosa aerial part	A	52.66	***	**
	M	60.94	*	**
	D	72.5	0.44	***
L. camara aerial part	A	52	***	*
	M	54.58	**	*
	D	66.78	0.1195	***
S. alata leaf	A	98.2	0.956	***
	M	82.03	0.9848	***
	D	58.08	**	**
M. paradisiaca leaf	A	25.8	***	***
	M	70.68	***	***
	D	9.49	***	0.93
T. citrifolia leaf	A	85	0.76	***
	M	76	**	***
	D	33.5	***	1
C. moschata seed	A	97	0.52	***
	M	93.6	0.98	***
	D	94	0.97	***
M.esculenta leaf	A	8.35	***	0.33
	M	42.41	***	***
	D	1.01	***	0.51

P/alb: P value for comparison between treatment and albendazole; P/PBS: P value for comparison between treatment and PBS; *P<0.05; **P<0.01; ***P<0.0001

Article n°2

In vitro effects of *Cucurbita moschata L.* seed extracts on *Haemonchus contortus*

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Abstract

This study was carried out to evaluate the *in vitro* effect of *Cucurbita moschata L.*seed against the parasitic nematode of small ruminants *Haemonchus contortus*. Three extracts (aqueous, methanolic and dichloromethane) of *Cucurbita moschata L.*seed were tested *in vitro* on four developmental stages of *Haemonchus contortus* using egg hatch assay (EHA), larval development assay (LDA), L3 migration inhibition assay (LMI) and adult worm motility assay (AWM). The highly significant ($P< 0.001$) ability to stop larval development (inhibition > 90% for each extract) and the negative effect of the dichloromethane and methanolic extracts on adult worm motility (inhibition of motility >59.2% after 24 hours of incubation) compared to the negative controls, suggest anthelmintic properties of *Cucurbita moschata L.*seed against *Haemonchus contortus*. The active principles responsible for the activity could be secondary metabolites such as amino acid compounds or terpenoid compounds present in the extracts.

Keywords: Gastrointestinal nematodes; Egg hatching; Larval development; L3 migration; Adult worm motility

1. Introduction

Gastrointestinal parasitism with nematodes (especially *Haemonchus contortus*) has been classified as a major health and welfare problem for small ruminants, particularly in the tropics (Aumont et al., 1997; Chandrawathani et al., 1999; Geerts and Dorny, 1996; Hoste et al., 2005; Jabbar et al., 2006a; Lefevre et al., 2003; Wolstenholme et al., 2004)**.** Parasitism with gastrointestinal nematodes impairs animal health and welfare and productivity since the presence of worms results in increased death rate and poor growth and reproduction (Aumont et al., 1997; Coop et al., 1982; Dakkak, 1995; Hoste et al., 2005). To date, the usual mode of control of gastrointestinal parasitism relied on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Aumont et al., 1997; Jabbar et al., 2006b; Jackson and Coop, 2000; Wolstenholme et al., 2004) in gastrointestinal nematode (GIN) populations has motivated investigation into alternative approaches. Phytotherapy is one of these alternative approaches currently explored (Athanasiadou and Kyriazakis, 2004; Githiori et al., 2006; Wolstenholme et al., 2004). Phytotherapy is mainly based on the use of preparations of fresh leaves and seed. In previous studies and data from the ethnoveterinary literature, *Cucurbitaceae* seed is indicated as a potential taenifuge and a schistosomicidal preparation (Duez et al., 1988; Mihranian and Abou-Chaar, 1968; Schenkel et al., 1992; Sun et al., 1961). Nevertheless, only a few studies have reported on the efficacy of the *Cucurbitaceae* against nematodes. Beloin (2005) reported an anthelmintic activity against *Caenorhabditis elegans* at 500µg/ml. Sharma (1971) tested water extract of *Cucurbita pepo* and *Momordica charantia L.* seed on adult *Haemonchus contortus* and found a significant activity at 1% level with a 1 in 50 concentration.

The anthelmintic activity of *Cucurbitaceae* is attributed to secondary metabolites, yet to be identified. The suspected secondary metabolites for anthelmintic activity in *Cucurbitaceae* seed are: a triterpenic compound named Cucurbitacin B, a non proteic amino acid named Cucurbitin (3-amino-pyrrolidine-3-carboxylic acid), saponins and sterols (TRAMIL, 1999, Mihranian and Abou-Chaar, 1968), but other compounds might not be excluded such as cucurmosin, a ribosome-inactivating protein present in the sarcocarp of the pumpkin but also in the seed (Morton, 1981). Secondary metabolites may be extracted from the raw plant material by different solvents according to the polarity of the molecules to be extracted. For example hot water will extract heterosides, iridoids and tannins (Balansard et al., 1991). Methanol will extract tannins, catechins, terpenoids for example, and a more apolar solvent such as dichloromethane will extract semi polar and more apolar compounds (Balansard et al., 1991).

Several methods are commonly used for testing nematicidal activity of both chemical drugs and plant extracts. Amongst them, *in vitro* assays have been revealed to be relevant and cheaper than *in vivo* methods. The Egg Hatch Assay (EHA), (Hubert et Kerboeuf, 1984) and Larval Development Assay (LDA), (Hubert et Kerboeuf, 1992) are currently used for the detection of anthelmintic resistance in GIN (Coles et al., 1992). The Larval Migration Inhibition assay (LMI) (Wagland et al. 1992, Rabel et al. 1994) and the Adult Worms Motility Assay (AWM) (Hounzangbe-Adote et al., 2005) allow a more realistic evaluation of the *in vivo* nematicidal activity. These four tests are based on the hypothesis that a nematicidal activity observed *in vitro* would be indicative for potential *in vivo* activity.

In the present study, an experiment was performed to test the efficacy of the commonly grown tropical *Cucurbita moschata L.*against the GIN *H. contortus*. Three different extraction solvents were used to evaluate the large range of secondary metabolites present in the seed, and all four *in vitro* tests were performed to increase the chances of detecting anthelmintic activity.

2. Materials and methods

2.1. Plant extracts

Plant materials were collected in Guadeloupe (French.West. Indies), at the beginning of the dry season. The seeds were removed from mature fruits and dried at 55°C in a ventilated oven, until a constant weight was reached. The seeds were then ground.

Thereafter three extractions were performed on the ground seed, either with water, methanol or dichloromethane, in order to extract most of the polar and semi-polar molecules depending on the eluant.

First, 50 g of ground seed was extracted with 1L of hot distilled water: 500 ml of boiling water mixed with the ground seed, simmered and filtered through a Büchner, and then 500 ml of boiling water mixed with the seed residue and simmered again. The filtrates were collected together and lyophilised to obtain a powdered extract.

Then, 50 g of ground seed was lixiviated by 500 ml of solvent (methanol or dichloromethane): the ground seed was moistened with 150 ml of solvent to cover it, and then macerated for 3 h in dark.

Thereafter, the filtrate was removed while the rest of the solvent (350 ml) was poured on the residue. The filtrates were collected together and evaporated at low pressure at 40°C. All the extracts were stored, in the dark in dessicator, at 22°C.

2.2. In vitro anthelmintic assays

The anthelmintic efficacy tests of the 3 seed extracts on the different life-cycle stages of *H. contortus* were performed, using four different procedures.

For each assay, the eggs or adult nematodes were obtained from feces and abomasum of Black Belly donor lambs which were experimentally infected with oral administration of a pure aqueous suspension of 10 000 *H. contortus* third stage larvae (L3).

2.2.1. Egg hatch assay

The in vitro anthelmintic activity of the three extracts of *C. moschata* seed on the egg hatching of *H. contortus* was carried out according to a modification of the method used for testing anthelmintic resistance (Assis et al., 2003), after eggs were extracted from feces according to the method described by Hubert and Kerboeuf, (1984).

After crushing the feces in water, and successive siftings (500, 250, 125, 63, 50 and 30µm sieves), eggs were collected and centrifuged for 10 min at 2000 rpm. The supernatant was removed and a sodium chloride solution (density 1.2) was added. After homogenization, the mixture was centrifuged for 15 min at 3000 rpm. The floating eggs were then extracted by pouring the supernatant on a 32µm sieve and then by abundant washing first with distilled, and then with sterilised water.

The egg suspension with a concentration of 400 eggs per ml, was distributed in 24 multiwell plates (0.5 ml per well). Concentrations of seed extracts (2400, 1200, 600 and 300 µg/ml), and albendazole (0.5%, 0.25%, 0.125%, used as positive control) were diluted in PBS (pH 7.2) buffer and were added to the wells (0.5 ml per well). In addition, negative controls in PBS were also included in the assay. Five replicates were run per concentration.

After 48h incubation at 25°C, egg hatching was stopped by adding Lugol's iodine solution. The number of L1 larvae and egg per well was then counted using a reverse microscope (at 10X magnification). The percentage of hatched eggs was determined using the ratio: number of L1 / (number of eggs + number of L1).

2.2.2. Larval development assay

The objective of this assay was to test the *in vitro* efficacy of the seed extracts to inhibit the larval development of *H. contortus* from the L1 stage to the infective L3 stage. The assay used was a modification (Assis et al., 2003) of the technique described by Hubert and Kerboeuf (1992).

L1 and L2 stage larvae were obtained through culture and extraction of eggs from feces. The eggs were incubated at room temperature for 48 h in 24-multiwell plates (0.5 ml per well with a concentration of 400 eggs/ml).

After hatching, 70µl of culture medium (for one plate: 10µg of autolytic yeast extract, 1.5 ml of distilled water and 170 µl of Earle's balanced solution, the mixture was stabilised to pH= 7.2 with a sodium bicarbonate solution at 50 g/L) was added to each well, followed by either one of four concentrations of seed extract (2400, 1200, 600 and 300 µg/ml), or albendazole (0.5, 0.25, 0.125%), all diluted in PBS buffer, compared with a negative control of PBS (0.5 ml per well and five replicates for each dose).

The larvae were incubated for 8 days at room temperature (25°C), in order to permit the larval development from the first stage to the third infective stage. Thereafter, several drops of a Lugol's iodine solution were added and the number of larvae was counted using inverse microscope (at 10 X magnification) by separating L3 infective larvae from L1-L2 larvae. The percentage of development was calculated as the ratio: number of L3/ total number of larvae per well.

2.2.3. Larval migration inhibition assay

This test is aimed at evaluating the anthelmintic effect of the seed extracts on the migration capacity of the L3 larvae. This test was performed according to Rabel et al. (1994). *H. contortus* L3 were obtained by fecal culture. Eggs reached the L3 stage after 10 days. The L3 were then collected by sedimentation using Baermann's devices. The anthelmintic effect of each seed extracts was tested using 150, 300, 600 µg/ml concentrations of PBS. Negative (in PBS) and positive controls (levamisole at 0.125, 0.25 and 0.5%) were also prepared in PBS and incorporated to the assay. Five replicates were run for each seed extract and for the controls.

After 3 hours of incubation at room temperature (25°C), the larval suspensions were rinsed out with PBS buffer, and then, each suspension was poured in a Falcon® tube fitted with a 20µm sieve. After 3 hours of incubation, the inserts were removed and the volumes were adjusted to 2 ml by adding PBS.

Migrating larvae through the sieve (N= number of migrating larvae through the sieve) were counted in a total volume of 500µl (10 drops of 50µl) using optical microscope (at 40X magnification). The total number of migrated larvae was calculated as N x 4. The percentage of L3 that migrated was then calculated as (N x 4/ 1000) x100.

2.2.4. Adult worms motility assay

The purpose of this assay was to test the anthelmintic effect of the seed extracts on adult worm motility. This test was performed according to Hounzangbe-Adote et al. (2005).

Adult worms were collected from an experimentally infected lamb, four weeks after infection. Immediately after slaughtering, the abomasum was removed, opened and placed in 37°C saline. The mobile worms were rapidly collected and put into 24-multiwell plates, 3 worms per well in 2 ml of 37°C saline. After one hour of incubation (37°C, 5% carbon dioxide), the saline was removed from each well and replaced by 1 ml of seed extract at varying concentrations (1200, 600, 300, 150 and 75 µg/ml) or levamisole (0.125, 0.25, 0.5, 1%) or PBS. All dilutions were performed in PBS with penicillin/streptomycin. The incubation media were changed after 24 h. Adult worm motility was evaluated by observation under magnifying glass and evaluated as the ratio: number of motile worms/ total number of worms per well.

2.2.5. Phytochemistry

A qualitative thin layer chromatographic (TLC) analysis on silica gel was performed on each *C. moschata* seed extracts, in three different migration systems. System 1, for amino acids analysis, was n-butanol/acetic acid /water (6:2:2, v/v/v). System 2, for terpenoids analysis, was: chloroform/methanol (95:10, v/v); and system 3, for saponosides analysis, was: chloroform/acetic acid/methanol/water (64:32:12:8, v/v/v/v). Ninhydrin reagent was employed for the development of colour of amino acid spots in system1. Cucurbitin appears as a characteristic brown spot when reacting with ninhydrin reagent (Duez et al., 1998). Vanillin-sulfuric acid reagent was employed for revealing terpenoid spots (triterpens and saponins) in systems 2 and 3.

Vanillin- phosphoric acid reagent was employed for specific development of cucurbitacin spots in system 2, developing blue-violet or brown spots (Wagner and Bladt, 1996). Presence of amino acids, terpenoids, or saponisides was determined by presence of indicator color.

2.2.6. Statistical analysis

The data for EHA, LDA and LMI assays were analysed by multiple comparisons (Bonferroni method) using orthogonal contrasts with the SAS software. Regression was used for evaluation of dose response relationship using Minitab® Release 14. For the AWM assay, the data were analysed by the mixed GLM procedures using the Glimmix macro of the SAS software.

3. Results

3.1. Egg hatch assay

The water and dichloromethane seed extracts showed no effect on egg hatching compared to the negative control ($P > 0.10$). Conversely, the methanolic seed extract was observed to inhibit hatching (Table 1) compared with the control PBS ($P < 0.001$). Regression analysis indicates that there is a dose dependent effect of the methanolic extract ($P = 0.021$; $R^2 = 26.1\%$; regression equation: hatch inhibition = 86.4% – 10.7 dose). The positive and negative controls differed ($P < 0.001$).

3.2. Larval development inhibition

The extracts of *C. moschata* seed exhibited larval development inhibition at all concentrations and extraction methods examined ($P < 0.001$) compared with negative control (Table 2). Regression analysis indicated a dose dependent effect within the concentration ranges tested, for aqueous and dichloromethane extracts (Aqueous: $P = 0.023$; $R^2 = 25.5\%$; larval inhibition = 92.3% + 7.97 dose. Dichloromethane: $P = 0.008$; $R^2 = 32.9\%$; larval inhibition = 86.1% + 14 dose). Moreover, the percent inhibitions of the seed extracts yielded by different extraction methods were not different from the positive control.

3.3. Larval migration

The aqueous and methanolic extracts of seed inhibited larval migration of L3 compared to negative control ($P < 0.001$), but less so than levamisole ($P < 0.001$; Table 3). Regression analysis indicated an absence of a dose dependent relationship of methanolic and aqueous extracts of *C. moschata* seed on larval migration. The dichloromethane extract was not effective in inhibiting larval migration.

3.4. Adult worm motility

Levamisole was associated with reduced worm motility compared with the negative control ($P < 0.001$). Dichloromethane extract of *C. moschata* seed reduced worm motility relative to negative control after 24 hours (Table 4). Nevertheless, worm motility remained lower than the positive control. Nevertheless, no dose effect response was observed for the three seed extracts ($P>0.10$).

3.5. Phytochemistry

The TLC analysis indicates that the aqueous, methanol and dichloromethane extracts of *C. moschata* seed contained terpenoid and amino acid compounds. The aqueous and methanolic extract contained saponins. Cucurbitacin B was identified only in the methanol extract in system 2 with vanillin phosphoric reagent: Cucurbitacin B glucoside at $Rf \approx 0.29$ and Cucurbitacin B aglycone at $Rf \approx 0.9$.

The presence of Cucurbitin was detected in methanol and aqueous extracts in system 1 with ninhydrin reagent. It appeared as a characteristic brown spot ($Rf \approx 0.3$) in system 1. The spot was predominant in aqueous extract. Many amino acids compounds were detected in the three extracts, mainly in aqueous and methanolic extracts. The chemical composition of the different extracts according to this TLC assay is presented in Table 5.

3. Discussion and conclusion

Given the overall result, *C. moschata* seed has an *in vitro* anthelmintic property on H*. contortus*. Moreover the *C. moschata* seed anthelmintic activity was observed for each of the *H. contortus* developmental stages. The methanolic extract gave more consistent results. The literature is void of studies on *C. moschata* seed anthelmintic activity on *H. contortus*. One trial has been published on *Cucurbita pepo* seed for which the aqueous extracts were only effective in reducing the adult worm motility (Sharma et al., 1971). One of the limitations of that trial was that only the aqueous extract was tested, which limits the quality and the quantity of the potential active compounds present in the seed. The present trials confirmed previous studies with a result of a 9% reduction in worm motility with the aqueous extract after six hours. Even so, this value was not considered sufficient compared to the controls. Nevertheless the present trial indicates the aqueous extract was less effective than methanolic and dichloromethane extracts on reduction of adult worm motility. Perhaps because the aqueous extract was exposed to high temperature, potentially bioactive molecules could have been denatured and influenced any anthelmintic activity.
Cucurbita genus seed are known to contain several secondary metabolites of medicinal interest: a tetracyclic triterpenic compound Cucurbitacin B, a non proteic amino acid named Cucurbitin (3-amino-pyrrolidine-3-carboxylic acid), saponins and sterols (Mihranian and Abou-Chaar, 1968; Morton, 1981; TRAMIL, 1999). It also contains cucurmosin, a ribosome-inactivating protein (RIP). Our phytochemical analysis confirmed that *C. moschata* seed contained amino acids and terpenoid compounds which could be suspected for anthelmintic activity.

Non-proteic amino acids in plants are aimed at protecting seed from predators by intoxicating the predators with an anti-metabolite action due to interference between non-proteic and normal amino-acids during biosyntheses of proteins by the predator (Bruneton, 1999). This toxicity occurs because the enzymatic system of the predators cannot distinguish these non proteic amino acids from the normal amino- acids due to their isostery so that the proteins then biosynthetized by the predator could not be functional. The non proteic amino acid cucurbitin is well known for its potential anthelmintic properties against *Taenia* in humans and against the growth of the immature trematode *Schistosomas japonicum* (Paik et al., 2000; Sun et al., 1961).

Furthermore, the chemical structure of the cucurbitin compound is close to that of the nematicidal compound named kainik acid (Bruneton, 1999). Kainik acid has a neurodegenerative action on nematodes by substituting for glutamate. The non proteic amino acid cucurbitin could have this nematicidal mode of action on *H. contortus* because of its chemical structure (Bruneton, 1999).

RIPs are RNA N-glycosidases that inactivate ribosomes with a site-specific deadenylation of the large ribosomal RNA (Shi et al, 2003). They are also capable of inactivating many non ribosomal nucleic acid substrates.

Some RIPs were shown to have antitumor or antiviral activities (Shi et al., 2003). The cucurmosin RIP, potentially present in the seed, could also act on the nematodes by inhibiting the synthesis of proteins and stopping developmental stage.

Terpenoid compounds are known to be active against a large range of organisms. Some of them can be bioactive whereas others can affect physical variables. The triterpenic Cucurbitacins can be toxic, purgative compounds, involved in insect resistance (Aharoni et al., 2005; Bruneton, 1999; Louis, 2004). Others (Molan et al., 2000a; Molan et al., 2003) have shown that tannins and terpenoids reduced the mobility and the consequent migration ability of ovine nematode larvae. The synergy of several terpenoids can be effective on several targets because they are a complex mixture of compounds. Terpenoid compounds present in the seed might be active against nematodes and helminthes (Acamovic and Brooker, 2005; Hoskin et al., 1999) by using a synergy mode of action. Sterols can stop the development of insects (Singh et al., 1982; Louis, 2004). Furthermore, triterpenoids, saponins and sterols are all antibacterial, antimicrobic, anticarcinogenic and antifungic molecules (Aharoni et al., 2005). These complexities could enable the compounds to interact with multiple molecular targets on the various developmental stages of the parasite.

The different trials were performed in order to screen the activity and to search for the mode of action of compounds which have the ability to disrupt the life cycle of *H. contortus*. By examining the effects of the different methods of extractions and the results of the TLC, several hypotheses could be made for the compounds acting on the different stages of *H. contortus* development. The egg hatching could have been inhibited by some saponins, potentially present in the methanolic extract. These molecules are known to stop the *H. contortus* from egg hatching (Camurça-Vasconcelos et al., 2007; Eguale et al., 2007a, b). The larval development could have been inhibited by terpenoids, potentially present in the aqueous, methanolic and dichloromethane extracts. These molecules are known to stop *H. contortus* larval development (Maciel et al., 2006, Camurça et al., 2007).

The hypothesis of the implication of some amino acid compounds (cucurbitin, cucurmosin) involved in the activity against the development should be considered, taking into account their high concentration in the aqueous and methanol extracts observed on the TLC. The larval migration might also have been inhibited either by saponins (present mainly in the methanolic extract which was the most effective) and other triterpens, or by some amino acid compounds (cucurbitin, cucurmosin) potentially present in the aqueous and methanolic extracts. The adult worm motility could have been inhibited by the terpenoid compounds, mainly present in the dichloromethane extract. Examining TLC results and bioassays results, Cucurbitin and cucurbitacin B do not seem to be involved in the efficacy of the extract on the adult stage of *H. contortus*. Furthermore, the cucurmosin RIP, potentially present in the dichloromethane extract, could also act on adult worms by inhibiting the synthesis of proteins.

According to the polarity of methanol, the methanolic extract of *C.moschata* seed could contain a large spectrum of compounds. This could explain the multiple target activity (on egg hatching, larval development and L3 mobility) of this extract.

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Table 1: Least square means of hatching percentages for the different extracts of *Cucurbita moschata L.*seeds (Cms), compared to negative control PBS and to positive control albendazole, with details for dose dependent extract Cms M

Products	**% hatching**	**P value/ albendazole**	**P value/ PBS**
Albendazole	20.6	-	0
PBS	98.0	0.00	-
Cms A	93.0	0.00	0.48
Cms M	80.4	0.00	0.00
Cms M doses (mg/ml)			
0.15	92.5		
0.30	77.8		
0.60	75.0		
1.20	76.7		
Cms D	98.1	0.00	0.99

A: Aqueous; M: Methanol; D: Dichloromethane

Table 2: Development inhibition least square means (% L1-L2) for the different extracts of *Cucurbita moschata L.*seeds (Cms), compared to negative control PBS and to positive control albendazole, with details for dose dependent extracts Cms A and Cms D.

Products	**% (L1-L2)**	**P value/ albendazole**	**P value/ PBS**
Albendazole	92.0	-	0.00
PBS	26.4	0.00	-
Cms A	97.0	0.52	0.00
Cms A doses			
0.15			
0.30	87.8		
0.60	99.5		
1.20	100		
Cms M	100		
Cms D	93.6	0.98	0.00
Cms D doses	94.0	0.97	0.00
0.15			
0.30			
0.60	82.4		
1.20	93.3		
	100		
	100		

A: Aqueous; M: Methanol; D: Dichloromethane

Table 3: L3 migration least square means for the different extracts of *Cucurbita moschata L.*seeds (Cms), compared to negative control PBS and to positive control levamisole

Products	**%L3 migration**	**P value/Levamisole**	**P value/ PBS**
Levamisole	0	-	0.00
PBS	83.5	0.00	-
Cms A	65.7	0.00	0.00
Cms M	55.5	0.00	0.00
Cms D	82.0	0.00	0.99

A: Aqueous; M: Methanol; D: Dichloromethane

Table 4: Least square means of adult worm percentage of motility for the different extracts of *Cucurbita moschata L.*seeds (Cms), compared to negative control PBS and to positive control levamisole

Products	Incubation time (hours)								
	6h			**24h**			**48h**		
	%mot	**P1**	**P2**	**%mot**	**P1**	**P2**	**%mot**	**P1**	**P2**
Levamisole®	53.4	-	0.00	4.2	-	0.00	0.0	-	0.04
PBS	94.2	0.00	-	56.2	0.00	-	5.8	0.05	-
Cms A	91.0	0.00	0.74	69.6	0.00	0.35	0.0	0.99	0.12
Cms M	79.7	0.02	0.24	40.8	0.00	0.08	0.0	0.99	0.27
Cms D	88.8	0.00	0.67	31.1	0.00	0.00	0.0	0.99	0.27

P1: P value compared with Levamisole, **P2**: P value compared with PBS control.

A: Aqueous; M: Methanol; D: Dichloromethane

Table 5: TLC distribution of compounds and class of secondary metabolites of importance in the different extracts of *Cucurbita moschata L.*seeds (Cms), developed in the three different migration systems 1, 2 and 3.

Extracts	**Terpenoids**	**Cucurbitacin**	**saponosides**	**Amino-acids compounds**	**cucrbitin**
Cms A	+	-	+	++++	++++
Cms M	++	++	+++	+++	++
Cms D	+++	-	-	++	-

System 1 for amino acids analysis: n-butanol/acetic acid /water (6:2:2, v/v/v). Ninhydrin reagent.

System 2 for terpenoids analysis: chloroform/methanol (95:10, v/v). Vanillin-sulfuric acid reagent

System 3 for saponosides analysis: chloroform/acetic acid/methanol/water (64:32:12:8, v/v/v/v). Vanillin-sulfuric acid reagent. Vanillin-phosphoric acid reagent for specific development of cucurbitacin.

+: weak intensity spots.

++: normal intensity spots, until 4 compounds.

+++: mean intensity spots, six compounds per class (for Cms D only one spot but with strong intensity).

++++: strong intensity spots, more than 6 compounds per class.

Article n°3

In vitro effects of *Tabernaemontana citrifolia L.* extracts on *Haemonchus contortus*

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Abstract

Tabernaemontana citrifolia L. (Apocynaceae) is traditionally used as an anthelmintic preparation for ruminants in Guadeloupe (French West Indies). This study was carried out to evaluate the *in vitro* effect of this plant against the parasitic nematode of small ruminants *Haemonchus contortus*. Three extracts (aqueous, methanolic and dichloromethane) of *T. citrifolia* fruit, leaf and root were tested on four developmental stages of the parasite, using egg hatch assay (EHA), larval development assay (LDA), L3 migration inhibition assay (LMI), and adult worm motility assay (AWM). Compared to the negative control, significant effects were observed for the different parts of *T. citrifolia* but with differences depending on the parasitic stage; efficacies on the larval development of *H. contortus* from 88.9% to 99.8 % for fruit, from72.1% to 83.8% for root and from 33.5% to 85 % for leaf with dose dependent effect for the methanolic extract. The root gave the best result on EHA (22.7% efficacy for dichloromethane extract) and AWM (56% efficacy, with dose dependent effect for dichloromethane extract) and the leaf on LMI (49.4% efficacy).These results suggest that *T. citrifolia* possess anthelmintic activity against *H.contortus*. The active ingredients responsible for the activity could be the alkaloid compounds present in the plant parts of the plant.

***Keywords*: Gastrointestinal nematodes*;* Egg hatch assay; Larval development; L3 migration; Adult worm motility; Alkaloids.**

1. Introduction

Gastrointestinal nematodes (GIN), especially *Haemonchus contortus,* had been classified as a major health and welfare problem for small ruminants particularly in the tropics (Aumont et al., 1997; Chandrawathani et al., 1999; Geerts and Dorny, 1996; Hoste et al., 2005; Jabbar et al., 2006; Wolstenholme et al., 2004)**.** Such parasites impair animal health and welfare and productivity since the GIN infection results in increased death rate and poor growth and reproduction (Coop et al., 1982; Dakkak, 1995; Aumont et al., 1997; Hoste et al., 2005). To date, the usual mode of control of gastrointestinal parasitism relied on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Aumont et al., 1997; Jabbar et al., 2006; Jackson and Coop, 2000; Wolstenholme et al., 2004) in gastrointestinal nematode populations has motivated investigation into alternative approaches. Phytotherapy is one of these alternative approaches currently explored (Athanasiadou and Kyriazakis, 2004; Githiori et al., 2006; Wolstenholme et al., 2004). Phytotherapy is mainly based on the use of preparations of leaves, roots and seeds.

Tabernaemontana citrifolia L. (Apocynaceae) is a plant of interest in human medicine: stem bark and leaves are used as an antipyretic preparation, the latex is anti hemorrhagic and the infusion of leaves is laxative (Lucrèce-Abaul, 1988). This tropical shrub is traditionally used as an anthelmintic preparation for ruminants in Guadeloupe (French West Indies). Previous studies (Rastogi *et al.*, 1998) reported that *T. citrifolia* leaves had antimycobacterial activity. Nevertheless, no studies have dealt with the efficacy of the plant against nematodes. The possible secondary metabolites with an anthelmintic activity of *T. citrifolia* are yet to be identified. Secondary metabolites may be extracted from the raw plant material by different solvents (water, methanol (CH_3OH) and dichloromethane (CH_2Cl_2)) according to the polarity of the molecules to be extracted.

For example hot water will extract heterosides, iridoids and tannins; methanol will extract tannins, catechins, terpenoids, and alkaloids, and a more apolar solvent such as dichloromethane will extract semi polar and more apolar (some heterosides, terpens, sterols, coumarins, carotenoids) compounds (Balansard, 1991).

Several methods are commonly used for testing nematicidal activity of both chemical drugs and plant extracts. Amongst them, *in vitro* assays revealed to be relevant and cheaper than *in vivo* methods.

The Egg Hatch Assay (EHA), (Hubert and Kerboeuf, 1984) and the larval development assay (LDA), (Hubert and Kerboeuf, 1992) are currently used for the detection of anthelmintic resistance in GIN (Coles et al., 1992). The larval migration inhibition assay (LMI), (Wagland et al., 1992; Rabel et al., 1994) and the adult worm motility assay (AWM) (Hounzangbe-Adote *et al.*, 2005) allow a more realistic evaluation of the *in vivo* nematicidal activity. These four tests are based on the hypothesis that a nematicidal activity observed *in vitro* would be indicative for a potential *in vivo* activity.

The current study aimed to explore the anthelmintic properties of the tropical shrub *T. citrifolia* against the gastrointestinal parasite *Haemonchus contortus*. Several *in vitro* tests were performed with several plant extracts, to increase the chances of detecting an anthelmintic activity.

2. Materials and methods

2.1. Plant extracts

Plant materials were collected in Guadeloupe (French West Indies) at the beginning of the dry season. The *T. citrifolia* leaves, fruits and roots were dried at 55°C in a ventilated oven, until constant weight. The different parts of the plant were then ground.

Thereafter three extractions were performed on the ground plant parts either with water, methanol (CH_3OH) or dichloromethane (CH_2Cl_2), in order to extract most of the polar and semi-polar molecules depending on the solvent agent.

First, 50 g of each ground plant parts were extracted with 1L of hot distilled water: 500 ml of boiling water mixed with the ground plant parts, simmered and filtered through a Büchner, and then 500 ml of boiling water mixed with the plant residue and simmered again. The filtrates were collected together and lyophilised to obtain a powdered extract.

Then, 50 g of each ground plant parts were lixiviated by 500 ml of solvent (methanol or dichloromethane): the ground plant part was moistened with 150 ml of solvent to cover it, and then let to maceration for 3 h sheltered from light. Thereafter, the filtrate was removed while the rest of the solvent (350 ml) was poured on the plant residue. The filtrates were collected together and evaporated at low pressure at 40°C. All the extracts were stored, in the dark in dessicator, at 22°C.

2.2. In vitro anthelmintic assays

The anthelmintic efficacy tests of the 3 extracts of each fruit, root and leaf, on the different life-cycle stages of *H. contortus* were performed, using four different procedures.

For each assay, the four parasitic stages were obtained from Black Belly donor lambs which were experimentally infected with oral administration of a pure aqueous suspension of 10 000 *H. contortus* third stage larvae (L3).

2.2.1. Egg hatch assay

The *in vitro* anthelmintic activity of the three extracts of *T. citrifolia* plant parts on the egg hatching of *H. contortus* was carried out according to a modification of the method used for testing anthelmintic resistance (Assis *et al.*, 2003). Eggs were extracted from faeces according to the method described by Hubert and Kerboeuf (1984).

After crushing the faeces in water, and successive siftings (500, 250, 125, 63, 50 and 30µm sieves), eggs were collected and centrifuged for 10 min at 2000 rpm. The supernatant was removed and a NaCl solution (density 1.2) was added. After homogenization, the mixture was centrifuged for 15 min at 3000 rpm. The floating eggs were then extracted by pouring the supernatant on a 32µm sieve and then by abundant washing first with distilled, and then with sterilised water.

The egg suspension with a concentration of 400 eggs per ml, was distributed in 24 multiwell plates (0.5 ml per well). Concentrations of extracts (2400, 1200, 600 and 300 µg/ml), and albendazole (0.5%, 0.25%, 0.125%, (w/v) used as positive control) were diluted in PBS (0.1 M phosphate, 0.05 M NaCl; pH 7.2) buffer and were added to the wells (0.5 ml per well). In addition, negative controls in PBS were also included in the assay. Five replicates were run per concentration.

After 48h incubation at 25°C, egg hatching was stopped by adding Lugol's iodine solution. The number of L1 larvae and egg per well was then counted using a reverse microscope (at 100X magnification). The percentage of hatched eggs was determined using the ratio: number of L1 / (number of egg + number of L1).

2.2.2. Larval development assay

The objective of this assay was to test the *in vitro* efficacy of the extracts to inhibit the larval development of *H. contortus* from the L1 stage to the infective L3 stage. The assay used was a modification (Assis et al., 2003) of the technique described by Hubert and Kerboeuf (1992).

L1 and L2 stages larvae were obtained through culture and extraction of eggs from faeces. The eggs were incubated at room temperature for 48 h in 24-multiwell plates (0.5 ml per well with a concentration of 400 eggs/ml). After hatching, 70µL of culture medium (for one plate: 10µg of autolytic yeast extract, 1.5 ml of distilled water and 170 µl of Earle's balanced solution, stabilised to pH= 7.2 with a sodium bicarbonate solution at 50 g/L) was added to each one of the wells, followed by either one of four concentration of extracts (2400, 1200, 600 and 300 µg/ml), or albendazole (0.5%, 0.25%, 0.125% (w/v)), all diluted in PBS buffer, compared with a negative control of PBS (0.5 ml per well and five replicates for each dose).

The larvae were incubated for 8 days at room temperature (25°C), in order to permit the larval development from the first stage to the third infective stage. Thereafter, several drops of a Lugol's iodine solution were added and the number of larvae was counted using inverse microscope (at 40 x magnification) by separating L3 infective larvae from L1-L2 larvae. The percentage of development was calculated as the ratio: number of L3/ total number of larvae per well.

2.2.3. Larval migration inhibition assay

This test is aimed at evaluating the anthelmintic effect of the extracts on the migration capacity of the infective larvae L3. This test was performed according to Rabel et al. (1994).

H. contortus L3 were obtained by fecal culture. Eggs reached the L3 stage after 10 days. The L3 were then collected by sedimentation using Baermann's devices. The anthelmintic effect of each extract was tested using 150, 300, 600 µg of extract/ml PBS with 1000 L3 per dose. Negative (in PBS only) and positive controls (levamisole at 0.125, 0.25 and 0.5%, (w/v)) were also prepared in PBS and incorporated in the assay. Five replicates were run for each plant extract and for the controls. After 3 hours of incubation at room temperature (25°C), the larval suspensions were rinsed out with PBS buffer, and then, each suspension was poured in a Falcon® tube fitted with a 20µm sieve.

After 3 hours of incubation, the inserts were removed and the volumes were adjusted to 2 ml by adding PBS.

Migrating larvae through the sieve (N= number of migrating larvae through the sieve) were counted in a total volume of 500µl (10 drops of 50µl) using optical microscope (at 40 x magnification). The total number of migrated larvae was calculated as N x 4. The percentage of L3 that migrated was then calculated as (N x 4/ 1000) x100.

2.2.4. Adult worms motility assay

The purpose of this assay was to test the anthelmintic effect of the extracts on adult worm motility. This test was performed according to Hounzangbe-Adote et al. (2005).

Adult worms were collected from an experimentally infected lamb, four weeks after experimental infection. Immediately after slaughtering, the abomasum was removed, opened and placed in 37°C saline. The mobile worms were rapidly collected and put into 24-multiwell plates, 3 worms per well in 2 ml of 37°C saline. After one hour of incubation (37°C, 5% carbon dioxide), the saline was removed from each well and replaced by 1ml of either one of the different concentrations of extracts (1200, 600, 300, 150 and 75 µg/ml) or levamisole (0.125, 0.25, 0.5, 1% (w/v)), or PBS.

All dilutions were performed in PBS with penicillin/streptomycin. The incubation media were changed after 24 h. Adult worm motility was evaluated by observation under magnifying glass after 6, 24 and 48h incubation and evaluated each time as the ratio: number of motile worms/ total number of worms per well.

2.3. Phytochemistry

A qualitative thin layer chromatographic (TLC) analysis on silica gel was performed on each *T. citrifolia* extracts, in the ethyl acetate: acetic acid: formic acid: water (100:11:11:26, v/v/v/v) migration system. Developed TLC was first inspected under UV-254 nm and UV-365 nm light. Dragendorff reagent was employed for specific development of alkaloid spots. Presence of alkaloids was determined by developing orange to brown spots (Wagner, 1996).

2.4. Statistical analysis

The data for EHA, LDA and LMI assays were analysed by multiple comparisons (Bonferroni method) using orthogonal contrasts with the SAS software. Regression was used for evaluation of dose response relationship, and IC50 was calculated by Probit analysis of doses and efficacies results, using Minitab® Release 14 software. For the AWM assay, the data were analysed by the mixed General Linear Model procedures using the Glimmix macro of the SAS software. Before analysis all data were Log10(X+1) transformed.

3. Results

3.1. Egg hatch assay

The extracts of fruit and leaf and the aqueous and CH_3OH extracts of root of *T. citrifolia* showed no significant effects on egg hatching compared to the PBS negative control ($P > 0.10$). Conversely, the dichloromethane extract of root was observed to inhibit hatching (Table 1) compared with the control PBS ($P < 0.01$). Regression analysis indicates that there is a dose dependent effect of the dichloromethane extract of root ($P<0.0001$; $R^2= 81.5\%$; regression equation: hatch inhibition = 9.45% + 23.6 dose); with IC50 =2.32 mg/ml. The positive and negative controls differed ($P < 0.001$).

3.2. Larval development inhibition

All the extracts of *T. citrifolia* (except the dichloromethane leaf extract) exhibited larval development inhibition ($P < 0.01$) compared with negative control (Table 2). The larval development least square means varied from 0.2% to 27.9% and regression analysis indicated a dose dependent effect within the concentration ranges tested, for the methanolic extract of leaf ($P= 0.006$; $R^2=35.4\%$; larval inhibition = 49.2% +47.6 dose); with IC50= 0.18 mg/ml. A dose dependent effect within the concentration ranges tested, was also observed for the aqueous, methanolic and dichloromethane extracts of root (aqueous: $P<0.0001$; $R^2=63.4\%$; larval inhibition = 58.8% +23.9 dose; with IC50= 0.07 mg/ml. Methanolic: $P<0.0001$; $R^2=52.9\%$; larval inhibition = 71.6% +21.2 dose; with IC50= 0.04 mg/ml. Dichloromethane: $P<0.0001$; $R^2=60.9\%$; larval inhibition = 72.3% +20.3 dose; with IC50= 0.03 mg/ml). There was a dose dependent effect for the aqueous and methanolic extracts of fruit (aqueous: $P<0.0001$; $R^2=54.3\%$; larval inhibition = 79.3% +16.9 dose; with IC50= 0.02 mg/ml. methanolic: $P<0.0001$; $R^2=58.8\%$; larval inhibition = 83.9% +14.3dose; with IC50= 0.02 mg/ml).

Moreover, the percent inhibitions of most of the extracts of *T. citrifolia* (except aqueous extract of root and methanolic extract of leaf) were not different from the positive control.

3.3. Larval migration

The methanolic extract of leaf of *T. citrifolia* inhibited larval migration of L3 compared to negative control ($P < 0.05$), but less so than levamisole ($P < 0.001$; Table 3). The efficacy of the methanolic extract of leaf was not dose-dependent within the concentration ranges tested. The other extracts of *T. citrifolia* were not effective in inhibiting larval migration.

3.4. Adult worm motility

Levamisole was associated with reduced worm motility compared with the negative control ($P <0.01$). The aqueous and dichloromethane extract of root had significant effect between 6h and 24h of incubation ($P<0.01$). The aqueous and methanolic extract of leaf, the aqueous and dichloromethane extract of root, and the dichloromethane extract of fruit had significant effect between 6h and 48h ($P<0.05$). Compared to the PBS negative control (Table 4), efficacy of the dichloromethane extract of fruit and of the aqueous and dichloromethane extract of root of *T. citrifolia* was observed after 24 hours ($P<0.05$). Nevertheless worm motility remained lower than the positive control. Regression analysis indicates that there is a dose dependent effect of the dichloromethane extract of root after 24h of incubation ($P= 0.002$; $R^2= 54.9\%$; regression equation : motility inhibition = 0.262% + 0.000608 dose); with IC50 = 0.3 mg/ml. The positive and negative controls differed ($P < 0.001$).

3.5. Phytochemistry

The TLC analysis indicates that the leaf (aqueous and methanolic extracts), root (aqueous, methanolic and dichloromethane extracts) and fruit (methanolic and dichloromethane extracts) of *T. citrifolia* contained alkaloid compounds (orange to brown spots with Dragendorff reagent). Observation under 254 nm UV light (quenching zones) indicates indole alkaloids in the root (aqueous and methanolic extracts), fruit (Dichloromethane extract) and leaf (aqueous and methanolic extracts). Observation under 365 nm UV light (yellow, green and orange fluorescent spots) indicates the presence of many flavonoid compounds in the fruit (methanolic and dichloromethane extracts), root (aqueous and methanolic extracts) and leaf (aqueous and methanolic extracts).

4. Discussion and conclusion

The results indicate that *T. citrifolia* has an anthelmintic property on *H. contortus*. Moreover the *T. citrifolia* nematicidal activity was observed for each of the *H. contortus* developmental stages. The root extracts gave the more consistent results. The three parts of the plant: fruits, leaves and roots had effect on the larval development of *H. contortus*.

To the authors knowledge, there are no published studies about the anthelmintic effect of *T. citrifolia* anthelmintic activity on *H. contortus*. The plant is known to contain a large amount of alkaloids (total alkaloids: 5.6 g/kg of dry matter of leaf, 180 g/kg of dry matter of root bark in Lucrèce-Abaul, 1988, Abaul et al., 1989), which is one of the most important groups of secondary metabolites of medicinal interest (Longuefosse, 2003). *T. citrifolia* contains a complex of mono-indoloterpenic alkaloid types: ibogane, aspidospermane and corynane alkaloids (Abaul et al., 1988), known for their neurotoxic and antimitotic activity (Bruneton, 1999). Indole compounds, related to the metabolism of tryptophan, constitute an extensive family. Indolic compounds possess significant and complex physiological roles, and especially indole alkaloids have historically constituted a class of major importance in the development of new plant derived drugs.

The different trials were performed in order to screen the activity and to search for the mode of action of compounds which have the ability to disrupt the life cycle of *H. contortus*. By examining the effects of the different methods of extractions and the results of the TLC, hypothesis could be made for the compounds acting on the different stages of *H. contortus* development. Taking into account the high level of alkaloid compounds detected in the different extracts, these compounds could be involved in the activity of *T. citrifolia* on *H. contortus*. The antimitotic and neurotoxic activity of these molecules and their ability in inhibiting the synthesis of proteins (Bruneton, 1999), could explain the activity of the different plant parts of the plant on the different development stages of the parasite. The complexity of structure of the different types of alkaloids present in the extracts could enable the compounds to interact with multiple molecular targets on the parasite different development stages. Nevertheless, the egg hatching was certainly stopped due to some specific compounds, present in the dichloromethane extract of roots, as no other extract can stop this developmental stage of the parasite. The larval migration might also be stopped by some specific alkaloids present in the methanolic extract of leaf as well. The larval development could be stopped by the indole

alkaloids present in the extracts but the hypothesis of some flavonoid compounds involved in the activity on the development has to be considered, taking into account their high concentration in the aqueous and methanolic extracts observed on the TLC. Studies on the anthelmintic activity of a tropical plant from the family of Apocynaceae showed efficacy against nematodes (Okpekon et al., 2004), it could be attributed to its alkaloid content. Eguale et al., (2007) suggested that alkaloids and/or flavonoids could be involved in an anthelmintic activity against *H. contortus*, it must be validated by further *in vitro* testing of the purified compounds.

In vitro tests are used as preliminary studies in the search for natural anthelmintics. Nevertheless, further investigations are needed to validate the activity of *T. citrifolia*, particularly *in vivo* studies. *In vitro* trials suppose direct action on parasite and cannot be sufficient to conclude for the anthelmintic efficacy because results obtained on *in vitro* conditions could be different from *in vivo* conditions. This might be due to the gastrointestinal tract of ruminants. This kind of contrast was obtained with condensed tannins for which *in vitro* and *in vivo* trials gave different results (Athanasiadou *et al.*, 2001; Paolini *et al.*, 2005). Moreover, indirect effects on parasite could also be observed *in vivo*. Furthermore, it is unlikely that the assays can be achieved *in vivo* by using directly *T. citrifolia* raw plant because of the bitterness of the plant. That is the reason why the *in vivo* trial must be conducted with the extracts of *T. citrifolia*. Toxicity level should also be evaluated and it could only be done by the way of *in vivo* studies.

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Table 1: Means of hatching percentages in the egg hatch assay, for the different extracts of *T. citrifolia* (*Tc*) organs, compared to PBS negative control and to albendazole positive control.

Products	**Hatching %**	**P value/Albendazole**	**P value/ PBS**
Albendazole	22.3	-	<0.0001
PBS	95.6	<0.0001	-
*Tc*L A	98.0	<0.0001	0.99
*Tc*L M	98.4	<0.0001	0.99
*Tc*L D	93.5	<0.0001	0.96
*Tc*F A	93.6	<0.0001	0.99
*Tc*F M	91.8	<0.0001	0.82
*Tc*F D	87.3	<0.0001	0.99
*Tc*R A	92.5	<0.0001	0.99
*Tc*R M	83.4	<0.0001	0.21
*Tc*R D	77.3	<0.0001	0.002

L: Leaf; F: Fruit; R: Root

A: Aqueous; M: Methanol; D: Dichloromethane

Table 2: Development means (development %) in the larval development assay, for the different extracts of *T. citrifolia* (*Tc*) organs, compared to PBS negative control and to albendazole positive control.

Products	**development%**	**P value/albendazole**	**P value/ PBS**
Albendazole	5.6	-	<0.0001
PBS	69.5	<0.0001	-
*Tc*L A	15.0	0.76	<0.0001
*Tc*L M	24.0	0.01	<0.0001
*Tc*L D	66.5	<0.0001	0.99
*Tc*F A	11.1	0.99	<0.0001
*Tc*F M	8.0	1.00	<0.0001
*Tc*F D	0.2	0.99	<0.0001
*Tc*R A	27.9	<0.0001	<0.0001
*Tc*R M	16.4	0.53	<0.0001
*Tc*R D	16.2	0.56	<0.0001

L: Leaf; F: Fruit; R: Root

A: Aqueous; M: Methanol; D: Dichloromethane

Table 3: L3 *H. contortus* migration means in the larval migration assay, for the different extracts of *T. citrifolia* (*Tc*) organs, compared to PBS negative control and to levamisole positive control.

Products	**%L3 migration**	**P value/Levamisole**	**P value/ PBS**
Levamisole	0	-	<0.0001
PBS	78.6	<0.0001	-
*Tc*L A	79.4	<0.0001	0.99
*Tc*L M	50.6	<0.0001	0.02
*Tc*L D	91.8	<0.0001	0.60
*Tc*F A	88.5	<0.0001	0.48
*Tc*F M	79.4	<0.0001	0.99
*Tc*F D	95.3	<0.0001	0.01
*Tc*R A	90.7	<0.0001	0.19
*Tc*R M	81.3	<0.0001	0.99
*Tc*R D	83.1	<0.0001	0.99

L: Leaf; F: Fruit; R: Root

A: Aqueous; M: Methanol; D: Dichloromethane

Table 4: Means of adult *H. contortus* worms percentage of motility after 24h incubation time for the different extracts of *T. citrifolia* (*Tc*) organs, compared to PBS negative control and to levamisole positive control.

Products	Incubation time (hours)					
	6h			24h		
	%mot	P1	P2	%mot	P1	P2
Levamisole	69.2	-	0.26	16.8	-	<0.0001
PBS	94.3	0.26	-	68.8	<0.0001	-
*Tc*L A	96.8	0.03	0.79	74.9	<0.0001	0.27
*Tc*L M	84.7	0.06	0.44	50.11	0.02	0.47
*Tc*L D	70.2	0.28	0.05	42.4	0.06	0.12
*Tc*F A	96.9	0.32	0.83	84.0	<0.0001	0.86
*Tc*F M	96.8	0.33	0.84	79.6	<0.0001	0.56
*Tc*F D	94.6	0.44	0.99	52.7	<0.0001	<0.0001
*Tc*R A	96.7	0.24	0.82	65.6	<0.0001	0.03
*Tc*R M	86.6	0.93	0.49	68.4	<0.0001	0.10
*Tc*R D	94.7	0.00	0.00	44.1	0.0004	<0.0001

P1: P value compared with Levamisole, **P2**: P value compared with PBS control.

*Tc*L: *Tc* Leaf; *Tc*F: *Tc* Fruit; *Tc*R: *Tc* Root; A: Aqueous; M: Methanol; D: Dichloromethane

Article n°4

In vitro effects of *Musa x paradisiaca L.*extracts on four developmental stages of *Haemonchus contortus*

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Soumis à Research in Veterinary Science

Abstract

This study was carried out to evaluate the *in vitro* effect of *Musa x paradisiaca L.*stem and leaf against the parasitic nematode of small ruminants *Haemonchus contortus*. Three extracts (aqueous, methanolic and dichloromethane) of *Musa x paradisiaca L.*stem and leaf were tested *in vitro* on four developmental stages of *Haemonchus contortus* using egg hatch assay (EHA), larval development assay (LDA), L3 migration inhibition assay (LMI) and adult worm motility assay (AWM). The highly significant ($P< 0.0001$) ability to stop larval development (inhibition > 67% for each extract) and the negative effect of the dichloromethane extract of leaf on adult worm motility (43% of inhibition of motility after 24 hours of incubation) compared to the negative controls, suggest anthelmintic properties of *Musa x paradisiaca L.*stem and leaf against *H. contortus*. The active principles responsible for the activity could be secondary metabolites such as terpenoid and flavonoid compounds present in the organs of the plant.

Keywords: Banana tree; Gastro-intestinal nematodes*;* Egg hatching; Larval development; L3 migration; Adult worm motility.

1. Introduction

Gastrointestinal nematodes, (especially *Haemonchus contortus*) had been classified as a major health and welfare problem for small ruminants particularly in the tropics (Aumont *et al.*, 1997; Chandrawathani *et al.*, 1999; Geerts & Dorny, 1996; Hoste *et al.*, 2005; Jabbar *et al.*, 2006; Wolstenholme *et al.*, 2004)**.** Such parasites impair animal health and welfare and productivity since gastrointestinal nematodes infection results in increased death rate and poor growth and reproduction (Aumont *et al.*, 1997; Coop *et al.*, 1982; Dakkak, 1995; Hoste *et al.*, 2005). To date, the usual mode of control of gastrointestinal parasitism relies on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Aumont *et al.*, 1997; Jabbar *et al.*, 2006; Jackson & Coop, 2000; Wolstenholme *et al.*, 2004) in GIN populations motivates investigation into alternative approaches. Phytotherapy is one of these alternative approaches currently explored (Athanasiadou & Kyriazakis, 2004; Githiori *et al.*, 2006; Wolstenholme *et al.*, 2004). Phytotherapy is mainly based on the use of preparations of leaves and seeds and the effects are attributed to plant secondary metabolites. *Musa x paradisiaca L.*is a plant of medicinal interest in human medicine in the Caribbean (TRAMIL, 1999; Longuefosse, 2003): the leaf and stem are used to treat diarrhoea; the stem is good for asthenia and wounds, and the leaf for the treatment of inflammation, headache and rheumatism. Previous studies (TRAMIL, 1999) reported that *M. Paradisiaca* had antimicrobial and healing activities. Nevertheless, only a few studies have reported on the efficacy of this plant against nematodes. Sharma et al. (1971) tested aqueous extracts of *M. Paradisiaca* root on adult *H. contortus* and found a significant activity at 5% level with a 1 in 25 concentration. Batatinha et al. (2005) tested the *in vitro* effect of aqueous extracts of leaves of *Musa cavendishii Linn*. on *Strongyloidea* larvae of goats and found a reduction of development of more than 95% at the concentration of 130.6 mg/ml. *Musa acuminata* was tested *in vivo* on goats experimentally infected with *H. contortus* (Vieira et al., 1999) and it did not significantly reduce the number of adult worms.

The possible secondary metabolites with an anthelmintic activity of *M. paradisiaca* are yet to be identified. As tannin compounds are a part of the metabolites of this plant (TRAMIL, 1999), they should be considered as potential anthelmintics.

Nevertheless, other compounds might not be excluded. Secondary metabolites may be extracted from the raw plant material by different solvents (water, dichloromethane, and methanol according to the polarity of the molecules to be extracted (Balansard, 1991). For example hot water will extract heterosides, iridoids and tannins; methanol will extract tannins, catechins, terpenoids and alkaloids, and a more apolar solvent such as dichloromethane will extract semi polar and more apolar compounds (Balansard, 1991).

Several methods are commonly used for testing nematicidal activity of both chemical drugs and plant extracts. Amongst them, *in vitro* assays revealed to be relevant and cheaper than *in vivo* methods. The Egg Hatch Assay (EHA), (Hubert and Kerboeuf, 1984) and the larval development assay (LDA), (Hubert & Kerboeuf, 1992) are currently used for the detection of anthelmintic resistance in GIN (Coles *et al.*, 1992). The larval migration inhibition assay (LMI), (Rabel *et al.*, 1994; Wagland *et al.*, 1992) and the adult worm motility assay (AWM), (Hounzangbe-Adote *et al.*, 2005) allow a more realistic evaluation of the *in vivo* nematicidal activity. These four tests are based on the hypothesis that a nematicidal activity observed *in vitro* would be indicative for a potential *in vivo* activity.

The current study aimed to explore the anthelmintic properties of the banana tree against the gastrointestinal parasite *H. contortus,* and to enquire on the phytochemical compounds involved in the activity. Several *in vitro* tests were performed with several plant extracts, to increase the chances of detecting an anthelmintic activity.

2. Materials and methods

2.1. Plant extracts

Plant materials were collected in Guadeloupe (French West Indies). The *M. Paradisiaca* leaves and stem were first lyophilised and then ground. Three different extractions were performed on leaf either with water, methanol (CH_3OH) or dichloromethane (CH_2Cl_2), in order to extract most of the polar and semi-polar molecules depending on the solvent. Two different extractions with water and methanol were performed on stem.

First, 50 g of ground leaf or stem were extracted with 1L of hot distilled water: 500ml of boiling water mixed with the powder, simmered and filtered through a Büchner, and then 500ml of boiling water mixed with the plant organ residue and simmered again. The filtrates were collected together and lyophilised to obtain a powdered extract.

Then, 50 g of each ground organ were lixiviated by 500 ml of solvent (methanol or dichloromethane): the ground plant organ was moistened with 150 ml of solvent to cover it, and then let to maceration for 3 h sheltered from light. Thereafter, the filtrate was removed while the rest of the solvent (350 ml) was poured on the residue. The filtrates were collected together and evaporated at low pressure at 40°C. All the extracts (aqueous, methanolic and dichloromethane) were stored, in dark in desiccators, at 22°C.

2.2. *In vitro* anthelmintic assays

The anthelmintic efficacy tests of the different extracts of each organ of *M. paradisiaca* on the different life-cycle stages of *H. Contortus,* were performed using four different procedures.

For each assay, the four parasitic stages were obtained from faeces and abomasums of Black Belly donor lambs which were experimentally infected by an oral administration of a pure 10 000 *H. contortus* third stage larvae (L3) suspension.

2.2.1. Egg hatch assay

The *in vitro* anthelmintic activity of the three extracts of the two plant organs on the egg hatching of *H. contortus* was carried out according to a modification of the method used for testing anthelmintic resistance (Assis *et al.*, 2003). Eggs were extracted from the faeces of the donor sheep according to the method described by Hubert and Kerboeuf (1984).

After crushing the faeces in water, and successive siftings (500, 250, 125, 63, 50 and 30µm sieves), eggs were collected and centrifuged for 10 min at 2000 rpm.

The supernatant was removed and a NaCl solution (density 1.2) was added. After homogenization, the mixture was centrifuged for 15 min at 3000 rpm. The ring with eggs was then extracted by pouring it on a 32µm sieve and then by abundant washing first with distilled, and then with sterilised water. The egg suspension with a concentration of 400 eggs per ml, was distributed in 24-well plates (0.5 ml per well). Concentrations of plant extracts (2400, 1200, 600 and 300 µg/ml), and albendazole (0.5%, 0.25%, 0.125%, used as positive control) were diluted in PBS (0.1 M phosphate, 0.05 M NaCl; pH 7.2) buffer and were added to the wells (0.5 ml per well). In addition, PBS negative controls were also included in the assay. Five replicates were run per concentration.

After 48h incubation at 25°C, egg hatching was stopped by adding Lugol's iodine solution. The number of L1 larvae and egg per well was then counted using a reverse microscope (at 100 X magnification). The percentage of hatched eggs was determined using the ratio: number of L1 / (number of egg + number of L1).

2.2.2. Larval development assay

The objective of this assay was to test the *in vitro* efficacy of the extracts to inhibit the larval development of *H. contortus* from the L1 stage to the infective L3 stage. The assay used was a modification (Assis et al., 2003) of the technique described by Hubert and Kerboeuf (1992).

L1-L2 stages larvae were obtained after culture of eggs extracted from the faeces of the donor lambs. The eggs were incubated at room temperature for 48 h in 24-multiwell plates (0.5 ml per well with a concentration of 400 eggs/ml). After hatching, 70µl of culture medium (for one plate: 10µg of autolytic yeast extract, 1.5 ml of distilled water and 170 µl of Earle's balanced solution, stabilised to pH= 7.2 with a $NaHCO_3$ solution at 50 g/L) was added to each one of the wells, followed by either each concentration of extracts (2400, 1200, 600 and 300 µg/ml), albendazole positive control (0.5%, 0.25%, 0.125%), all diluted in PBS buffer, and PBS negative control (0.5 ml per well and five replicates for each dose). All dilutions were made in PBS.

The larvae were incubated for 8 days at room temperature (25°C), in order to permit the larval development from the first stage to the third infective stage. Thereafter, several drops of a Lugol's iodine solution were added and the number of larvae was counted using inverse microscope (at 40 X magnification) by separating L3 infective larvae from L1-L2 larvae. The percentage of development was calculated as the ratio: number of L3/ total number of larvae per well.

2.2.3. Larval migration inhibition assay

This test is aimed at evaluating the anthelmintic effect of the extracts on the migration capacity of the infective larvae L3. This test was performed according to Rabel et al. (1994) in Hounzangbe-Adote et al. (2005).

H. contortus L3 were obtained by larval culture from the faeces of donor lambs. Eggs reached the L3 stage after 10 days. The L3 were then collected by sedimentation using Baermann's devices. The anthelmintic effect of each extract was tested using 150, 300, 600, 1200 and 2400 µg of extract/ml PBS with 1000 L3 per dose. Negative (in PBS only) and positive controls (levamisole at 0.125, 0.25 and 0.5%) were also prepared in PBS and incorporated in the assay. Five replicates were run for each plant extract and for the controls. After 3 h of incubation at room temperature (25°C), the larval suspensions were rinsed out with PBS buffer, by 3 successive centrifugations (5 min, 4500 rpm). After the last washing, the suspensions were given out in Falcon® tubes fitted with 20µm sieves in such a way that the larval crossing through the sieve was a dynamic phenomenon. After 3h of incubation, the inserts were removed and the volumes were adjusted to 2 ml by adding PBS.

Larvae having passed through the sieve (N= number of migrating larvae) were counted in a total volume of 500µl (10 drops of 50µl) using optical microscope (at 40 X magnification). The total number of migrant larvae was calculated as = N x 4. The percentage of migrant L3 was then calculated as = (N x 4 / 1000) x100.

2.2.4. Adult worm motility assay

The purpose of this assay was to test the anthelmintic effect of the extracts on adult worm motility. This test was performed according to Hounzangbe-Adote et al. (2005).

Adult worms were collected from an experimentally infected lamb, four weeks after infection. Immediately after slaughtering, the abomasum was removed, opened and placed in 37°C saline. The mobile worms were rapidly collected and put into 24-multiwell plates, 3 worms per well in 2 ml of 37°C saline. After one hour of incubation (37°C, 5% carbon dioxide), the saline was removed from each well and replaced by 1ml of either one of the different concentrations of extracts (1200, 600, 300, 150 and 75 µg/ml) or of the levamisole positive control (0.125, 0.25, 0.5, 1%), or of the PBS negative control. All dilutions were performed in PBS plus penicillin/streptomycin. The incubation media were replaced after 24 h. Adult worm motility was evaluated by observation under magnifying glass and evaluated as the ratio: number of motile worms/ total number of worms per well.

2.3. Phytochemistry

2.3.1. Phytochemical screening

The phytochemical tests were accomplished following classical methodologies (Dohou et al., 2003). These tests are based on the addition of specific reagents to the ground leaf and stem of *M. paradisiaca*, directly or after an infusion, and observing changes of the solution colours, or precipitate formation. The presence of the several chemical compounds was evaluated: alkaloids, coumarins, saponosides, tannins, triterpens and sterols, of free quinones, anthraquinones, anthraquinone heterosides, flavonoids, flavonols, flavans, and anthocyanins.

2.3.2. TLC analysis

A qualitative thin layer chromatographic (TLC) analysis on silica gel (Al coated Kieselgel 60 F_{254} (Merck) plates), was performed on each plant extracts, in chloroform/acetic acid/methanol/water (64:32:12:8, v/v/v/v) migration system. After migration, TLC was first inspected under UV-254 nm and UV-365 nm light to detect flavonoid and phenolic fluorescent or coloured spots. Vanillin-sulphuric acid reagent was then employed for revealing terpenoids (triterpens and saponins) and phenolic spots (Wagner, 1996). Naphtoresorcinol reagent was employed for detection of heterosides.and ninhydrine reagent for detection of amino acid compounds.

2.3.3. Spectrophotometric determination of condensed tannins

The condensed tannins (proanthocyanidin) content was determined by the vanillin-HCL method (Nakamura et al., 2003). Quebracho is a plant extract with high condensed tannins (CT) concentration, with direct activity against *H.contortus* larval survival (Athanasiadou et al., 2001). Quebracho CT was used as standard for determination of CT, as indicated by Hagerman and Butler (1989).

2.4. Statistical analysis

The data for EHA, LDA and LMI assays were analysed by the general linear model (GLM) procedures using Minitab® Release 14 software. Efficacy was determined by multiple comparisons procedure using the Bonferroni method. Dose response level was analysed using simple regression procedure for dose response analysis and probit method for IC50 determinationwith Minitab® Release 14 software. For the AWM assay, the data were analysed by the mixed procedures using the Glimmix macro of the SAS software to determine the significance level of difference between the extracts and the positive and negative controls.

3. Results

3.1. *In vitro* Anthelmintic assays

3.1.1. Egg hatch assay

The mean hatching rate was 94.6% ±3.8 for the PBS negative control. The aqueous, methanol and dichloromethane extracts of leaf and the aqueous and methanol extracts of stem showed no significant effects on egg hatching (93.9% ±0.6 mean of hatching), compared with negative control ($P > 0.10$). For the albendazole positive control, the mean hatching rate measured was 22% ±4.4 (from 48.5% ±1.7for the lowest 0.6 mg/ml dose, up to 6.0% ±2.0 for the highest 2.5 mg/ml dose). This value was significantly different from the PBS negative control ($P <0.0001$).

3.1.2. Larval development inhibition assay

The extracts of *M. paradisiaca* leaf and stem exhibited larval development inhibition at all extraction methods examined ($P < 0.0001$) compared with negative control (Table 1). Regression analysis indicated a dose dependent effect, within the concentration ranges tested, for aqueous extracts of leaf and stem (leaf: $P= 0.002$; $R^2= 44.6\%$; larval inhibition = 47.1% + 0.0260 dose. Stem: $P< 0.0001$; $R^2= 69.4\%$; larval inhibition = 25.7% + 0.0365 dose); and for dichloromethane extract of leaf ($P< 0.0001$; $R^2= 54\%$; larval inhibition = 59.5% + 10.9 dose). Moreover, the percent inhibitions of the aqueous extract of leaf and methanolic extracts of leaf and stem were not different from the positive control. Conversely, their IC50 values were different from leaf to stem and from the positive control IC50 value.

3.1.3. Larval migration inhibition assay

The mean migration rate was 80.6% ±1.5 for the PBS negative control. The extracts of *M. paradisiaca* leaf and stem (73.0% ±0.2 mean of migration) exhibited no significant effects on migration at all extraction methods examined ($P > 0.10$) compared with negative control. For the levamisole positive control, the mean migration rate measured was 0%. This value was significantly different from the PBS negative control ($P < 0.0001$).

3.1.4. Adult worm motility assay

The levamisole positive control was associated with reduced worm motility compared with the negative control ($P < 0.001$) after 24hours. There was a time dependent effect of the treatments. Compared to the PBS negative control, efficacy of the dichloromethane extract of leaf of *M. Paradisiaca* was observed after 24 hours (Table 2). Nevertheless this efficacy remained lower than that observed with the positive control. No dose effect response was observed for the dichloromethane extract of leaf after 24h of incubation ($P>0.05$), within the concentration ranges tested.

3.2. Phytochemistry

3.2.1. Phytochemical screening

The phytochemical screening indicates that both leaf and stem of *M. Paradisiaca* contained alkaloids, saponosides and triterpens and sterols. The stem also contained anthraquinon heterosides, benzoquinon, flavonols, flavans and anthocyans. The leaf also contained tannins and flavonoids. The chemical composition of *M. Paradisiaca,* according to this phytochemical screening is presented in Table 3.

3.2.2. TLC

Observation under 365 nm UV light (yellow, green and orange fluorescent spots) indicates the presence of many flavonoid compounds in the stem and leaf of *M. paradisiaca* (aqueous and methanolic extracts). Aqueous and methanol extract of leaf and stem contained terpenoid and heteroside compounds. Amino acid compounds were detected in the aqueous extract of leaf and major spots of saponosides compounds were detected in the methanol extracts of stem and leaf.

3.2.3. Condensed tannins determination

Referring to quebracho CT as standard, the extracts of *M. paradisiaca* leaf and stem exhibited low levels of CT. The results of the CT spectrophotometric determination are presented in Table 4. No detection was possible for the aqueous and methanolic extracts of stem.

4. Discussion and conclusion

The results indicate that *Musa x paradisiaca L.*has an *in vitro* anthelmintic property on *H. contortus*. The two organs stem and leaf had efficacy on the larval development of *H. contortus*. The methanolic extract of the two organs was the most efficacious. These results confirm the previous results obtained with aqueous extracts of Musa sp. Leaf (Batatinha et al., 2005) on nematodes.

The low level of CT and other tannins observed in the present study suggests that others compounds are involved in the nematicidal activity of *M. Paradisiaca.* This also suggests that the results observed in previous studies (TRAMIL, 1999) about tannins content of *M. Paradisiaca* was certainly due to non specific interactions between non-tannic phenolic compounds of the plant (detected in the screening of the present study) and the reagent employed, or perhaps was it due to inappropriate choice of standards in the assaying method. The present study showed that *M. paradisiaca* aqueous and methanolic extracts contained flavonoid, sterols and terpenoid compounds, which could be involved in the activity. TLC analysis indicated that the methanolic extract, contained a majority of saponosides, which is an important group of secondary metabolites of medicinal interest (Longuefosse, 2003).

Terpenoid compounds are known to be active against a large range of organisms. Some of them can be bioactive whereas others can affect physical variables. Terpenoids and flavonoids are known to stop *H. contortus* larval development (Ademola et al., 2005; Maciel et al., 2006; Camurça-Vasconcelos et al., 2007). Furthermore, triterpenoids, saponins and sterols are all antibacterial, antimicrobic, anticarcinogenic and antifungic molecules (Aharoni et al., 2005). These complexities could enable the compounds to interact with multiple molecular targets on the various developmental stages of the parasite.

The saponoside compounds could be involved in the nematicidal activity of the plant and it could be hypothesis that they are responsible for the efficacy of the plant as they represent the major part of the most efficacious extract. Saponosides compounds usually act by binding to surface molecules

(proteins or sterols) inducing inhibition of the protein expression, and/or lyses of the cell (Bruneton, 1999). The expression of surface proteins of nematodes is stage specific (Rhoads and Fetterer, 1994). Moreover, studies have reported on a dose dependent muscle paralysing effect of an extract from the stem of the banana tree (Singh and Dryden, 1990). The neuromuscular blockade was reversed by calcium, suggesting that banana stem juice had a labilizing effect on cell calcium. The saponosides in the methanolic extract could specifically bind to the surface proteins of the larval developmental stage of the parasite, explaining the specific major efficacy of this extract. These compounds could act as described by Singh et al. (1990), inhibiting the larval development of *H. contortus* and causing their death. The best efficacy observed in the LDA could also be due to a longer incubation of the larvae with the extracts, conversely to EHA and LMI. Moreover, these two developmental stages possess a sheath that protects them from external agents such as the potentially active secondary metabolites of the plant. Hypothesis could be made on a hydrophilic structure of the anthelmintic compounds of *M. paradisiaca*, as lipophilic anthelmintics have a greater capability to cross the external surface of the helminths than the hydrophilic compounds (Geary et al., 1999).

Concerning the adult worm motility, it was certainly stopped due to some specific compounds, present in the dichloromethane extract of leaf, as no other extract could stop this developmental stage of the parasite. According to the phytochemical screening, probably phenols or other specific terpenoid compounds could have been extracted by the dichloromethane solvent. These hypotheses must be validated by further *in vitro* testing of the purified compounds or class of compounds.

In vitro tests are used as preliminary studies in the search for natural anthelmintics. Nevertheless, further investigations are needed to validate the activity of *M. paradisiaca*, particularly *in vivo* studies. *In vitro* trials suppose direct action on parasite and cannot be sufficient to conclude for anthelmintic efficacy as results obtained on *in vitro* conditions could be different from *in vivo* conditions, particularly due to the gastrointestinal tract of ruminants.

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Table 1: L1 to L3 stage larvae development inhibition (% L1-L2) for the different extracts of *M. paradisiaca* (Mp) leaf and stem, compared to PBS (Phosphate Buffer Sample) negative control and to albendazole positive control.

Products	**% L1-L2 ± SE**	**P value/albendazole**	**P value/ PBS**	**IC_{50} (µg/ml)**
Albendazole	88.0 ±0.2	-	<0.0001	0.8
PBS	15.9 ±0.1	<0.0001	-	-
MpL A	77.4 ±1.7	0.99	<0.0001	370.8
MpL M	98.7 ±0.1	0.06	<0.0001	-
MpL D	79.0 ±1.8	<0.0001	<0.0001	364.6
MpS A	67.8 ±2.4	0.12	<0.0001	511.7
MpS M	95.1 ±0.3	0.24	<0.0001	-

L: Leaf; S: Stem; A: Aqueous; M: Methanol; D: Dichloromethane.

IC_{50}: Inhibition concentration of 50% of a population.

Table 2: *H. contortus* adult worm percentage of motility for the different extracts of *M. paradisiaca* (Mp) organs, compared to PBS (Phosphate Buffer Sample) negative control and to levamisole positive control.

Products	Incubation time (hours)								
	6h			**24h**			**48h**		
	%mot ±SE	**P1**	**P2**	**%mot ±SE**	**P1**	**P2**	**%mot ±SE**	**P1**	**P2**
Levamisole	83.2±0.1	-	0.05	9.2±0.2	-	<0.0001	0.8±0.5	-	0.2
PBS	96.2±0.0	0.05	-	62.6±0.1	<0.0001	-	1.9±0.3	0.2	-
MpL A	92.5±0.1	0.70	0.32	61.3±0.1	0.86	<0.0001	12.9±0.2	<0.0001	<0.0001
MpL M	96.8±0.1	0.15	0.95	68.6±0.1	0.47	<0.0001	0.0	0.99	0.99
MpL D	88.2±0.1	0.40	0.58	43.0±0.1	0.01	<0.0001	1±0.1	0.99	0.99
MpS A	96.9±0.1	0.94	0.15	80.2±0.1	0.03	<0.0001	3.8±0.4	0.23	0.04
MpS M	96.9±0.1	0.94	0.15	63.5±0.1	0.91	<0.0001	4.3±0.4	0.14	0.02

MpL: Mp Leaf; MpS: Mp Stem; A: Aqueous; M: Methanol; D: Dichloromethane

Table 3: Results of the phytochemical screening of *M. paradisiaca* leaf and stem

Compounds	**Leaf**	**Stem**
Alkaloids	+	+
Coumarins	-	
Saponosides	++	++
Tanins	+	-
Triterpens and sterols	+	+
Free anthraquinones	-	-
Anthraquinons heterosides	-	+
Quinones	-	+ (benzo-quinones)
Phenols	+	-
Flavonoids	+	++ (flavonols)
Anthocyanins	-	+
Flavans	-	+

- : Negative

+: Weak coloration

++: Positive

Table 4: Condensed tannins content in *Musa x paradisiaca L.* extracts, using the vanillin–HCl method.

***M. paradisiaca* extracts**	**CT content (%)**	**±S.E.**
Methanolic extract of leaf	2.54	±0.81
Aqueous extract of leaf	0.05	±0.50
Methanolic extract of stem	No detection	-
Aqueous extract of stem	No detection	-

Article n°5

In vitro effects of Cassava (*Manihot esculenta)* leaf extracts on four development stages of *Haemonchus contortus*

C. Marie-Magdeleine et al.

Soumis à Veterinary Parasitology

Abstract

Three extracts (aqueous, methanolic and dichloromethane) of *Manihot esculenta Crantz* (Cassava) leaf were tested *in vitro* on four development stages of *Haemonchus contortus* using egg hatch assay (EHA), larval development assay (LDA), L3 migration inhibition assay (LMI) and adult worm motility assay (AWM). Compared to the negative control, significant effects (P <0.0001) were observed for the methanolic extract of leaf against larval development (57.6% ±7.6), with a dose dependent effect. These results suggest that Cassava possess anthelmintic activity against *H.contortus*. The active principles responsible for the activity could be the terpenoid and condensed tannin compounds present in the leaf.

Keywords: gastrointestinal nematodes*;* egg hatch assay; larval development; larval migration; adult worm motility; condensed tannins.

1. Introduction

Gastrointestinal nematodes, (especially *Haemonchus contortus*) had been classified as a major health and welfare problem for small ruminants particularly in the tropics (Aumont *et al.*, 1997; Chandrawathani *et al.*, 1999; Geerts & Dorny, 1996; Hoste *et al.*, 2005; Jabbar *et al.*, 2006; Wolstenholme *et al.*, 2004)**.** Such parasites impair animal health, welfare and productivity since the gastrointestinal nematodes (Beauchemin et al.) infection results in increased death rate and poor growth and reproduction (Aumont *et al.*, 1997; Coop *et al.*, 1982; Dakkak, 1995; Hoste *et al.*, 2005). To date, the usual mode of control of gastrointestinal parasitism relies on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Aumont *et al.*, 1997; Jabbar *et al.*, 2006; Jackson & Coop, 2000; Wolstenholme *et al.*, 2004) in GIN populations motivates investigation towards alternative approaches. Phytotherapy is one of them, which is currently explored (Athanasiadou & Kyriazakis, 2004; Githiori *et al.*, 2006; Wolstenholme *et al.*, 2004). It is mainly based on the use of preparations of leaf and seed and the effects are attributed to secondary metabolites of plants. *M. Esculenta* (Cassava) is a highly productive tropical crop that is traditionally cultivates to produce roots for human consumption or for industrial extraction of starch. Cassava is also a plant of medicinal interest in human medicine in the Carribean (TRAMIL, 1999; Longuefosse, 2003): the leaf is used against headache and mycosis and also as an haemostatic. Buds and leaves are used as internal and external vermifugals. Previous studies (TRAMIL, 1999) reported that ethanolic extracts of leaf of Cassava had antiviral antimycobacterial activities and that fresh leaf was an inhibitor of protein synthesis. *In vivo* studies showed that Cassava foliage relieved gastrointestinal nematode egg excretion (Sokerya and Rodriguez, 2001; Nguyen et al., 2003; Sokerya and Preston, 2003; Nguyen et al., 2005). But no explanations were made about the active compounds. Depending on the varieties, the plant could contain variable quantities of acetone, oxalic acid, saponins, tryptophane. Aerial parts contain linamarin and lotaustralin, two cyanogenic glycosides. Leaf contains free quercetin, an anti oxidative, anti inflammatory, renal protective and venotonic flavonoid (TRAMIL, 1999); vitamin C, flavonic glucosides, alkans and sulfhydric acid (Longuefosse, 2003) and condensed tannins (Dung et al., 2005).

The possible secondary metabolites of *Manihot esculenta Crantz* (Cassava) with an anthelmintic activity are yet to be identified. As condensed tannin compounds are part of the secondary metabolites of Cassava leaf (estimated to 23g/kg of dry matter in Dung et al. 2005); they should be considered as potential anthelmintics. Nevertheless, other compounds might not be excluded.

Secondary metabolites may be extracted from the raw plant material by different solvents (water, dichloromethane (CH_2Cl_2), and methanol (CH_3OH)) according to the polarity of the molecules to be extracted (Balansard, 1991). For example hot water will extract heterosides, iridoids and tannins; methanol will extract tannins, catechins, terpenoids and alkaloids, and a more apolar solvent such as dichloromethane will extract semi polar and more apolar compounds (Balansard, 1991).

Several methods are commonly used to test nematicidal activity of both chemical drugs and plant extracts. Among them, *in vitro* assays revealed to be significant and cheaper than *in vivo* methods. The Egg Hatch Assay (EHA), (Hubert and Kerboeuf, 1984) and the larval development assay (LDA), (Hubert & Kerboeuf, 1992) are currently used for the detection of anthelmintic resistance in GIN (Coles *et al.*, 1992). The larval migration inhibition assay (LMI), (Rabel *et al.*, 1994) and the adult worm motility assay (AWM), (Hounzangbe-Adote *et al.*, 2005) allow a more realistic evaluation of the *in vivo* nematicidal activity. These four tests are based on the hypothesis that a nematicidal activity observed *in vitro* would be indicative for a potential *in vivo* activity.

The current study aimed to explore the anthelmintic properties of Cassava leaf against the gastrointestinal parasite *Haemonchus contortus* and to enquire on the phytochemical compounds involved in the activity. Several *in vitro* tests were performed with several plant extracts, to increase the chances to detect an anthelmintic activity.

2. Materials and methods

2.1. Plant extracts

Plant materials were collected in Guadeloupe (French West Indies). Cassava leaves were dried at 55°C in a ventilated oven, until constant weight. This operation allowed removing of toxic cyanogenic glycosides (Guillermo Gómez, 1985). The leaves were then lyophilised and ground. Thereafter, in order to separate the molecules depending on their polarity, three successive extractions were performed on the powder, first with dichloromethane (CH_2Cl_2), then with methanol (CH_3OH) and finally water, in order to extract separately most of the polar and semi-polar molecules depending on the solvent.

First, 50 g of powder of leaf were lixiviated by 500 ml of dichloromethane: the powder was moistened with 150 ml of solvent to cover it, and then let to maceration for 3 h sheltered from light.

Thereafter, the filtrate was removed while the residue was washed with the rest of the solvent (350 ml). The filtrates were collected together and evaporated at low pressure at 40°C.

The dichloromethane extraction residue of leaf was dried under vacuum and the same operation was performed on it, with methanol as extraction solvent.

Then, the residual leaves were dried under vacuum and extracted with 500ml of hot distilled water: 250ml of boiling water mixed with the powder, put to simmer, and filtered through a Büchner, and then 250ml of boiling water mixed with the following residue, put to simmer and filtered. The filtrates were lyophilised to obtain a powdered extract.

All the extracts (aqueous, methanolic and dichloromethane) were stored, sheltered from light and humidity, at 22°C.

2.2. *In vitro* anthelmintic assays

The anthelmintic efficacy tests of the 3 extracts of leaf of Cassava, on the different life-cycle stages of *H. contortus* were performed, using four different procedures.

For each assay, the four parasitic stages were obtained from Black Belly donor lambs which were experimentally infected by an oral administration of a pure 10 000 *H. contortus* third stage larvae (L3) suspension.

2.2.1. Egg hatch assay

The *in vitro* anthelmintic activity of the three extracts of leaf on the egg hatching of *H. contortus* was carried out according to a modification of the method used for testing anthelmintic resistance (Assis *et al.*, 2003). Eggs were extracted from the faeces of the donor sheep according to the method described by Hubert and Kerboeuf (1984).

After crushing the faeces in water, and successive siftings (500, 250, 125, 63, 50 and 30µm), eggs were collected and centrifuged for 10 min at 2000 rpm. The supernatant was removed and a NaCl solution (density 1.2) was added. After homogenization, the mixture was centrifuged for 15 min at 3000 rpm. The ring with eggs was then extracted by pouring it on a 32µm sieve and then by abundant washing first with distilled, and then with sterilised water. The egg suspension with a

concentration of 400 eggs per ml, was distributed in 24-well plates (0.5 ml per well). The leaf extracts and albendazole positive were diluted in PBS buffer (0.1 M phosphate, 0.05 M NaCl; pH 7.2) and were added to the wells (0.5 ml per well), to reach final tested concentrations of: 2400, 1200, 600, 300 and 150 µg/ml for the extracts; and of 0.5%, 0.25%, 0.125% for albendazole. In addition, PBS negative controls were also included in the assay. Five replicates were run per concentration.

After 48 hours of incubation at 25°C, egg hatching was stopped by adding Lugol's iodine solution. The number of L1 larvae and egg per well was then counted using a reverse microscope (at 100 X magnification). The percentage of hatched eggs was determined using the ratio: number of L1 / (number of egg + number of L1).

2.2.2. L1 to L3 stage development inhibition assay

The objective of this assay was to test the *in vitro* efficacy of the extracts to inhibit the larval development of *H. contortus* from the L1 stage to the infective L3 stage. The assay used was a modification (Assis et al., 2003) of the technique described by Hubert and Kerboeuf (1992).

L1-L2 stages larvae were obtained after culture of eggs extracted from the faeces of the donor lambs. The eggs were incubated at room temperature for 48 h in 24-multiwell plates (0.5 ml per well with a concentration of 400 eggs/ml). After hatching, 70µL of culture medium (for one plate: 10µg of autolytic yeast extract, 1.5 ml of distilled water and 170 µl of Earle's balanced solution, stabilised to pH= 7.2 with a $NaHCO_3$ solution at 50 g/L) was added to each one of the wells, followed by either each concentration of extracts (2400, 4800, 2400, 1200, 600 and 300 µg/ml), albendazole positive control (1%, 0.5%, 0.25%), all diluted in PBS buffer, and PBS negative control (0.5 ml per well and five replicates for each dose). Final tested concentrations for extracts were: 2400, 1200, 600, 300, and 150µg/ml. Final tested concentrations for albendazole were: 0.5%, 0.25%, and 0.125%.

The larvae were incubated for 8 days at room temperature (25°C), in order to permit the larval development from the first stage to the third infective stage. Thereafter, several drops of a Lugol's iodine solution were added and the number of larvae was counted using inverse microscope (at 40 X magnification) by separating L3 infective larvae from L1-L2 larvae. The percentage of development was calculated as the ratio: number of L3/ total number of larvae per well.

2.2.3. L3 migration inhibition assay

This test is aimed to evaluate the anthelmintic effect of the extracts on the migration capacity of the infective larvae L3. It was performed according to Rabel et al. (1994) in Hounzangbe-Adote et al. (2005).

Haemonchus contortus L3 were obtained by larval culture from the faeces of donor lambs. Eggs reached the L3 stage after 10 days. The L3 were then collected by sedimentation using Baermann's devices.

The anthelmintic effect of each extract was tested using final doses of 150, 300, 600, 1200 and 2400 µg of extract/ml PBS with 1000 L3 per dose. Negative (in PBS only) and positive controls (levamisole at final doses: 0.125, 0.25 and 0.5%) were also prepared in PBS and incorporated in the assay. Five replicates were run for each plant extract and for the controls. After 3 h of incubation at room temperature (25°C), the larval suspensions were rinsed out with PBS buffer, by 3 successive centrifugations (5 min, 4500 rpm). After the last washing, the suspensions were given out in Falcon® tubes fitted with 20µm sieves in such a way that the larval crossing through the sieve was a dynamic phenomenon. After 3h of incubation, the inserts were removed and the volumes were adjusted to 2 ml by adding PBS.

Larvae which have passed through the sieve (N= number of migrating larvae) were counted in a total volume of 500µL (10 drops of 50µL) using optical microscope (at 40 X magnification). The total number of migrant larvae was calculated as = N x 4. The percentage of migrant L3 was then calculated as = (N x 4 / 1000) x100.

2.2.4. Adult worm motility assay

The purpose of this assay was to test the anthelmintic effect of the extracts on adult worm motility. This test was performed according to Hounzangbe-Adote et al. (2005).

Adult worms were collected from an experimentally infected lamb, four weeks after infection. Immediately after slaughtering, the abomasum was removed, opened and placed in 37°C saline. The mobile worms were rapidly collected and put into 24-multiwell plates, 3 worms per well in 2 ml of 37°C saline. After one hour of incubation (37°C, 5% carbon dioxide), the saline was removed from each well and replaced by 1ml of either one of the different concentrations of extracts (1200, 600, 300, 150 and 75 µg/ml) or of the levamisole positive control (0.125, 0.25, 0.5, 1%), or of the PBS negative control. All dilutions were performed in PBS plus penicillin/streptomycin. The incubation

media were replaced after 24 h. Adult worm motility was evaluated by observation under magnifying glass and evaluated as the ratio: number of motile worms/ total number of worms per well.

2.3. Phytochemistry

2.3.1. Phytochemical screening

The phytochemical tests were accomplished following classical methodologies (Dohou et al., 2003). These tests are based on the addition of specific reagents to the ground leaf and stem of *M. paradisiaca*, directly or after an infusion, and observing changes of the solution colours, or precipitate formation. The presence of the several chemical compounds was evaluated: phenols, proanthocyanidols, anthocyans, alkaloids, coumarins, saponosides, tannins (catechic and galic), triterpens and sterols, quinones, anthracenic compounds, flavonoids and flavans.

2.3.2. TLC analysis

A qualitative thin layer chromatographic (TLC) analysis on silica gel (Al coated Kieselgel 60 F_{254} (Merck) plates), was performed on each plant extracts, in chloroform/acetic acid/methanol/water (64:32:12:8, v/v/v/v) migration system. After migration, TLC was first inspected under UV-254 nm and UV-365 nm light to detect flavonoid and phenolic fluorescent or coloured spots. Vanillin-sulfuric acid reagent was then employed for revealing terpenoids (triterpens and saponins) and phenolic spots (Wagner, 1996).

2.3.3. Determination of condensed tannins content

The condensed tannins (proanthocyanidin) content was determined by the vanillin-HCL method (Nakamura et al., 2003). Condensed tannins from Cassava were isolated using Sephadex LH-20 (Giner-Chavez et al., 1997) and used as standard in the vanillin-HCL method. Quebracho CT was also used as standard for determination of CT, as indicated by (Hagerman and Butler, 1989).

2.3.4. Reversed phase HPLC analysis

High performance liquid chromatography (HPLC) analysis was performed, for condensed tannins (CT) separation in the extracts, using: a liquid chromatograph (Varian® Prostar pump 210/18), a rheodyne

injection valve with a 20 µl loop, a diode array detector (DAD, Varian® Prostar 335) and the Galaxy 1.9 software for integration. The extracts were analysed using a Microsorb 100-5 C18, S250 x 4.6 column and operated at room temperature (23°C). The assay was performed according to a modification of the method of (Marin-Martinez et al., 2008). The CT were eluted for 15 min at 1 ml/min using an isocratic system. The mobile phase consisted of a mixture acetic acid: acetonitrile: water (2:28:70, v/v/v). The solution was degassed using inline Degassit Power Supply (Varian® Prostar 210 to 240 V, 10ml/min). Quebracho is a plant extract with high CT concentration, with direct activity against *H.contortus* larval survival (Athanasiadou et al., 2001).

Quebracho CT and extracted CT from Cassava leaf were used as reference for qualitative estimation of the different quebracho-like CT in the different extracts of Cassava leaf. The separated compounds were identified at 280 nm (CT absorption wavelength zone is around 280-300 nm) using the DAD on the basis of chromatographic retention times, UV spectra and co elution with the quebracho CT.

2.4. Statistical analysis

The data for EHA, LDA and LMI assays were analysed by the general linear model (GLM) procedures using Minitab® Release 14 software. Efficacy was determined by multiple comparisons procedure using the Bonferroni method. Dose response level was analysed using regressions procedures (simple and probit method) with Minitab® Release 14 software. For the AWM assay, the data were analysed by the mixed procedures using the Glimmix macro of the SAS software to determine the significance level of difference between the extracts and the positive and negative controls.

3. Results

3.1. *In vitro* anthelmintic assays

3.1.1. Egg hatch assay

For the PBS negative control, the mean hatching rate measured was 88.6% (±0.6). The extracts of leaf of Cassava showed no significant effects ($P > 0.05$) on egg hatching compared to the PBS negative

control (Aqueous extract: 87.6% ±2.1; methanolic extract: 92.2% ±1.1; dichloromethane extract: 86.2% ±3.4). For the albendazole positive control, the mean hatching rate measured was 23.07% (±1.4). This value was significantly different from the PBS negative control (P <0.0001).

3.1.2. L1 to L3 stage development inhibition assay

The mean development rate was 95.6% (±0.9) for the PBS negative control (table 1). The methanolic extract (57.6% ±7.6) showed significant efficacy (P <0.0001) compared to the negative control. Regression analysis showed that a dose dependent effect was observed, within the concentration range tested (P=0.002; R^2=97.5%; larval development = 96.2% - 0.0417 dose). The calculated IC50 was 0.85 mg/ml of methanolic extract of leaf. Nevertheless, the methanolic extract was less efficacious (P<0.0001) than the albendazole positive control (86.5% ± 1.8 of mean development inhibition rate) for which IC50 was 0.78 µg/ml.

3.1.3. L3 migration inhibition assay

For the PBS negative control, the larval migration mean was 89.1% (±3.7). The extracts of leaf of Cassava showed no significant effects (P > 0.05) on larval migration compared to the negative control (86.2% ±5.5 migration rate mean for the different extracts of Cassava leaf). For the levamisole positive control, the mean larval migration rate measured was 0.39% (±0.25). This value was significantly different from the PBS negative control (P <0.0001).

3.1.4. Adult worms motility assay

The levamisole positive control exhibited less motility than the negative control (P <0.001), after 24h incubation time. The mean percentages of motility measured after 6 and 24 hours was 78.1% (±0.1) and 36.3% (±0.2) respectively. There was a time dependent effect of the treatments: after 48h incubation time, no difference of motility was observed between the several treatments and controls. Compared to the PBS negative control, no efficacy of the extracts of Cassava leaf was observed (P>0.05) between 6h and 24h incubation times (motility rate mean after 24h for PBS: 68.9% ±5.3; motility rate mean for Cassava leaf extracts: 75.5% ±5.1).

3.2. Phytochemistry

3.2.1. Phytochemical screening

The phytochemical screening indicates that Cassava leaf contained phenols, catechic tannins: proanthocyanidols (condensed tannins), flavonoids, flavans, coumarins and triterpens and sterols. Polyphenolic compounds were mainly detected, with amajority of catechic tannins. No galic tannins (hydrolysable tannins) were detected. The chemical composition of Cassava leaf, according to this phytochemical screening is presented in Table 2.

3.2.2. Thin layer chromatography

Within the migration system used, some compounds of the two aqueous and methanolic extracts of leaf possess the same Retention factor (Rf). Nevertheless the aqueous extract contains two fluorescent compounds (blue spots at 365 nm) absent from the methanolic extract of leaf. This last one also countains terpenoid compounds revealed by blue spot with Vanillin sulfuric reagent and UV detection.

Observation under 365 nm UV light (yellow, blue and red fluorescent spots) indicates the presence of many flavonoid and phenolic compounds in the leaf (methanolic and aqueous extracts). Observation under 254 nm UV light (quenching zones) indicated the presence of three compounds both in the aqueous and methanolic extracts of Cassava leaf. These compounds are yellow in visible and are majority compounds. These are certainly flavonoid compounds. The aqueous and methanolic extracts of leaf contained condensed tannin compounds (red spots with Vanillin sulfuric reagent).

3.2.3. Condensed tannins content

Cassava condensed tannins (CT) content with quebracho CT as standard were lower than with Cassava CT standard. No detection was possible for dichloromethane extract because of the formation of a precipitate. The results are presented in Table 3.

3.2.4. HPLC analysis

HPLC analysis indicates that several compounds from the three Cassava leaf extracts absorbed at 280 nm. UV spectra analysis of quebracho and Cassava leaf CT indicates that most compounds had max absorbances around 280-300 nm. UV spectral data of the three Cassava extracts and 280-300 nm spectra were extracted and compared to UV spectral data from Cassava extracted CT and from quebracho CT. The results are presented in Table 4.

The UV spectra of the Cassava leaf extracts indicate that several compounds had max absorbance around 280-300 nm (Table 4), with overlapping retention times due to the presence of many isomers with similar polarity. Cassava leaf had CT family compounds in common with quebracho CT. Analysis of max absorbance of the UV spectra showed that the methanolic extract of Cassava leaf contained a specific family of condensed tannins-like compounds (CT compound H, table 4).

4. Discussion and conclusion

The results indicate that Cassava has an anthelmintic property on *H. Contortus* and particularly on the larval development of the parasite for which the methanolic extract of leaf gave the more consistent result. The literature is void of *in vitro* studies on Cassava anthelmintic activity against *H. Contortus* parasite.

According to literature (Longuefosse, 2003) and the current study with TLC analysis and phytochemical screening, Cassava leaf contains several secondary metabolites of medicinal interest: terpenoids, flavonoids, condensed tannins and other phenolic compounds. Mainly phenolic compounds were detected with a majority of condensed tannins. Both aqueous and methanolic extracts of leaf contained condensed tannins (CT), and the methanolic extract showed the best efficacy. The methanolic extract of leaf was different from the aqueous extract as it contained terpenoid compounds and specific CT compounds.

Terpenoid compounds are known to be active against a large range of organisms. Some of them can be bioactive whereas others can affect physical variables (Bruneton, 1999). In the current study, the larval development could have been stopped by terpenoids present in the methanolic extract of leaf; as this class of molecules has already been shown to stop the *H. contortus* larval development (Maciel et al., 2006; Camurça-Vasconcelos et al., 2007). Molan et al. (2003) have shown that terpenoids reduced the mobility and the consequent migration ability of ovine nematode larvae.

Forages containing condensed tannins have biological effects on the control of gastrointestinal nematodes of ruminants (Sokerya and Rodriguez, 2001; Nguyen et al., 2003; Sokerya and Preston, 2003; Nguyen et al., 2005). The vanillin-HCl assay showed that Cassava leaf contained CT. The use of two different standards showed the limitation of the method as the quebracho standard underestimated the CT content. This is due to multiple structure possibilities of CT between different plant species. That is the reason why CT standard directly purified from the plant to analyse is better for determination of CT content. Nevertheless, the present study allows ascertaining that Cassava leaf contains flavan-3-ol and procyanidin polymers, because the vanillin-HCl reagent specifically interacts with these molecules (Nakamura et al., 2003).

Two non exclusive hypotheses have been proposed to explain the anthelmintic effect of CT against GI nematodes, either directly, or indirectly, through the stimulation of the local host mucosal response. However, most current data supports the hypothesis of direct effect, mediated through condensed tannins - nematode interactions, which affect the physiological functions of different stages of the gastrointestinal parasites (Molan et al., 1999; Athanasiadou et al., 2000; Molan et al., 2000; Athanasiadou et al., 2001; Molan et al., 2002; Brunet and Hoste, 2006; Hoste et al., 2006; Brunet et al., 2007; Brunet et al., 2008). The direct mechanism of action of the CT plant resources is also strongly supported by *in vitro* trials. CT from *Leucaena leucocephala* (Ademola and Idowu, 2006) were effective at reducing the migration of ex-sheathed larvae, and the development of nematode eggs to the third larval stage was found to be reduced in the presence of CT (Kahn and A.Diaz-Hernandez, 2000; Alonso-Diaz et al., 2008a; Alonso-Diaz et al., 2008b). Condensed tannins have been shown to disrupt larval development of the nematode *Trichostrongylus colubriformis* (Molan et al., 2003). Therefore, condensed tannins may counteract parasites by one or more mechanisms, and this might differ depending on the nature and composition of condensed tannins from different forage species (Nguyen et al., 2005, Brunet and Hoste, 2006; Brunet et al, 2007). In the current study, HPLC analysis showed that Cassava leaf contained several families of CT common and not common with CT of quebracho.

Quebracho CT do not have effect on larval development but on larval viability (Athanasiadou et al., 2001), whereas Cassava leaf had effect on larval development. The common CT with quebracho were present in the dichloromethane extract of leaf, which is not efficacious against larval development either. As these CT are from the same families, hypothesis could be made that they have the same target and that the dichloromethane extract of Cassava leaf could be efficacious on *H. Contortus* larvae viability too.

Furthermore, the methanolic extract of Cassava leaf contains a specific CT family, which could explain its specific nematicidal activity against the larval developmental stage of *H. Contortus.*
The different trials were also performed in order to screen the activity and to search for the mode of action of compounds which have the ability to disrupt the life cycle of *H. contortus*. The fact that only larval development stage was affected shows that the active compounds are not capable to cross or to damage the cuticle of eggs, L3 larvae and adult worms. It has been shown that transcuticular diffusion was a common route for the uptake of major broad-spectum anthelmintics such as levamisole, benzimidazole and ivermectin (Geary et al., 1999). Hypothesis could be made on a hydrophilic structure of the anthelmintic compounds of Cassava, as lipophilic anthelmintics have a greater capability to cross the external surface of the helminths than the hydrophilic compounds (Geary et al., 1999), which comforts the hypothesis of CT as active principles in Cassava leaf.

Further *in vivo* investigations are needed to validate the activity of Cassava. *In vitro* trials suppose direct action on parasite and cannot be sufficient to conclude for the anthelmintic efficacy because results obtained on *in vitro* conditions could be different from *in vivo* conditions. This might be due to the gastrointestinal tract of ruminants. This kind of contrast was obtained with condensed tannins for which *in vitro* and *in vivo* trials gave different results (Athanasiadou *et al.*, 2001; Paolini *et al.*, 2005). Moreover, *in vivo* study will allow studying indirect effects of the plant on parasitism and animal.

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Table 1: Larval development assay results: L1 to L3 *H. contortus* larval development means (% L3) for the different extracts of cassava leaf, compared to PBS (Phosphate buffer sample) negative control and to albendazole positive control.

Products	**% L3 (±SE)**	**P value/albendazole**	**P value/ PBS**
Albendazole	13.5 (±1.8)	-	<0.0001
PBS	95.6 (±0.9)	<0.0001	-
Cassava leaf A	91.7 (±2.2)	<0.0001	0.33
Cassava leaf M	57.6 (±7.6)	<0.0001	<0.0001
Cassava leaf D	99.0 (±0.4)	<0.0001	0.51

A: Aqueous; M: Methanolic; D: Dichloromethane

Table 2: Results of the phytochemical screening of Cassava leaf

Compounds	**Score**
Phenols	+
Tannins	++++
Galic tannins	-
Catechic tannins	++++
Flavonoids	+++
Anthocyanins	-
Flavans	+++
Proanthocyanidols	++++
Quinones	-
Triterpens and sterols	++
Saponins	-
Alkaloids	-
Coumarins	+
Anthracenic compounds	-

- : Negative; +: Weak coloration; ++: Moderate coloration; +++: strong coloration; ++++: very strong coloration

Table 3: Condensed tannins content in cassava leaf extracts, with the vanillin–HCl method using either quebracho or cassava condensed tannins as standard.

Cassava extracts	**CT content (%±SE)** **with quebracho CT as standard**	**CT content (%±SE)** **with cassava CT as standard**
Methanolic extract of leaf	4.3 (± 0.2)	10.0 (± 0.7)
Aqueous extract of leaf	14.1 (± 1.0)	25.0 (± 0.8)
Dichloromethane extract of leaf	No detection possible	No detection possible

Table 4: Comparisons for condensed tannins compounds families(obtained by HPLC) between Quebracho and Cassava leaf extracts, with relative retention times for the different families, and UV spectral data.

Unknown CT families	**Relative retention time (min ± SE)**	**UV spectral data**	**Plant extracts (number of compounds)**
A	3.0	230-280-600	Quebracho CT (1)
B	3.4 ±0.3	230-280-350	Quebracho CT(3), Cassava D(2), Cassava CT(2)
C	3.5	215-240-280	Cassava CT(1)
D	3.5 ±0.2	210-265-280	Cassava(1), Cassava D(1), Cassava CT(1), Cassava M(1)
E	3.6	215-280-350	Cassava D(1)
F	4.7	198-228-248-279	Cassava D (1)
G	5.4	230-280-320	Cassava A (1)
H	5.6 ± 0.9	230-250-280	Cassava M (3)
I	6.2	230-280-400	Cassava A (1)

CT: condensed tannins; A: aqueous; D: dichloromethane; M: methanol

Condensed tannins compounds families: absorption zone at 280-300 nm.

UV spectral data for the different families: 190 to 400 nm or more for differentiation.

Article n°6

Effect of banana foliage (*Musa x paradisiacal)* on nutrition, parasite infection and growth of lambs

C. Marie-Magdeleine et al.

Soumis à Livestock Science

Abstract:

Two successive completely randomized feeding trials were performed to test the nutritional value and the anthelmintic properties of 12-month-old banana foliage in lambs. In the first trial forty 5-month-old Martinik lambs (body weight: 17.3 ± 4.1 kg) were divided into four groups and placed in individual pens: 1) two groups of eight control lambs fed hay (1000 g/lamb/day) + commercial pellet (250 g/lamb/day) or banana foliage (7000 g/lamb/day) + commercial pellet (300 g/lamb/day); 2) two groups of 12 infected lambs fed hay (1000 g/lamb/day) + commercial pellet (250 g/lamb/day) or banana foliage (7000 g/lamb/day) + commercial pellet (300 g/lamb/day). Diets were formulated to be isoenergetic and isonitrogenous. In the second trial, twenty 6-month-old Martinik lambs (body weight: 21.2 ± 4.9 kg) were divided into two groups and placed in individual pens. The two groups were artificially infected with *Haemonchus contortus* and fed the following diet for 21 days: hay (1000 g/lamb/day) + commercial pellet (250 g/lamb/day). Then a group continued on the hay diet and the other group was fed banana foliage (7000 g/lamb/day) + commercial pellet (300 g/lamb/day) + CMV block.

Low growth rates were recorded in lambs, which were linked to low intake of digestible dry matter. During the first trial there were no differences between groups in the indicators of parasitism infestation, illustrating the lack of effect of banana foliage against *Haemonchus contortus* in lambs. In the second trial a significant positive effect of banana foliage on faecal egg count was observed, illustrating the probable effect of banana foliage on the fecundity of adult worms.

Keywords: anthelmintic properties, banana foliage, gastrointestinal nematodes, growth, nutritional values, small ruminants.

1. Introduction

Gastrointestinal parasitism by nematodes (especially *Haemonchus contortus*) has been classified as a major health and welfare problem for small ruminants, particularly in the tropics (Chandrawathani et al., 1999; Fournet, 1993; Geerts and Dorny, 1996; Hoste et al., 2005; Jabbar et al., 2006a; Lefevre et al., 2003; Wolstenholme et al., 2004), impairing animal productivity (Aumont et al., 1997; Coop et al., 1982; Dakkak, 1995). To date, the usual mode of control of gastrointestinal parasitism has relied on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Jabbar et al., 2006b; Jackson and Coop, 2000; Wolstenholme et al., 2004) in gastrointestinal nematode (GIN) populations has motivated investigation into alternative approaches such as phytotherapy (Athanasiadou and Kyriazakis, 2004; Githiori et al., 2006; Wolstenholme et al., 2004). Protein supply can reduce the effect of parasitism on small ruminants. Moreover, consumption of plants containing condensed tannins (CT) may affect the resistance and resilience of ruminant livestock to parasitic gastrointestinal (GI) nematodes in both indirect and direct ways (Kahn and Diaz-Hernandez, 2000). Consequently resources that are rich in condensed tannins and proteins, like legumes, are good targets for studies on the anthelmintic properties of plants. Moreover these resources could be included in a sustainable system of production.

*Musa x paradisiaca L.*is a plant of medicinal interest in human medicine in the Caribbean (TRAMIL, 1999; Longuefosse, 2003): the leaf and stem are used to treat diarrhoea, the stem is good for asthenia and wounds, and the leaf for the treatment of inflammation, headache and rheumatism. Previous studies (TRAMIL, 1999) have reported that *M. paradisiaca* has antimicrobial and healing activities. Nevertheless, only a few studies have reported on the efficacy of this plant against nematodes. Sharma et al. (1971) tested aqueous extracts of *M. paradisiaca* root on adult *H. contortus* and found significant activity at a 5% level with a 1 in 25 concentration. Batatinha et al. (2005) tested the *in vitro* effect of aqueous extracts of leaves of *Musa cavendishii Linn*. on *Strongyloidea* larvae of goats and found a reduction of development of more than 95% at a concentration of 130.6 mg/ml. Marie-Magdeleine (2009) tested extracts of *Musa x paradisiacal* foliage *in vitro*. The author reported the ability of some extracts to reduce adult worm motility and suggested the anthelmintic properties of *Musa x paradisiaca L.*stem and leaf against *H. contortus*.

Nevertheless, few studies have been realized *in vivo*. *Musa acuminata* was tested *in vivo* on goats experimentally infected with *H. contortus* (Vieira et al., 1999) and it did not significantly reduce the number of adult worms. Sokerya and Rodriguez (2001) reported the ability of banana leaves to reduce the number of Trichostrongyle eggs in faecal samples of goats.

The first objective of this study was thus to test *in vivo* the effect of banana foliage against *H. contortus* in lambs and to explain the observed action. The second objective was to evaluate banana foliage as a source of forage.

2. Materials and methods

2.1. Experimental site and feed ingredients

The study was conducted in the fields and facilities of the experimental unit ("Plateforme Tropicale d'Expérimentation sur l'Animal") of an INRA research unit (Unité de Recherches Zootechniques) in Guadeloupe, French West Indies. The ingredients used to prepare the experimental diets were based on hay, *Musa paradisiaca* foliage (stem and leaf), and a commercial supplement pellet. The hay used comprised a spontaneous mix based on *Dichanthium* spp., the original forage of which was grown with irrigation and inorganic fertilization in Grande Terre, northeast Guadeloupe. Banana foliage was collected daily from a 12-month-old banana plantation after the harvesting of the fruits. The foliage was coarsely chopped before distribution to animals.

2.2. Animals, experimental diets, management and design

Animals were reared following European Union recommendations for animal welfare in accordance with the regulations of the Animal Care Committee of INRA. Two successive trials were performed.

Trial 1

Forty Martinik lambs (body weight (BW): 17.3 ± 4.1 kg; 5-months-old) were used in a completely randomized design for a feeding trial lasting 57 days (21 days of adaptation and 35 days of data collection). Animals were divided into four groups (two containing 8 control lambs and two containing 14 lambs that were later infected) and placed in individual pens. The different groups of lambs were selected according to their weight at weaning, their body weight at the beginning of the study, and their average daily growth between 30 and 70 days during suckling, ensuring similar values in each group. To ensure the experiment began worm-free, lambs were all previously orally administered Cestocure® and Hapadex®.

Diets consisted of *ad libitum* 45-day-old *Dichanthium spp.* hay or banana foliage with commercial supplement pellets according to the experimental treatment as follows (fresh matter basis):

- Control 1: Hay (1000 g/lamb/day) + commercial pellet (250 g/lamb/day) + CMV block
- Control 2: Banana foliage (7000 g/lamb/day) + commercial pellet (300 g/lamb/day) + CMV block
- Hay infected: Hay (1000 g/lamb/day) + commercial pellet (250 g/lamb/day) + CMV block
- Banana infected: Banana foliage (7000 g/lamb/day) + commercial pellet (300 g/lamb/day) + CMV block

Diets were formulated to be isoenergetic and isonitrogenous. The amounts of hay and banana foliage provided (*ad libitum*) were calculated to allow at least 10% refusal per lamb. The commercial supplement pellets were offered daily at 07.00 h and were completely consumed before 08.00 h. The hay and banana foliage were distributed at 08.30 h. Animals had free access to water and to a mineral block.

At the end of the 21-day adaptation period, all lambs of the two infested groups were orally infected with a single dose of 5,000 third-stage larvae (L3) of *Haemonchus contortus*. The same operation was repeated on day 35. The L3 infective larvae of *H. contortus* were obtained 42 days before challenge, from cultures of faeces taken from lambs monospecifically infected with isolates previously obtained from sheep reared on pasture at different farms in Guadeloupe (Aumont and Cabaret, 1999).

Trial 2

The lambs in the control group in trial 1 were used in trial 2. They were 6 months old and weighed 21.2 ± 4.9 kg. Animals were divided into two groups of eight, and placed in individual pens. The groups of lambs were selected according to their weight at weaning, their body weight at the beginning of the study, their average daily growth between 30 and 70 days during suckling, and their average daily growth during trial 1, ensuring similar values in the two groups. During 21 days of adaptation, the diets of all the lambs consisted of *ad libitum* 45-day-old *Dichanthium spp.* hay with commercial supplement pellets as follows (fresh matter basis): Hay (1000 g/lamb/day) + commercial pellet (250 g/lamb/day) + CMV block. At the end of the 21-day adaptation period, all lambs of the two infested groups were orally infected with a single dose of 5,000 third-stage larvae

(L3) of *Haemonchus contortus*. The same operation was repeated on day 35. Twenty-one days after the first challenge of L3, one group of 8 lambs received a new diet: Banana foliage (7000 g/lamb/day) + commercial pellet (300 g/lamb/day) + CMV block.

2.3. Measurements

The lambs were individually weighed at the beginning of the experiment, and then fortnightly until the end of the trial (57 days). Daily live weight gain (LWG, g/day), estimated from final minus initial body weight, was then calculated fortnightly.

Daily dry matter intake for each ingredient, total intake of dry matter (DM), organic matter intake (OM), Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and crude protein (CP) were determined and expressed either on a daily or metabolic weight basis. The Feed Conversion Ratio (FCR = Feed intake / Daily live weight gain) was estimated for each treatment. Feed intake was recorded from Monday to Friday throughout the experiment and for each lamb. It was considered to be the difference between the daily amount of food offered and refused. Samples of offered forage (two sub-samples of 200 g) and refusals for each animal were taken daily. One of the sub-samples was kept for daily dry matter determination.

All the samples of the feed provided during one week were mixed together for each animal and a new sub-sample (200 g) was used for chemical analyses. The same operation was carried out for the refusals for each animal. Feed intake measurements and analyses were carried out for all 30 lambs.

The lambs were fitted with faecal bags in order to study the digestibility of the diet. Two measurement periods of total tract digestibility (each one lasting 5 days), were carried out during the experiment. The bags were emptied every 12 h. The daily quantity of faeces was then weighed. After weighing, ten percent of the total faeces was removed to make up a weekly sample for each lamb. This sample was used for dry matter determination and for chemical analyses. Total tract digestibility was estimated as: (Feed intake – excreted faeces)/Feed intake.

Faecal samples were collected weekly for 6 weeks to determine faecal egg count (FEC) using a modified McMaster method for rapid determination (Aumont, 1997). Monitoring started 2 weeks before artificial infection with L3 larvae of *H. contortus*.

Faecal samples of every lamb, sampled on last week, were cultured for 10 days at room temperature each month, following the Roberts and O'Sullivan (1952) procedure to determine the development of faecal eggs. The identification of the third stage larvae was based on the key described by Gruner and Raynaud (1980).

Blood was sampled (EDTA-coated tubes, Becton Dickinson, Plymouth, UK) from each animal through the jugular vein puncture method, at the same time as the faecal samples were taken. The number of circulating eosinophils was measured according to the method of Dawkins et al. (1989). Eosinophils were counted with a Malassez cell counter. Packed cell volume (PCV) was also estimated using a capillary microhaematocrit (centrifuged for 5 min at 5000 rpm).

Thirty-five days after infection, all lambs were slaughtered for worm recovery and enumeration (Gaba et al., 2006). The abomasum was removed from the animal less than 15 min after slaughter and immediately processed. Formalin preservative (35% ethanol, 2.5% formol, qsp 1L distilled water) was added to each aliquot of worms removed (5% v/v). Worms were counted in each aliquot under a binocular magnifying glass, separating the males from females.

Female worms were stored in formalin preservative (5% v/v) for later size measurement and *in utero* egg count (Kloosterman et al., 1978).

Liver, kidney, and intestinal mucous membranes were examined to identify necrotic damage and gastritis, as an indicator of eventual toxicity.

In trial 2, the measurements were the same as in trial 1, except for growth.

2.4. Chemical analyses and analytical procedures

The DM content of feeds and faeces was determined by oven-drying to a constant weight at 60 °C for 48 h (AOAC, 1997) while ash content was determined by heating samples at 550 °C for 4 h (AOAC, 1997). Dry samples were obtained for further chemical analyses and were ground (to 1 mm). The CP content was calculated after N determination by combustion using the micro-Dumas method.

The methods of Van Soest et al. (1991) were followed to determine NDF and ADF (sequentially) on an ash-free basis using the Ankom^{2000} Fiber Analyser incubator. Total polyphenol content was determined using the Folin-Ciocalteu method (Singleton et al., 1998). Condensed tannins (CT)

content was determined by the vanillin-HCl assay (Nakamura et al., 2003), using extracted banana foliage CT as the standard (Giner-Chavez et al., 1997).

2.5. Statistical analysis

Intake, total tract digestibility, ADG PCV, FEC, and eosinophil data were analysed using the GLM procedure of SAS (SAS software, V8.01, SAS Institute Inc., Cary, NC, USA). PCV and FEC data were also log-transformed (log (FEC+1)) before analysis. In the first trial, the statistical model included the fixed effect of diet, infestation status, time and the interaction diet*infestation status. The percentage of faecal dry matter was taken into account as a covariable for FEC analysis. Differences between means were tested using the pdiff option. For all traits, significance was declared at probability levels of ≤ 5%, while tendencies were discussed between 5 to 15%; comparisons between means were tested using the LSMEANS test. In the second trial, the statistical model included the fixed effect of diet, time and the interaction diet*time.

In trial 1 and 2, abomasal worm count variables were analysed as a completely randomized design using the GLM procedure with diet as a fixed effect.

3. Results

3.1. Trial 1

3.1.1. Chemical composition of ingredients

Table 1 shows the mean values for the chemical composition of the ingredients of the diets. The CP content of the hay was lower than that of the banana foliage. The total polyphenol content of banana foliage was high whereas condensed tannin content was negligible.

3.1.2. Blood parameters (blood eosinophil counts, packed cell volume) and faecal egg count

Eosinophil counts were similar in the four groups of lambs. There was no difference in PCV values between the four groups of lambs (Table 2) over the entire experimental period. PCV ranged between 26 and 32%. Values recorded in infected lambs were lower than those of non-infected lambs but no difference was observed between the two groups of infected lambs. There was no difference in egg counts between the two groups of infected lambs.

3.1.3 Abomasal worm count

There was no significant difference in male, female and total worm count in the abomasum between the three groups of lambs, although numbers were higher in animals fed banana foliage. With regard to female worm size, there was no difference between groups (Table 2).

3.1.4. Intake

Table 3 shows the mean values for feed intake for the different diets and groups of lambs. The total dry matter intake was significantly higher in the group fed the diet containing banana foliage than in the hay groups, whereas there was no significant difference within the same diet. The tendency was the same for organic matter. The intake of NDF was similar for the four groups of lambs whereas ADF and ADL intakes were lower in groups of lambs fed hay. CP intakes were also lower for groups of lambs fed hay. There was no difference in digestible organic and digestible protein expressed per $LW^{0.75}$ between the four groups of lambs.

3.1.5. Total tract digestibility

Table 4 shows the mean values for feed total tract digestibility. There were no significant differences in the digestibility of DM, OM NDF ADF and CP between groups of lambs fed the same diet. The digestibility of NDF and ADF was significantly lower in groups of lambs fed banana foliage.

3.1.6. Growth and feed conversion

The effect of the treatments on live weight gain (LWG) is shown in Table 5. No significant difference was recorded between the treatments. There was no significant difference in the Feed Conversion Ratio (FCR) between the different diets.

3.2. Trial 2

3.2.1. Intake

The lamb intakes recorded in trial 2 were similar to those in trial 1. There was no difference (*P* = 0.43) in organic matter intake (g/kg $LW^{0.75}$) between groups of lambs fed banana foliage versus hay (54 *vs.* 55 ± 2.0). Crude protein intake was also similar (*P* = 0.10) in groups of lambs fed banana foliage versus hay (6.8 *vs.* 6.4 ± 0.2). Digestible organic matter intakes were also similar (*P* = 0.86) in the two groups of lambs (39 *vs*. 37 ± 3.5) and there was no difference (*P* = 0.45) in digestible crude protein intake (4.1 *vs*. 4.8 ± 0.6). The intake of hay was low (35g kg $LW^{0.75}$**)** but was consistent with the low quality of the forage.

3.2.2. Blood parameters (blood eosinophil counts, packed cell volume), faecal egg count and egg development

Eosinophil count tended (*P* = 0.13) to be higher in the groups of lambs fed hay (Table 6). There was no difference between the two groups of lambs in PCV values (Table 6) over the entire experimental period.

At the beginning of egg excretion (21 days after larval infection), just before the change of diet, there was no difference (*P* = 0.80) between the two groups of lambs in terms of FEC. The mean values of FEC were 5034 and 4176 (±2485) for the groups that were to be fed banana foliage and hay respectively. After 21 days (Table 6), the mean values (28 and 35 day) of FEC were significantly lower in lambs fed banana foliage compared with those fed hay. The rate of egg development was significantly higher in the group of animals fed with hay compared to banana foliage.

3.2.3. Abomasal worm count

There was no significant difference between the two groups of lambs in terms of male, female and total worm count in the abomasum, although numbers were higher in those fed banana foliage. With regard to female worm size, there was no difference between groups (Table 6).

4. Discussion

4.1. Nutritive value of banana foliage

4.1.1. Chemical composition of feeds

The experimental hay used in this experiment was very poor quality, as illustrated by its crude protein content. The chemical composition of banana foliage used in this experiment was close to values reported in the literature for leaves (Geoffroy, 1980). The CP content of the leaves was significantly higher than that of the stem. The CP value reported in this study for the foliage (stem + leaves) was close to that of the leaves. Few data are available on the secondary compounds present in banana foliage. Concerning tannins, Simmonds (cited by Greoffroy, 1980) indicated that banana trees may mainly contain phenolic compounds. Marie-Magdeleine (2009) also reported significant levels of total polyphenols. Only some of these polyphenols were tannins. In this study a very low level of condensed tannins was found in the banana foliage. Marie-Magdeleine (2009) indicated that these tannins were mainly found in the leaves.

4.1.2. Feed intake and digestibility

The voluntary diet intake of lambs during this study was low to medium. The low hay quality on the one hand and the low level of banana foliage dry matter on the other hand, explain this result. Previous studies (Ffoulkes and Preston, 1977; Martinez et al., 1980; Ruiz and Rowe, 1980) on intake of banana foliage fed to a young bull (200 kg) reported values ranging from 18–36 g/kg $LW^{0.9}$ and 13–21 g/kg $LW^{0.9}$ for leaves and stem respectively. Geoffroy and Despois (1978) reported values of 11 and 22 g/kg $LW^{0.9}$ in milking goats for stem and leaves respectively. No data exist for lambs. Nevertheless, comparative studies between cattle and sheep did not indicate differences in voluntary intake when expressed as $LW^{0.9}$ (Graham, N. McC, 1972). Consequently it can be hypothesized that the DM voluntary intakes of the banana foliage (25–27 g/kg $LW^{0.9}$) recorded for our experimental lambs were close to the highest values and probably correspond to the maximum value of intake for this diet. Based on reported data for banana leaves it can be hypothesized that banana leaf selection could increase intake by 60%. This low level of intake relative to better quality forage is probably due to the low level of dry matter in the banana foliage.

The dry matter total tract digestibility recorded for the mixed banana diet was similar to values recorded in cattle fed banana stem as the only ingredient of the diet (Ffoulkes and Preston, 1977). This result seemed coherent because the experimental diet contained banana leaves, which are known for their lower digestibility compared with the stem (Ffoulkes and Preston, 1977; Ruiz and

Rowe, 1980). The lower total tract digestibility recorded for the total NDF and ADF of the banana diet is probably due to the high level of polyphenol compounds in banana foliage.

The balance between intake and total tract digestibility allowed a similar level of digestible dry matter intake and consequently of energy by the four groups of lambs. For the first trial, based on a value of 3600 kcal per kg of digestible organic matter (INRA, 1988), the mean energy intake of the experimental lambs was 1340 kcal. As mentioned, the diets were isoenergetic. In contrast, nutritional protein, based on digestible protein intake, was higher for lambs fed the banana foliage diet compared with the hay diet (207 *vs.* 126 g/day).

4.1.3. Growth performance

The growth potential for Martinik sheep is 150–200g /d (Archimède et al., 2008). The growth recorded in this study was low in comparison to the potential of these animals. Based on the energy intake and animal requirements of low growth European breeds (INRA, 1988), the expected growth was 150 g/d. In comparison to energy, the amounts of protein were not a limiting factor for growth. Nevertheless, the energy intake in this study was 10 to 30% lower than the energy intake of similar young lambs (infected Martinik lambs) in which 40 to 60% more daily growth was recorded (Marie-Magdeleine, 2009). Consequently energy intake could be the limiting factor for growth.

4.2. Effect of banana foliage on parasitism

The first trial was performed to test the effect of banana foliage on establishment of *H. contortus* in lambs. The second experiment was performed to test the effect of banana foliage on adult *H. contortus.* In the first trial, in which FEC was recorded 21 and 28 days after infection, there was no significant effect of banana foliage, compared with hay, on establishment of *H. contortus.*

This result should be confirmed by further studies, but it could reflect the lack of effect of the banana foliage on the establishment of worms. Analysis of the kinetics of excretion of eggs after 28 days, which was not carried out in trial 1, would be pertinent. In trial 2, after an absence of effect at 21 days, the significant effect of banana foliage on the FEC reduction at later times (28–35 days) could reflect an effect on adult worms. At the same time, the number of adult female worms was similar in the two treatments. It is possible that the effect of the banana foliage could be linked to direct action on worm fecundity. The number of female worms was the same, whatever the treatment, whereas total egg excretion was significantly lower in lambs fed banana foliage. However, the lack of effect of banana on reducing the number of eggs *in utero* calls for caution with the latter

hypothesis. Some authors estimate fecundity as the number of eggs *in utero* (Terefe et al., 2005). This method does not take into account the turnover of total eggs *in utero*, and consequently it could be unrepresentative of total egg production. Trial 2 also demonstrated a significant effect of the banana foliage on reducing the ability of *H. contortus* eggs to develop. Our results obtained *in vivo* are consistent with *in vitro* studies using extracts from banana leaves, which showed anthelmintic effects on larval development (Marie-Magdeleine, 2009) that were attributed to saponosides. However, the low level of CT and other tannins observed in the present study suggests that other compounds are involved in the nematicidal activity of *M. paradisiaca.* A qualitative thin layer chromatographic analysis indicated that banana contained, as secondary compounds, mainly terpenoids and flavonoids (Marie-Magdeleine, 2009). Within the terpenoids family, the saponosides are an important group of secondary metabolites that are found in banana foliage (Marie-Magdeleine, 2009). Moreover, the saponosides are important for their medicinal properties (Longuefosse, 2003). Terpenoid compounds are known to be active against a large range of organisms. Some of them are bioactive whereas others affect physical variables. Terpenoids and flavonoids are known to stop *H. contortus* larval development (Ademola et al., 2005; Maciel et al., 2006; Camurça-Vasconcelos et al., 2007). Furthermore, terpenoids can be antibacterial, antimicrobial, anticarcinogenic and antifungal (Aharoni et al., 2005). Saponosides usually act by binding to surface molecules (proteins or sterols), inducing inhibition of protein expression, and/or cell lysis (Bruneton, 1999). The stage-specific expression of surface proteins of nematodes (Rhoads and Fetterer, 1994) could explain why saponoside compounds specifically target this developmental stage. Moreover, studies have reported a dose-dependent muscle paralysing effect of an extract from the stem of the banana tree (Singh and Dryden, 1990). The neuromuscular blockade was reversed by calcium, suggesting that banana stem juice has a labilizing effect on cell calcium. Saponoside compounds could be involved in the nematicidal activity of the plant and it is possible that they are responsible for the ability of banana foliage to reduce the FEC.

Terpenoids and flavonoids were well represented in the stem and leaf (Marie-Magdeleine, 2009) and the nutritive value of the leaves was higher compared to that of the stem. Thus the use of leaves only could be a good strategy for achieving the best nutritive value and nematicidal effect. **The absence of effect of banana foliage on worm establishment in lambs could explain why there was no difference between diets in the blood packed cell volume (PCV) or eosinophils.**

5. Conclusion

In conclusion, the anthelmintic properties of banana foliage were demonstrated. These properties could be linked to the terpenoid and flavonoid compounds of the banana foliage. These compounds were present in both the stem and leaves. The nutritive value of banana foliage (stem + leaves) was low compared to the potential in some tropical breeds. To optimize the anthelmintic and nutritional properties, the use of leaves only is recommended. However, complementary data will be required to develop the use of banana foliage as a diet and an anthelmintic.

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Table 1 Chemical composition of feed ingredients of Martinik lambs artificially infected or not with *H. contortus* for Banana foliage, *Dichantium* hay and commercial pellets

	Hay	Banana foliage	Commercial pellets
Dry Matter (g/kg)	841.0	101.0	950.0
Organic Matter (g/kg DM)	928.0	852.0	902.0
Crude Protein (g/kg DM)	65.1	104.0	162.0
Neutral Detergent Fibre (g/kg DM)	812.0	680.0	159.0
Acid Detergent Fibre (g/kg DM)	450.0	450.0	40.0
Acid Detergent Lignin (g/kg DM)	63.0	122.0	10.0
Total Polyphenols (g/kg DM)	84.0	80.0	0
Condensed Tannins (g/kg DM)	0	0.007	0

DM: dry matter

Table 2

Trial 1: Effect of diet on faecal egg count, blood packed cell volume, eosinophils, and number of worms in abomasum of Martinik lambs artificially infected or not with *Haemonchus contortus* after ingestion of Banana foliage and *Dichantium* hay (Least square means and standard error of mean)

	Banana foliage		*Dichantium* hay		S.E.M.	Diet*treat effect *P* - value
	Non-infected lambs	Infected lambs	Non-infected lambs	Infected lambs		
Mean faecal egg count	0[a]	3725.0[b]	0[a]	3394.0[b]	657.0	0.69
Log mean faecal egg count	0[a]	7.9[b]	0[a]	7.8[b]	0.2	0.70
Blood packed cell volume (%)	31.7[a]	26.3[b]	28.4[c]	27.0[bd]	0.5	0.003
No. eosinophils	205584.0[ab]	210253.0[ab]	181370.0[b]	259298.0[ab]	22409	0.8
Log eosinophils	11.9[a]	12.0[a]	12.0[a]	12.1[a]	0.9	0.73
No. female worms		856.0[a]		568[a]	183	0.29
No. male worms		755.0[a]		407.0[a]	167	0.16
Total no. worms		1611.0[a]		974.0[a]	348	0.22
Female worm size (cm)		0.85[a]		0.71[a]	0.05	

a,b,c,d means that within rows, values with different letters differ significantly ($P < 0.05$). Treat: treatment. Diet*treat: interaction between diet and treatment

SEM: standard error of mean

Table 3

Trial 1: Mean values of feed intake in lambs infected or not with *Haemonchus contortus* and fed *Dichanthium* spp., or banana foliage added to a commercial pellet

	Banana foliage		*Dichantium* hay		S.E.M.	Diet*treat effect *P* - value
	Non-infected lambs	Infected lambs	Non-infected lambs	Infected lambs		
Dry matter intake (DMI) of ingredients (g/day)						
Dichantium hay or Banana foliage	353[a]	380[a]	319[b]	322[ab]	14.5	0.78
Commercial pellet	285[a]	285[a]	216[b]	213[b]	2.5	0.61
Total Intake (g/day)						
Dry Matter	638[a]	665[a]	535[b]	535[b]	14.1	0.23
Organic Matter	576[a]	595[a]	501[b]	501[b]	12.7	0.77
Neutral Detergent Fibre	299[a]	294[a]	294[a]	298[a]	10.8	0.77
Acid Detergent Fibre	171[a]	170[a]	150[b]	154[b]	6.4	0.77
Acid Detergent Lignin	46.0[a]	50.0[a]	21.0[b]	22.0[b]	1.6	0.93
Crude Protein	88.9[a]	92.1[a]	57.9[b]	57.9[b]	1.3	0.71
OMI / $LW^{0.75}$	61.0[a]	64.0[b]	56.0[c]	54.0[c]	1.00	0.43
Digestible OMI (g/ $LW^{0.75}$)	40.0[a]	44.0[a]	40.0[a]	41.0[a]	1.5	0.13
Digestible Crude Protein Intake (g/ LW0.75)	5.9[a]	6.6[a]	3.8[b]	4.1[b]	0.2	0.38

a,b,c,d means that within rows, values with different letters differ significantly ($P < 0.05$). Treat: treatment.

Diet*treat: interaction between diet and treatment; SEM: standard error of mean;

Table 4

Trial 1: Mean values of total tract digestibility in lambs infected or not with *Haemonchus contortus* and fed *Dichanthium* spp., or banana foliage added to a commercial pellet

	Banana foliage		*Dichantium* hay		S.E.M.	Diet*treat effect
						P - value
	Non-infected lambs	Infected lambs	Non-infected lambs	Infected lambs		
Total tract digestibility (%)						
DM	66.0[a]	70.0[ab]	68.0[ab]	72.0[b]	1.8	0.86
OM	66.0[a]	70.0 [ab]	70.0[ab]	74.0[b]	1.7	0.79
Neutral detergent fibre	56.0[a]	55.0[a]	69.0[b]	72.0[b]	2.0	0.29
Acid detergent fibre	52.0[a]	48.0[a]	67.0[b]	68.0[b]	2.8	0.64
Crude protein	62.0[a]	65.0[ab]	58.0[ac]	64.0[a]	2.2	0.74
Faecal output (g /kg LW0.75)						
Dry matter	23.3[a]	21.8[a]	18.3[b]	17.0[b]	1.0	0.64
Lignin	2.8[a]	2.9[a]	1.9[b]	1.6[b]	1.3	0.37

a,b,c,d means that within rows, values with different letters differ significantly ($P < 0.05$). Treat: treatment. Diet*treat: interaction between diet and treatment

Table 5

Trial 1: Effect of diets (*Dichanthium* spp., or banana foliage added to a commercial pellet) on daily weight gain of lambs and feed conversion ratio (Least square means and standard error of mean)

	Banana foliage		*Dichantium* hay		S.E.M.	Diet*treat effect *P* - value
	Non-infected lambs	Infected lambs	Non-infected lambs	Infected Lambs		
Initial LW (kg)	17.9[a]	17.4[a]	16.6[a]	17.3[a]	1.2	0.60
Final LW (kg)	21.2[a]	20.8[a]	19.5[a]	20.2[a]	1.5	0.72
Live weight gain LWG (g/day)	96.0[a]	98.0[a]	82.0[a]	83.0[a]	10.4	0.40
Feed Conversion Ratio (FCR) (kg feed/kg LWG)	6.6[a]	6.8[a]	6.5[a]	6.4[a]	1.8	0.47

a,b,c,d means that within rows, values with different letters differ significantly ($P < 0.05$). Treat: treatment.

Diet*treat: interaction between diet and treatment

Table 6

Trial 2: Effect of diet on faecal egg count, blood packed cell volume, eosinophils, and number of worms in abomasum of lambs artificially infected with *Haemonchus contortus* and fed *Dichanthium* spp., or banana foliage added to a commercial pellet (Least square means and standard error of mean)

	Banana foliage	*Dichantium* hay	S.E.M.	Diet effect *P*- value
Mean faecal egg count	1933[a]	8780[b]	1563	0.0005
Log mean faecal egg count	7.6[a]	8.1[b]	0.12	0.05
Blood packed cell volume (%)	28.0[a]	27.2[a]	0.53	0.29
No. eosinophils	248829[a]	423511[b]	72777	0.13
log eosinophils	12.1[a]	12.7[a]	0.13	0.07
No. female worms	572[a]	524[a]	113	0.76
No. male worms	488[a]	413[a]	104	0.61
Total no. worms	1061[a]	938[a]	215	0.69
Female worm size (cm)	2.3[a]	2.1[b]	0.05	0.03
No. worms in utero	537[a]	601[a]	80	0.59
Percentage of eggs hatching	14.6[a]	31.2[b]	4.2	0.0001

a,b means that within rows, values with different letters differ significantly ($P < 0.05$).

No: number of

Article n°7

Effect of cassava (*Manihot esculenta*) foliage on nutrition, parasite infection and growth of lambs

C. Marie-Magdeleine et al.

Soumis à Small Ruminant Research

Abstract

A completely randomised design for a 78-day feeding trial was performed to test the nutritional value and the anthelmintic properties of 12-month-old cassava foliage. Thirty 6-month-old Martinik lambs (body weight: 20.3 ± 1.6 kg) were divided into three groups of ten animals and placed in individual pens. The lambs were fitted with faecal bags in order to study the digestibility of the diet. The diets consisted of 45-day-old *Dichanthium spp.* hay with a supplementation regime according to the experimental treatment as follows (as fed basis): **1)** hay + lucerne pellets (450 g/lamb/day) + ground wilted cassava tuber (450 g/lamb/day); **2)** hay + wilted cassava foliage (650 g/lamb/day) + ground wilted cassava tuber (450 g/lamb/day); **3)** hay + wilted cassava foliage (650 g/lamb/day) + ground wilted cassava tuber (450 g/lamb/day) + polyethylene glycol (40 g/lamb/day). The polyethylene glycol (PEG) was dissolved in water and given *per os* to the lambs. At the end of a 14-day period of adaptation to the diets, all lambs were artificially infected with L3 larvae of *H. contortus*. Intake and total tract digestibility of the diets were monitored throughout the trial. Growth of lambs, faecal egg count, packed cell volume (PCV), and abomasal adult worm count (FEC) were also monitored. The anthelmintic properties of wilted cassava leaves, which are linked to tannins , were demonstrated. FEC was lower when lambs fed cassava foliage compared with Lucerne. The hatching rate of faecal eggs coming from lambs fed cassava foliage was lower compared with Lucerne. Due to the high fill value (ADL), the consumption of wilted cassava leaves provides limited total energy intake in lambs and has a depressive action on growth compared to the lucerne diet. An alternative strategy would be to use younger cassava foliage to optimise the anthelmintic properties and nutritional value.

Keywords: **Anthelmintic properties, Cassava leaves, Gastrointestinal nematodes, Growth, Nutritional values, Small ruminants**

1. Introduction

Gastrointestinal parasitism with nematodes (especially *Haemonchus contortus*) has been classified as a major health and welfare problem for small ruminants, particularly in the tropics (Geerts and Dorny, 1996; Chandrawathani et al., 1999; Fournet, 2002; Lefevre et al., 2003; Wolstenholme et al., 2004; Hoste et al., 2005; Jabbar et al., 2006a). Parasitism with gastrointestinal nematodes impairs animal health, welfare and productivity since the presence of worms results in increased death rate and poor growth and reproduction (Coop et al., 1982; Dakkak, 1995; Aumont et al., 1997). To date, the usual mode of control of gastrointestinal parasitism has relied on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Jackson and Coop, 2000; Wolstenholme et al., 2004; Jabbar et al., 2006b) in gastrointestinal nematode (GIN) populations has motivated investigation into alternative approaches such as phytotherapy (Athanasiadou and Kyriazakis, 2004; Wolstenholme et al., 2004; Githiori et al., 2006). Moreover there is a positive effect of protein supply on reducing the effect of parasitism on small ruminants (Hoste et al., 2005). Consumption of plants containing condensed tannins (CT) may affect the resistance and resilience of ruminant livestock to parasitic gastrointestinal (GI) nematodes in both indirect and direct ways (Kahn and A.Diaz-Hernandez, 2000). Consequently resources rich in condensed tannins and proteins, are good targets for studies on the anthelmintic properties of plants. Moreover these resources have the potential to be included in a sustainable system of production.

Cassava foliage is rich in condensed tannins and proteins (Phengvichith and Ledin, 2007a, c). Moreover some studies (Nguyen et al., 2003; Sokerya and Preston, 2003; Dung et al., 2005; Nguyen et al., 2005; Bunyeth and Preston, 2006; Sokerya, 2007) on goats indicate a protective action of cassava foliage against nematodes. The first objective of this study was thus to test the effect of cassava foliage against *H. contortus* in lambs and to explain the observed action. The second objective was to evaluate cassava foliage as a source of protein.

2. Materials and methods

2.1. Experimental site and feed ingredients

The study was conducted in the fields and facilities of the experimental unit ("Plateforme Tropicale d'Expérimentation sur l'Animal") of an INRA research unit (Unité de Recherches Zootechniques) in Guadeloupe, French West Indies. The ingredients used to prepare the experimental diets were based on hay, cassava (*Manihot esculenta*) foliage, cassava tuber and lucerne *(Medicago sativa).* The hay used comprised a spontaneous mix based on *Dichanthium* spp., the original forage of which was grown with irrigation and inorganic fertilization in Grande Terre, northeast Guadeloupe. Cassava tubers were collected daily from a 12-month-old cassava, and each entire tuber was coarsely ground. The ground tubers were air-wilted for one day before being distributed to animals. The foliage and tubers were harvested at the same time. The leaves plus petioles were separated from the stems and air-wilted in the same conditions as tubers before distribution to animals.

2.2. Animals, experimental diets, management and design

Animals were reared following European Union recommendations for animal welfare in accordance with the regulations of the Animal Care Committee of INRA.

Thirty Martinik male lambs (body weight (BW): 20.3 ± 1.6 kg; 6-months-old) were used in a completely randomised design for a feeding trial lasting 78 days (14 d of adaptation and 64 d of data collection). Animals were divided into three groups of ten lambs each and placed in individual pens. The groups of lambs were balanced according to their weight at weaning, their body weight at the beginning of the study, and their average daily growth between 30 and 70 d during suckling. To ensure the experiment began worm-free, lambs were all previously orally administered Cestocure® and Hapadex® and FEC was controlled after treatment.

Polyethylene glycol (PEG) is an inert and non-absorbed molecule that can form a stable complex with condensed tannins, preventing binding between tannins and proteins. To demonstrate the possible activity of condensed tannins in cassava leaves, PEG was added to the diet of a treatment group.

Diets consisted of *ad libitum* 45-day-old *Dichanthium spp.* hay with supplementation according to the experimental treatment as follows (as fed basis for each lamb):

Control group: Hay (600 g/d) + lucerne pellets (450 g/ d) + chips of cassava tuber (450 g/ d).
Treatment group: Hay (600 g/ day) + cassava foliage (650 g/ day) + chips of cassava tuber (450 g/ d).
PEG group: Hay (600 g/ d) + cassava foliage (650 g/ d) + chips of cassava tuber (450 g/ d) + PEG (40 g/ d). The PEG was dissolved in water and given *per os* to each animal.

The amount of hay provided (*ad libitum*) was calculated to allow at least 10% refusal per lamb. Cassava tuber, lucerne and wilted cassava foliage (WCF) were offered daily at 07:00 h and were completely consumed before 11:00 h. The hay was distributed at 11:00 h. Animals had free access to water and to a mineral block.

At the end of the 14-day adaptation period, all lambs were orally infected with a single dose of 10,000 third-stage larvae (L3) of *Haemonchus contortus*. The L3 infective larvae of *H. contortus* were obtained 42 days before challenge, from cultures of faeces taken from lambs monospecifically infected with isolates previously obtained from sheep reared on pasture at different farms in Guadeloupe (Aumont and Cabaret, 1999).

2.3. Measurements

The lambs were individually weighed at the beginning of the experiment, and then fortnightly until the end of the trial (78 d). Daily live weight gain (LWG, g/day), estimated from final minus initial body weight, was then calculated fortnightly.

Daily dry matter intake for each ingredient, total intake of dry matter (DM), organic matter intake (OM), Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and crude protein were determined and expressed either on a daily or metabolic weight basis.

The Feed Conversion Ratio (FCR = Feed intake / Daily live weight gain) was estimated for each treatment. Feed intake was recorded from Monday to Friday throughout the experiment and for each lamb.

It was considered to be the difference between the daily amount of food offered and refused. Samples of offered forage (two sub-samples of 200 g) and refusals for each animal were taken daily. One of the sub-samples was kept for daily dry matter determination. All the samples of the feed provided during one week were mixed together for each animal and a new sub-sample (200 g) was used for chemical analyses. The same operation was carried out for the refusals for each animal.

Feed intake measurements and analyses were carried out for all 30 lambs.

The lambs were fitted with faecal bags in order to study the digestibility of the diet. Four measurement periods of total tract digestibility (each one lasting 5 d), were carried out during the experiment. The bags were emptied every 12 h. The daily quantity of faeces was then weighed. After weighing, ten percent of the total faeces were removed to make up a weekly sample for each lamb. This sample was used for dry matter determination and for chemical analyses. Total tract digestibility was estimated as: (Feed intake – faeces excreted)/Feed intake.

Faecal samples were collected weekly for 10 weeks to determine faecal egg count (FEC) using a modified McMaster method for rapid determination (Aumont, 1997). Monitoring started 2 weeks before artificial infection with L3 larvae of *H. contortus*.

Faecal samples of every lamb, sampled on week 10, were cultured for 10 days at room temperature each month, following the Roberts and O'Sullivan (1952) procedure to determine the development of faecal eggs. The identification of the third stage larvae used the key described by Gruner and Raynaud (1980).

Blood was sampled (EDTA-coated tubes, Becton Dickinson, Plymouth, UK) from each animal through the jugular vein puncture method, at the same time as the faecal samples were taken. The number of circulating eosinophils was measured according to the method of Dawkins et al. (1989). Eosinophils were counted with a Malassez cell counter. Packed cell volume (PCV) was also estimated using a capillary microhaematocrit (centrifuged for 5 min at 5000 rpm).

Seven weeks after infection, all lambs were slaughtered for worm recovery and enumeration (Gaba et al., 2006). The abomasum was removed from the animal less than 15 min after slaughter and immediately processed.

Formalin preservative (35% ethanol, 2.5% formol, qsp 1L distilled water) was added to each subsample of removed worms (5% v/v). About 5 or 10% of the subsample was examined when aliquots were taken from 2 or 1 L, respectively. In all aliquots, worms were counted under a binocular magnifying glass, separating the males from females. Female worms were stored in formalin preservative (5% v/v) for later size measurement and *in utero* egg count (Kloosterman et al., 1978).

Liver, kidney, and intestinal mucous membranes were observed to identify eventual necrotic damage and gastritis, as an indicator of eventual toxicity.

2.4. Chemical Analyses and Analytical Procedures

The dry matter content of feeds and faeces was determined by oven-drying to a constant weight at 60 °C for 48 h (AOAC, 1997) while ash content was determined by heating samples at 550 °C for 4 h according to AOAC (1997). Dry samples were obtained for further chemical analyses and were ground (to 1 mm). The CP content was calculated after N determination by combustion using the micro-Dumas method.

The methods of Van Soest *et al*. (1991) were followed to determine NDF and ADF (sequentially) on an ash-free basis using the Ankom^{2000} Fiber Analyser incubator. Cyanogen content (HCN determination) was determined using the method of Hogg and Ahlgren (1942). Condensed tannins (CT) content was determined by the vanillin-HCl assay (Nakamura et al., 2003), using extracted cassava leaf CT as the standard (Giner-Chavez et al., 1997).Total polyphenol content was determined with the Folin-Ciocalteau reagent, according to the method of Singleton et al. (1998) the results were expressed in g of gallic acid equivalent per 100 g DM.

2.5. Statistical Analysis

Intake, total tract digestibility, ADG PCV, FEC, and eosinophil data were analysed as a completely randomized design using the mixed procedure of SAS (SAS, 2000)(SAS software, V8.01, SAS Institute Inc., Cary, NC, USA). The statistical model included the fixed effect of diet, measurement time (linear, quadratic and cubic), the interaction between diet and time and the random effect of animal. Comparisons between means were tested using the Bonferroni. For all traits, significance was declared at probability levels of ≤ 5%, while tendencies were discussed between 5 to 15%. PCV and FEC data were also log-transformed (log (FEC+1)) before analysis. The results were similar to the raw data so the latter were used in the discussion.

Concerning the curve of faecal eggs excretion the Gamma model was used to compare the treatments with the mixed procedure of SAS. The model was (log (FEC+1)) = log (a) – c * time + c*b*log (time) where a b and c are the scale parameter (the beginning of the curve), the value of time corresponding to the maximum value of FEC and the rate of decrease after the maximum value of FEC. The same effects that the previous model were used.

Abomasal worm count variables were analysed as a completely randomized design using GLM procedure of SAS. The statistical model included the fixed effect of diet. Differences between means were tested using the pdiff option.

3. Results

3.1. Chemical Composition of Ingredients

Table 1 shows the mean values for the chemical composition of the ingredients of the diets. The CP content of wilted cassava foliage (WCF) was higher than that of lucerne. Wilting caused HCN contents (representing the cyanogen contents) of fresh cassava foliage and cassava tuber to decrease by 78% and 74% respectively. The HCN contents of fresh cassava foliage and wilted cassava foliage were 1064 and 272 mg/kg DM, respectively. The condensed tannin content of cassava foliage was 40 g/kg DM.

3.2. Blood Parameters (Blood Eosinophil Counts, Packed Cell Volume)

Eosinophil counts in blood significantly increased after infection ($P < 0.001$). A peak occurred the week of the larval challenge (Figure 1). A slight peak also occurred between 4 and 5 weeks after the larval challenge. No significant difference was found between the kinetics of the eosinophils of the three groups of lambs. An interaction between diet and time was recorded illustrating significant differences between the group of lambs fed cassava compared with the two others group at days 42 and 49 after the artificial infection. There was a tendency (P=0.08) of PCV to be lower for the group of lambs fed with cassava compared with the two other groups (Table 2). PCV ranged between 24 and 32%. However, as egg counts were rapidly increasing (figure 3), there were correspondingly decreasing trajectories of PCV in all groups (Figure 2).

3.3. Faecal Egg Count and Egg Development

The mean faecal egg count tended (P=0.09) to be significantly higher for lucerne compared with the two other groups (Table 2). The log transformed data of faecal was significantly (P=0.04) higher for lucerne compared with the two other groups (Table 2). The curve of FEC according to time is illustrated in Figure 3. Comparing the main parameters (initial FEC value, value of time corresponding to the maximum FEC excretion, and the rate of decrease after the maximum) the curves were not significantly different. Twenty eight days after the artificial infection, the FEC tended (P=0.09) to be lower with cassava compared with the two other diets. The total FEC during the kinetic of eggs excretion tended (P=0.08) to be lower with cassava compared with the two other diets.

The rate of egg development was significantly higher in the group of animals fed with lucerne compared to WCF.

3.4. Abomasal Worm Count

The worm establishment rates and the number of worms in the abomasum were not significantly (*P* = 0.16) different between the three groups of lambs. With regard to female worm size and fecundity (*in utero* egg count), there was no difference between groups (Table 2).

3.5. Intake

Table 3 shows the mean values for feed intake for the different diets. The total dry matter intake was significantly higher in the group fed the diet containing lucerne than in the other groups, whereas there was no significant difference between groups fed wilted cassava foliage. The intake of CP was not significantly different among treatments. The intake of NDF and ADF was significantly higher in the control group fed lucerne than in the groups supplemented with WCF and WCF-PEG. Conversely, the intake of ADL was significantly higher with the WCF diets. The intake of hay was significantly higher with the lucerne diet. Moreover, whatever the diet, intake of hay increased over time except during the week following infection during which a decrease was recorded.

3.6. Total Tract Digestibility

Table 4 shows the mean values for feed total tract digestibility. DM and OM digestibility tend to be higher with Lucerne compared with the two others diets. The digestibility of CP was significantly higher for the diet containing lucerne than for the WCF diets without PEG. When PEG was added to the WCF, the total tract digestibility of CP was higher than the diet of lucerne. Whatever the diet, a decrease in total tract digestibility was recorded during the week following infection (Figure 4, 5). The digestible dry matter intake was significantly higher with the lucerne diet whereas the highest digestible crude protein intake was recorded for the WCF-PEG diet.

3.7. Growth and Feed Conversion

The effect of the diets on live weight gain (LWG) is shown in Table 5. The LWG was highest in the treatment with lucerne, but there was no significant difference between the treatments with WCF. There were no significant differences in the Feed Conversion Ratio (FCR) with the different diets.

4. Discussion

4.1. Chemical Composition of Feeds

The CP content of cassava foliage estimated in the current study was consistent with results reported in the literature (Ravindran et al., 1987; Ravindran, 1993 ; Van and Ledin, 2002; Khang and Wiktorsson, 2004; Dung et al., 2005), where CP content of cassava foliage ranged from 190 to 250 g/kg DM. This range of variation can be explained by harvesting time, climatic conditions, plant variety, and soil fertility. Wilting drastically reduced the cyanogen content in cassava foliage. However the initial concentration of cyanogens was close to the lower value reported in the literature. The cyanogen content usually ranges from 200 mg to 900 mg/kg DM in fresh leaves (Ravindran et al., 1987; Ravindran, 1993; Man and Wiktorsson, 2001; Borin et al., 2005). In the present experiment, the air wilting effect on the reduction in the cyanogen content of WCF was more efficient than that reported by Phengvichith and Ledin (2007a, b) and Du and Preston (2005). Nevertheless the value recorded in this experiment was higher than the value of 100 mg HCN indicated by Tewe (1994, in Sokerya et al., 2007), which is the maximum allowed to guarantee the safety of cassava for livestock. With regards the cassava tuber, the efficiency of wilting was similar to that for the foliage.

4.2. Feed Intake

In cattle and sheep, HCN can be lethal at 2–4 mg HCN kg^{-1} BW (Kumar, 1992). In the present study the average daily intake of HCN (cyanogen content) was around 5.8 mg/d^{-1} / kg^{-1} BW, which was higher than the lethal dose stated above. Systemic tannin-related toxicity is induced by necrotic damage to the liver and kidney in monogastric and ruminant animals (Kumar and Singh, 1984). None of these symptoms were observed after the necroscopy of the lambs. Moreover, neither gastritis nor damage to the intestinal mucous membrane was observed.

The lower total dry matter intake recorded with WCF compared to lucerne could not be explained by the condensed tannins content in WCF. The negative effect of tannins on intake (Nguyen et al., 2005) was generally explained by: i) reduced palatability as a consequence of stringency; ii) systemic toxicity; iii) low rate of evacuation of digesta from the rumen (gut-fill effect). In the current study, the mean concentration of condensed tannins, 15% and 40% of the total diet and cassava respectively, did not limit the total intake of the proposed amount of cassava foliage. No toxic effect was recorded.

The rumen turnover was not evaluated in the current study. Nevertheless, it is possible that the lower intake of diets containing cassava foliage could be due to the higher fill value of cassava foliage diet compared with lucerne diet. The daily organic matter and lignin faecal excretion were close to maximum values recorded in animals fed *ad libitum*. Moreover lignin intake was higher with the WCF diet and consequently the efficiency of ingestive and merycic rumination could be lower, thus explaining the lower intake.

4.3. Digestibility

The decrease in total tract digestibility of crude protein (CP) and fibre (NDF and ADF) observed with the cassava diet compared with lucerne disappeared when PEG was added to the diet, as a result of the formation of CT-PEG complexes, which prevent binding between CT and proteins.

The lower CP digestibility recorded without PEG was the consequence of a depressive effect of condensed tannins (CT). The most impressive effect of CT was the depression of CP digestibility (Silanikove et al., 1996).

The anti-nutritional effect of CT is exerted through the reduction of food protein availability and depression of digestive tract enzyme activities. One of the main adverse effects of CT on nutrient utilisation is caused by a depression in pancreatic enzyme activities (Silanikove *et al*., 1996). CT may reduce cell-wall digestibility by binding bacterial enzymes and/or forming indigestible complexes with cell-wall carbohydrates (Silanikove et al., 1996). Improvement in NDF digestibility following PEG supplementation reflected (i) a decrease in the protein−tannin complex determined as NDF in faeces and (ii) enhanced fermentation of rumen fibre (Silanikove et al., 1996).

4.4. Parasitism

In the present study, the statistical analysis can not conclude that *H. contortus* was affected when infected lambs were fed cassava leaves. The great variability recorded on the main parameters studied partly explains this result. Probably more data enable trends in FEC to become significant. Cassava foliage diet mainly affected faecal egg excretion and egg development to L3. This effect could be due to condensed tannins (CT) because, although the differences were not always significant, the positive effect of cassava reduced or disappeared when PEG was added to the diet.

This effect of PEG on tannin-rich plants has previously been observed (Kabasa et al., 2000). It is known that the effects of PEG are mediated through the formation of competitive complexes with dietary condensed tannins in the rumen. As a result, the amount of tannin-precipitated forage proteins passing through to the abomasum and small intestines (bypass proteins) for digestion will be reduced. Nevertheless, CT are reported to enhance the availability and utilization of proteins and amino acids in ruminants at concentrations below 5% (Waghorn, 1994).

In the present experiment, the main effect of cassava compared with lucerne was the decreased nematode egg count and lower establishment of abomasal nematodes. The lower egg excretion cannot be explained by lower numbers of female worms as data on prolificacy for female worms were well correlated with the number of eggs *in utero*. Moreover, the latter also correlated well with the length of the female worms.

The present study also demonstrated a significant effect of cassava foliage in reducing the capacity of *H. contortus* eggs to develop. These findings lend support to *in vitro* studies using extracts from cassava leaves, which showed anthelmintic effects on larval development (Marie-Magdeleine, 2009), attributed to condensed tannins.

The effect of fresh, dry or ensiled cassava foliage and its suppression of FEC has previously been indicated in goats (Nguyen et al., 2003; Sokerya and Preston, 2003; Bunyeth and Preston, 2005; Dung et al., 2005; Bunyeth and Preston, 2006 in Sokerya et al., 2007). There was a significant reduction in FEC (0.5 to 5 times the control level) after, depending on the experiment, a 3-week to 3-month feeding period with cassava foliage in a ration. The positive effect of cassava foliage on suppression of FEC has also been reported for large ruminants (Netpana et al., 2001; Granum et al., 2003). The FEC values recorded in lambs in the present experiment (mean decrease of 1.7 times during the sampling period) were included in this range. There was no difference between treatments in terms of the blood packed cell volume (PCV). However, the haematophagous action of *H. contortus* was reflected by a general decrease in PCV, 21 days after infection. This may be due to the better level of nutrition of the lucerne-fed lambs compared with the cassava-fed lambs. *H. contortus*-infected animals exhibited higher requirements for energy, proteins and minerals due to loss of endogenous nitrogen (blood, sloughed cells…). The higher amount of nutrients in the lucerne diet may have counterbalanced the haematophagous action of *H. contortus* through improved turnover of the lost cells. A similar effect is possible for eosinophils.

4.5. Growth Performance

Growth on the lucerne diet was close to the potential for Martinik sheep (Archimède et al, 2008). The lower growth on cassava leaves may be due to the lower energy intake. On one hand, the intake of digestible crude protein was similar in the group of lambs fed with lucerne and WCF. Comparing WCF and WCF-PEG fed lambs, the level of digestible crude protein was 25% higher in the latter, although there was no difference in growth between the two treatments. On the other hand, digestible dry matter intake was higher with the lucerne diet compared with the two other treatments.

Based on a energetic requirement for 3600 kcal / kg of growth of low growth lambs (INRA, 1988), the theoretical energy cost for 43 g of growth (the difference between lucerne and WCF) was 150 kcal. Based on the value of 3600 kcal per kg of digestible organic matter (INRA, 1988), the difference in energy intake between the two diets was 242 kcal. Moreover, the metabolic efficiency of these diets was similar, as illustrated by the index of feed conversion.

This experiment illustrates that WCF can be used as feed and as an anthelmintic for small ruminants however more data are necessary to confirm this. One of the remaining questions is how to optimize these two functions. With regard to feed value, the results indicate that a 4% DM concentration of condensed tannins in the cassava foliage has a detrimental effect on protein and energetic values. Consequently, a 25% decrease in growth was recorded compared to control animals. However, the latter were more highly infected by *H. contortus*. Moreover the growth of the control lambs was close to the potential of the breed. This result illustrates the resilience (ability of a parasitized animal to perform to a level similar to an unparasitized control) of the lucerne-fed lambs (the control group) to GI nematode infections. A number of reports (cited by Kahn and Diaz-Hernandez, 2000) suggest that an increase in the supply of digestible protein (DP) would improve the resilience and resistance (ability of an animal to resist parasite establishment and to impair the development of, and/or expel, previously established parasites) of sheep to GI nematodes. This resilience probably also exists with the WCF diet because of the similar level of digestible protein. So, the question is how to increase energy, and thus intake of digestible organic matter, with WCF.

One solution would be to decrease the NDF and ADF content by using younger foliage. Wanapat (2003), working with 3-month-old cassava foliage, reported values of 30 and 44% NDF for cassava leaves and cassava hay respectively.

This level could be related to higher intake. Moreover, the level of CT is lower in younger forage but remains within the range (2–4%) required to protect crude proteins against microbial rumen degradation (Wanapat, 2003) without adverse effects on the digestibility of fibre and retaining the anthelmintic properties. Nevertheless, research on the nutritional impact of CT in temperate forages in New Zealand has shown that the results obtained depend on the structure or type of CT as well as on their concentration (Makkar, 2000; Makkar, 2003).

5. Conclusion

In conclusion, the anthelmintic properties of wilted cassava foliage were suspected. These properties were linked to the condensed tannin content of the foliage. Condensed tannins restricted the development of *H. contortus* eggs. Unfortunately, as a result of a high fill effect, the intake of old wilted cassava foliage limited the total energy intake of the animals, with a subsequent depressive effect on growth. An alternative strategy would be to use younger foliage to optimize the anthelmintic properties and nutritional values.

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Table 1. Chemical composition of feed ingredients of lambs fed *Dichanthium* spp., and cassava tuber add to the different diets: Lucerne, cassava foliage

	Hay	**Lucerne**	**Cassava leaves**	**Cassava tubers**
Dry Matter (g/kg)	899.0	965.0	517.0	463.0
Organic Matter (g/kg DM)	918.0	888.0	927.0	963.0
Crude Protein (g/kg DM)	82.0	159.0	224.0	28.0
Neutral Detergent Fibre (g/kg DM)	740.0	534.0	498.0	77.0
Acid Detergent Fibre (g/kg DM)	392.0	350.0	341.0	49.0
Acid Detergent Lignin (g/kg DM)	50.0	109.0	194.0	15.1
(1) Total polyphenols	8.4	12.2	18.7	0
Condensed Tannin (g/kg DM)	0	0	40.0	0
(2) HCN (mg/kg DM)			1064	1367
(3) HCN (mg/kg DM)			272	288

(1) in g of gallic acid equivalent per100 g DM.

(2) in fresh leaves, (3) in wilted leaves

Table 2. Effect of diet on faecal egg count, blood packed cell volume, eosinophils, and number of worms in abomasum (Least square means and standard error of mean) in lambs fed *Dichanthium* spp., and cassava tuber add to the different diets: Lucerne, cassava foliage and cassava foliage plus Polyethylenglycol (PEG)

	Lucerne	**Cassava**	**Cassava + PEG**	**SEM**	**P-values**		
					Treat	**Time**	**Treat*Time**
Mean faecal egg counts (eggs/ g of faeces)	6918a	4082b	5384b	1092	0.09	0.03	0.88
Log mean faecal egg count	8.3a	7.6b	7.9b	0.3	0.04	0.002	0.01
Blood packed cell volume (%)	27.7a	27.1a	27.7a	1.1	0.08	0.004	0.15
No. Eosinophils	345950a	390487a	354887a	61724	0.49	0.0001	0.001
log Eosinophils	12.5a	12.5a	12.0a	0.2	0.17	0.0001	0.001
No. Female worms	499.0a	315.0a	347.0a	127	0.6		
No. Male worms	440.0a	145.0a	226.0a	112	0.07		
No. Total worms	939.0a	460.0a	573.0a	236	0.16		
Female worm size (cm)	2.0a	2.1a	2.0a	0.06	0.24		
No. Eggs in utero	337a	324a	360a	235	0.95		
Ratio male /female	0.71a	0.37b	0.54a	0.1	0.02		
Worm establishment rates	9.4a	4.6a	5.7a	2.4	0.16		
Percentage of hatching	40a	16b		4.1	0.0001		

a,b,c,d means that within a row, values with different letters differ significantly ($P < 0.05$). Treat: treatment.

Treat*Time: interaction between time and treatment

Table 3. Mean values for feed intake in lambs fed *Dichanthium* spp., and cassava tuber add to the different diets: Lucerne, cassava foliage and cassava foliage plus Polyethylenglycol (PEG).

	Lucerne	Cassava	Cassava+PEG	SEM	P-values		
					Treat	Time	Treat*Time
Dry matter intake (DMI) of ingredients (g/day)							
Hay	379.3a	362.9a	335.0b	16.3	0.57	0.0001	0.57
Lucerne	441.8						
Cassava leaves		336.4a	331.7a	4.2	0.95		
Cassava tuber	202.0	202.0	202.0	2.4	1		
Total Intake (g/day)							
Dry matter	1022.8a	897.3b	869.0b	17.5	0.05	0.0001	0.34
Organic Matter	934.4a	835.2b	809.3b	16.1	0.08	0.0001	0.35
Neutral Detergent Fibre	532.0a	450.4b	429.2b	12.8	0.04	0.70	0.42
Acid Detergent Fibre	313.5a	265.8b	254.5b	6.9	0.04	0.03	0.32
Acid Detergent Lignin	70.1a	85.6b	84.3b	1.3	0.001	0.5	0.5
Crude Protein	107.4a	110.2a	108.4a	1.7	0.16	0.23	0.06
DMI / LW0.75	89.9a	78.4b	76.6b	0.9	0.05	0.001	0.45
Digestible DMI / LW0.75	57.6a	51.0b	49.5b	0.7	0.12	0.0001	0.13
Digestible Crude Protein/ LW0.75	4.7a	4.6a	6.0b	0.1	0.0001	0.0001	0.0001

a,b,c,d means that within a row, values with different letters differ significantly ($P < 0.05$). Treat: treatment.

Treat*Time: interaction between time and treatment

Table 4. Mean values for total tract digestibility in lambs fed *Dichanthium* spp., and cassava tuber add to the different diets: Lucerne, cassava foliage and cassava foliage plus Polyethylenglycol (PEG)

	Lucerne	Cassava	Cassava+PEG	SEM	P- values		
					Treat	Time	Treat*Time
Total tract digestibility (%)							
DM	63.1a	62.8a	62.6a	0.4	0.06	0.0001	0.05
OM	64.6a	64.2a	63.8a	0.4	0.08	0.0001	0.92
Neutral detergent fibre	57.0a	54.9b	59.2c	0.6	0.03	0.0004	0.33
Acid detergent fibre	52.6a	45.3b	50.7c	0.7	0.03	0.0001	0.02
Crude protein	51.3a	45.2b	60.1c	0.6	0.0001	0.0001	0.0001
Faecal output (g /kg LW0.75)							
Dry matter	26.6a	27.1b	26.7b	0.5	0.13	0.78	0.13
Lignin	4.7a	7.7b	6.4c	0.14	0.50	0.60	0.67

a,b,c,d means that within a row, values with different letters differ significantly ($P < 0.05$). Treat: treatment.

Treat*Time: interaction between time and treatment

Table 5. Effect of diets fed to lambs (*Dichanthium* spp., and cassava tuber add to the different diets: Lucerne, cassava foliage and cassava foliage plus Polyethylenglycol (PEG)) on daily weight gain and feed conversion ratio (Least square means and standard error of mean)

	Lucerne	**Cassava**	**Cassava+PEG**	**SEM**	**P-values**
Initial LW (kg)	19.8a	20.7a	20.5a	0.9	0.27
Final LW (kg)	32.7a	30.2a	30.8a	1.1	0.11
Live weight gain LWG (g/day)	163.5a	120.8b	134.8b	7.7	0.001
FCR (kg feed/kg LWG)	5.7a	5.6a	6.8a	3.5	0.55

a,b,c,d means that within a row, values with different letters differ significantly ($P < 0.05$).

Figure captions :

Figure1: Effect of basal diet on blood eosinophils of Martinik lambs artificially infected with *H. contortus* after ingestion of lucerne, cassava foliage or cassava foliage plus Polyethylenglycol (PEG)

Figure 2: Effect of basal diet on blood packed cell volume of Martinik lambs artificially infected with *H. contortus* after ingestion of lucerne, cassava foliage or cassava foliage plus Polyethylenglycol (PEG)

Figure 3: Effect of basal diet on faecal egg count of Martinik lambs artificially infected with *H. contortus* after ingestion of lucerne, cassava foliage or cassava foliage plus Polyethylenglycol (PEG)

Figure 4: Effect of basal diet on dry matter total tract digestibility of Martinik lambs artificially infected with *H. contortus* after ingestion of lucerne, cassava foliage or cassava foliage plus Polyethylenglycol (PEG)

Figure 5: Effect of basal diet on crude protein tract digestibility of Martinik lambs artificially infected with *H. contortus* after ingestion of lucerne, cassava foliage or cassava foliage plus Polyethylenglycol (PEG)

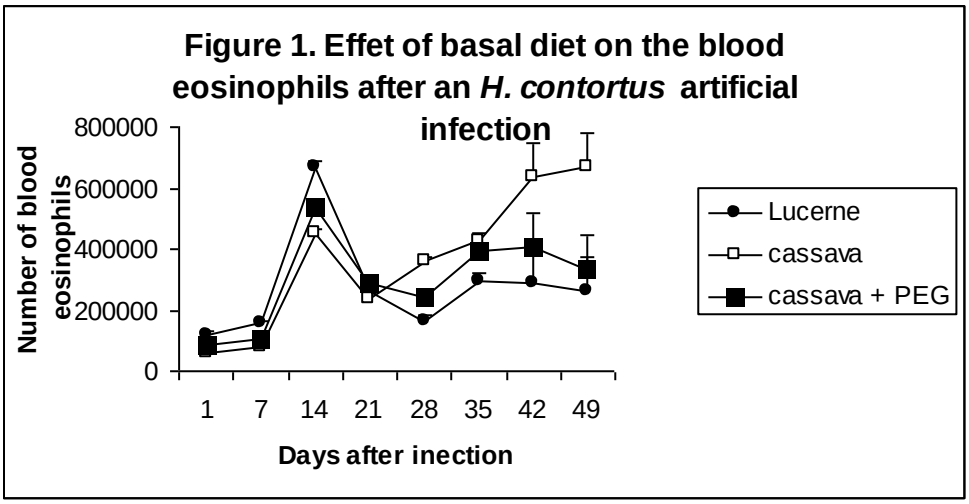


Figure 1. Effet of basal diet on the blood eosinophils after an H. contortus artificial infection
Number of blood eosinophils
800000
600000
400000
200000
0
1
7
14
21
28
35
42
49
Days after inection
Lucerne
cassava
cassava + PEG

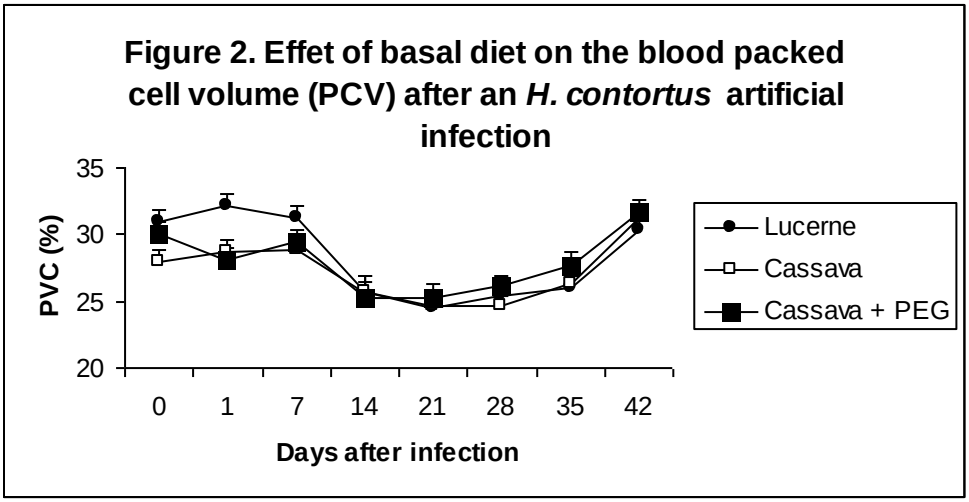
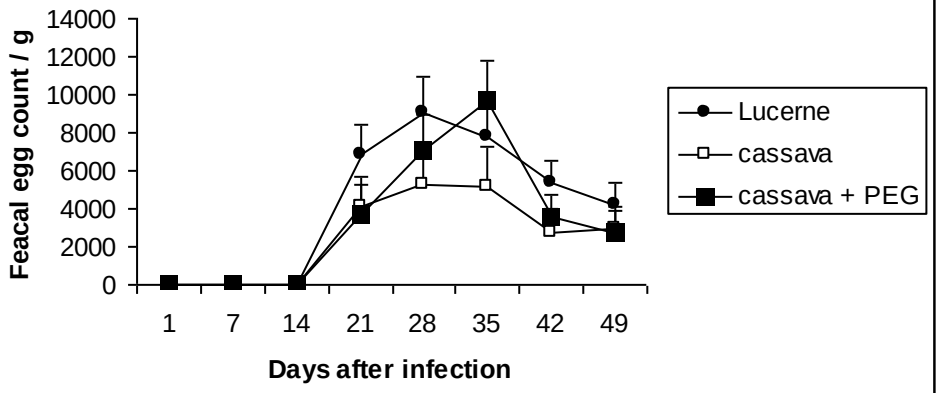


Figure 2. Effet of basal diet on the blood packed cell volume (PCV) after an *H. contortus* artificial infection
PVC (%)
35
30
25
20
0
1
7
14
21
28
35
42
Days after infection
Lucerne
Cassava
Cassava + PEG

Figure 3. Effet of basal diet on the faecal egg count after an *H. contortus* artificial infection



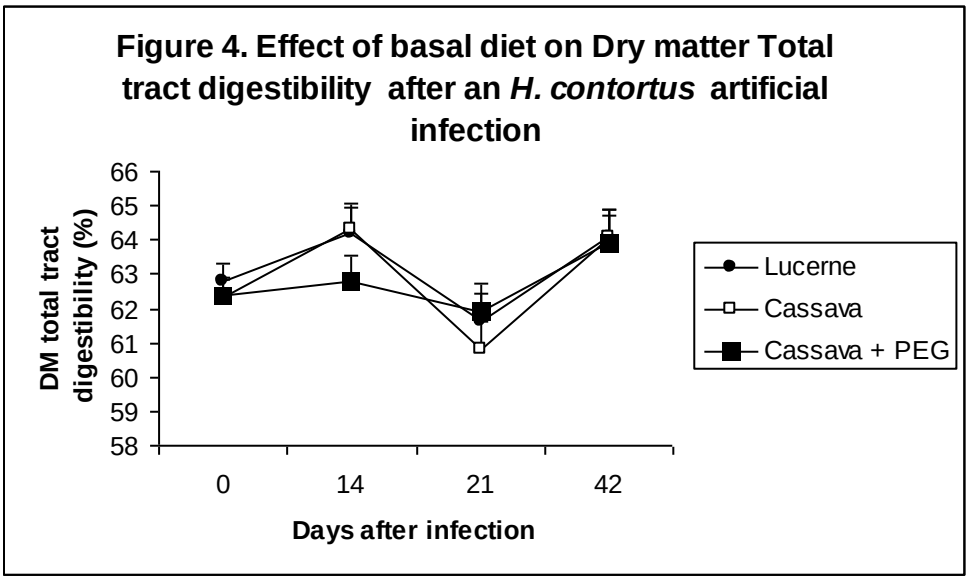


Figure 4. Effect of basal diet on Dry matter Total tract digestibility after an H. contortus artificial infection
DM total tract digestibility (%)
66
65
64
63
62
61
60
59
58
0
14
21
42
Days after infection
Lucerne
Cassava
Cassava + PEG

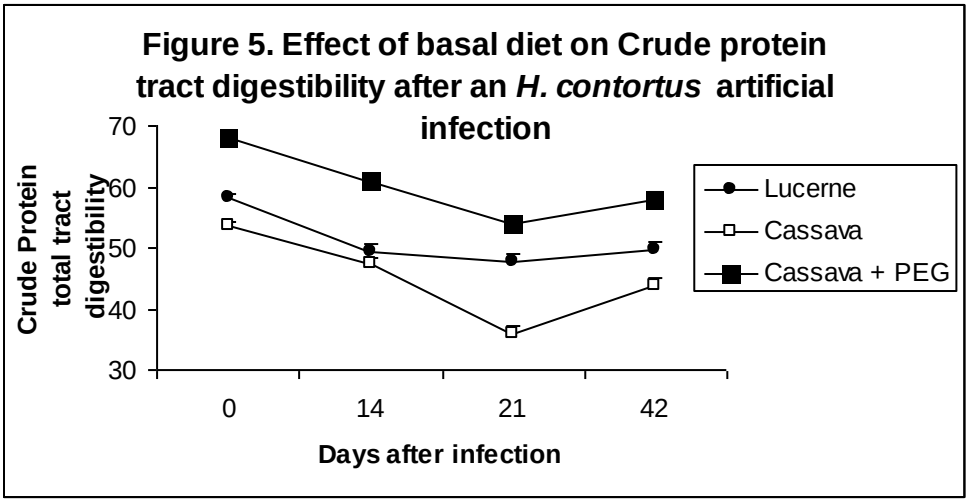


Figure 5. Effect of basal diet on Crude protein tract digestibility after an H. contortus artificial infection
Crude Protein total tract digestibility
70
60
50
40
30
0
14
21
42
Days after infection
Lucerne
Cassava
Cassava + PEG

IV. DISCUSSION GENERALE

Les objectifs de ce travail de thèse étaient d'étudier l'activité nématicide de certaines ressources végétales contre le parasite *Haemonchus contortus*; secondairement, de caractériser les classes chimiques responsables de l'activité ; puis d'expliquer le mode d'action. Il sera donc discuté, dans leur ensemble, des résultats obtenus avec les incertitudes et les perspectives qui s'y attachent.

1. Nos principaux acquis

1.1. Les ressources d'intérêt

Nos travaux ont permis de dresser un inventaire, dans la zone Caraïbe, des ressources d'intérêt pour un usage en santé animale et en particulier celles ayant une activité anthelminthique potentielle pour les ruminants. En effet, l'inventaire bibliographique réalisé (article 1) lors de cette étude a permis de recenser 194 plantes appartenant à 74 familles botaniques; potentiellement utilisables en médecine vétérinaire. Les familles les plus représentées sont: les *Asteraceae* (7.7% des pathologies), les *Euphorbiaceae* et les *Fabaceae* (4.6% chacune), et enfin les *Cucurbitaceae* et les *Rutaceae* (4.1% chacune). La famille la mieux répartie sur les différentes pathologies était celle des *Euphorbiaceae.* En ce qui concerne les pathologies liées aux helminthes, ce sont les *Asteraceae* (14%) qui ont été le plus souvent citées.

Nous avons réalisé la première évaluation sur le parasite *H. contortus* de 9 d'entre elles (Annona *muricata, Rauvolfia viridis, Tabernaemontana citrifolia L., Neurolena lobata, Cucurbita moschata, Lantana camara, Senna alata, Crescentia cujete L.et Bidens pilosa*). Les 14 plantes testées *in vitro* (article 1) sur le parasite *Haemonchus contortus* ont confirmé leur activité anthelminthique (réduction du taux d'éclosion et du développement larvaire).

Elles ont, en particulier, toutes montré un effet sur la réduction du développement larvaire du stade L1 vers le stade L3. Parmi ces 14 plantes, 5 ont révélé des activités anthelminthiques supérieures (P< 0,05) à celle du témoin positif de la pharmacologie de synthèse: *Carica papaya L.*(extrait aqueux), *Annona muricata, Lantana camara, Senna alata L.*(extraits aqueux et méthanolique) et *Cucurbita moschata L.*(extraits aqueux, méthanolique et dichlorométhane). Le niveau d'efficacité *in vitro* de ces 14 plantes varie entre 20% et 55% sur l'éclosion des œufs; et de 26% à 98% pour le développement larvaire. Ces résultats sont le reflet de la variabilité d'efficacité des substances d'origine végétale, ce qui peut constituer un véritable atout. En effet, le faible niveau d'efficacité comparativement aux anthelminthiques de synthèse permet d'avoir une moindre pression de sélection sur le parasite. De plus, la variété des composés actifs dans une même plante, fait supposer la mise en œuvre de plusieurs mécanismes d'action sur le parasite. Ces deux phénomènes limiteraient les risques de développement de résistances comparativement à ce qui est observé avec les composés de synthèse.

1.2. Les familles de molécules d'intérêt

Nos essais *in vitro* associés à la phytochimie ont permis de cibler plusieurs familles de molécules d'intérêt pour lutter contre le parasite *H. contortus*. Les essais *in vivo*, quant à eux, ont permis de valider les effets en conditions réelles. Les principales familles de composés identifiées sont : des composés aminés (l'acide aminé cucurbitine et la protéine cucurmosine); des terpénoïdes (saponosides, cucurbitacine B entre autres); des alcaloïdes indoliques; des composés phénoliques (tanins condensés, flavonoïdes).

L'efficacité *in vitro* des graines de *Cucurbita moschata L.*(*Cucurbitaceae*) sur le développement larvaire (94% à 97% d'inhibition), l'éclosion des œufs (20%) et sur La migration des larves infestantes (34% à 45% d'inhibition), serait due à des composés aminés (l'acide aminé cucurbitine et la protéine cucurmosine) et/ou terpénoïdes (saponosides, cucurbitacine B entre autres) (article 2).

Cette hypothèse est appuyée par des études antérieures qui ont montré un effet de terpénoïdes sur le développement larvaire et l'éclosion des oeufs du parasite *H. contortus* (Camurça-Vasconcelos et al., 2007; Eguale et al., 2007a, b; Maciel et al., 2006). Concernant la motilité du ver adulte (inhibition de 69%), d'après les résultats obtenus lors de nos travaux, seuls les composés terpénoïdes semblent être impliqués.

L'efficacité *in vitro* des feuilles, fruits et racines de *Tabernaemontana citrifolia L.* (*Apocynaceae*) sur l'éclosion (23% d'inhibition), sur le développement larvaire (72% à 100% d'inhibition), sur la migration des larves L3 (49% d'inhibition) et sur la motilité du ver adulte (34% à 56% d'inhibition) serait due à la présence d'alcaloïdes indoliques (article 3). Les alcaloïdes indoliques de *Tabernaemontana citrifolia L.* pourraient, compte tenu de leur complexité structurale, globalement agir sur tous les stades parasitaires, avec une spécificité pour chacun des stades en fonction des structures moléculaires des différents alcaloïdes à l'intérieur de la classe. Des composés phénoliques de type flavonoïdes sont également suspectés d'agir sur le développement larvaire, certains flavonoïdes étant connus pour avoir un effet sur ce stade de développement chez *H. contortus* (Ademola et al., 2005; Camurça-Vasconcelos et al., 2007; Maciel et al., 2006).

L'efficacité *in vitro* des feuilles de *Manihot esculenta Crantz*(*Euphoriaceae*) sur le développement larvaire (58% d'inhibition) est imputée à des composés terpénoïdes et/ou aux tanins condensés (article 5). Des travaux antérieurs sur les tanins condensés ont montré une réduction du développement des œufs de nématodes, de l'éclosion jusqu'au stade L3 (Alonso-Diaz et al., 2008a; Alonso-Diaz et al., 2008b; Kahn et A. Diaz-Hernandez, 2000). Il a également été montré qu'ils perturbaient le développement de *Trichostrongylus colubriformis*, autre nématode parasite des petits ruminants (Molan et al., 2003b).

Enfin, l'efficacité *in vitro* des stipes et feuilles de *Musa x paradisiaca L.*(*Musaceae*) sur le développement larvaire du parasite (68 à 99% d'inhibition) et sur la motilité des vers adultes (43% d'inhibition) serait, quant à elle, due à des composés terpénoïdes et/ou flavonoïdes (article 4).

Les résultats des essais *in vitro* réalisés sur différents stades de développement du parasite ont donc permis de mettre en évidence l'action ciblée des substances présentes dans les ressources végétales testées.

Les sources de molécules (tanins condensés et autres polyphénols, alcaloïdes, acides aminés, saponosides et autres composés terpéniques), mais également leur nature biochimique et physicochimique (structure, possibilité de synergie), peuvent expliquer la variabilité de l'efficacité sur les différents stades de développement du parasite. En effet, à chaque stade parasitaire sont associées des modifications au niveau des protéines de surface du parasite (Rhoads et Fetterer, 1994), modulant ainsi leurs interactions avec le milieu extérieur. De plus, à l'intérieur de chaque classe de métabolites secondaires, il existe des variations de structure entrainant ainsi des possibilités multiples d'interactions avec les protéines des différents stades parasitaires. C'est le cas, par exemple, des tanins condensés, qui peuvent être différents d'une plante à l'autre, en termes de nature moléculaire et de rapports de concentration (article 5; Brunet et al., 2007; Brunet and Hoste, 2006; Nguyen et al., 2005).

1.3. Tests *in vivo* et *in vitro*

L'analyse bibliographique réalisée lors du travail de thèse montre qu'en ce qui concerne l'effet nématicide sur *H. contortus*, 17% de ces ressources ont été évaluée *in vitro,* 22% *in vivo et* seulement 4% à la fois *in vitro* et *in vivo.* Les tests *in vitro* décrits dans la littérature sont surtout axés sur un ou deux stades de développement du parasite. Il apparaît nécessaire d'appliquer l'évaluation *in vitro* à un maximum de stades parasitaires pour une meilleure évaluation de l'effet nématicide. C'est l'approche qui a été adoptée dans nos travaux, en ciblant 4 stades clés: l'éclosion, le développement larvaire de L1 à L3, le stade infestant L3 et le vers adulte.

Cependant, la nature des tests *in vitro* est tellement diversifiée qu'il peut encore être discuté du type de test à réaliser sur les différents stades de développement, chacun relevant d'un aspect particulier du fonctionnement du parasite ou de l'anthelminthique (Geary et al., 1999). Notre choix s'est porté sur les deux tests appliqués pour la détection de résistances aux anthelminthiques chimiques (Hubert et Kerboeuf, 1992), ainsi que ceux évaluant la migration des L3 infestantes (Wagland et al., 1992), et la motilité des vers adultes (Hounzangbe-Adote et al., 2005). Ces tests sont assez pertinents car ils constituent une référence en parasitologie pour les deux premiers (Coles et al., 1992) et d'autre part, ils visent des mécanismes clés de la vie du parasite. D'autre part, la question du choix des doses à tester *in vitro* se pose. Elle a été, pour notre part, résolue en faisant dans un premier temps une étude bibliographique, suivie d'essais préalables afin d'affiner l'intervalle de doses à tester. Les doses choisies pour les essais *in vitro* se situant dans l'intervalle de la bibliographie. La plus forte dose a étée choisie en tenant compte non seulement de l'effet (plateau), mais également de l'aspect pratique dans la lecture du test (turbidité). Ainsi, pour les TC par exemple, les doses testées *in vitro* sont en deçà de celles utilisées pour les essais *in vivo*, qui sont elles-mêmes déjà non toxiques pour l'animal.

De plus, les tests *in vitro* sur les stades de vie libre du parasite (éclosion, développement larvaire, migration des L3), peuvent être un reflet de l'effet *in vivo*. Il est, en effet, possible que la plante ingérée ait des répercussions sur les stades de vie libre, par action dans les fèces ou dans le tractus gastro-intestinal. Nous avons l'exemple dans nos travaux, de L'effet *in vivo* sur le développement larvaire après ingestion des fourrages bioactifs par le ruminant (articles 6 et 7).

Les résultats de l'analyse de la littérature mettent en avant d'autre part, la nécessité de développer les tests *in vitro* et *in vivo* en parallèle pour une plante donnée. Le test *in vitro* est, certes, un moyen rapide et peu coûteux d'évaluer une efficacité, mais il ne reflète pas toujours l'effet observé *in vivo*. Ce travail de thèse a été l'occasion d'établir un rapport entre l'*in vivo* et l'*in vitro* pour lesquels il n'est pas toujours simple d'expliquer, de comparer, les résultats observés (Athanasiadou et Kyriazakis, 2004).

Dans le cas de nos travaux, les résultats obtenus lors des tests *in vivo* et *in vitro* avec les feuilles de manioc (*Manihot esculenta)* d'une part et le bananier (*Musa x paradisiaca)* d'autre part, vont dans le même sens. Il semble donc d'une part, que les substances ont des effets directs (développement du parasite de l'œuf à la larve L3) et indirects (production d'œufs donc fécondité des vers femelles) sur le parasite.

Et d'autre part, que les substances actives ne subissent pas de transformation dans le tractus digestif des ruminants, susceptible d'inhiber leur efficacité sur le parasite. Cette hypothèse doit être confirmée par des travaux ultérieurs concernant le devenir des substances actives dans le tube digestif des petits ruminants et leur impact *in situ* sur le parasite, notamment les œufs. Des travaux sont en cours à l'URZ, pour l'étude de ces phénomènes.

Nos résultats laissent supposer que les essais *in vivo* et *in vitro* sont complémentaires. En effet, l'essai *in vivo* est un moyen d'appréhender le mode d'action des principes actifs en conditions réelles et ainsi d'étudier la pharmacologie (mécanismes d'interaction entre le principe actif et l'organisme) et la pharmacodynamique (effets des principes actifs sur l'organisme). Mais l'essai *in vitro* a l'avantage de permettre d'étudier directement l'effet des substances actives sur les stades parasitaires de manière individuelle et d'aller étudier les interactions moléculaires. La complémentarité de ces deux moyens d'étude, comme entendu ici, nécessite bien sûr que les effets aillent dans le même sens dans les deux cas. Il devient alors possible d'établir, pour un composé donné, des corrélations entre les effets *in vitro* et *in vivo*, comme cela se fait dans le cas des tests de résistance aux anthelminthiques chimiques avec l'analogie entre l'effet *in vitro* sur le développement larvaire et l'effet *in vivo* sur les vers adultes (Geary et al., 1999).

Cependant, si dans le cas présent les résultats vont dans le même sens, il n'en est pas toujours ainsi (Athanasiadou et al., 2001). Sous certaines conditions, la disponibilité de la substance active pour atteindre le parasite *in vivo* n'est pas la même qu'*in vitro.* En effet, des interactions moléculaires dans l'hôte peuvent empêcher le contact direct entre le principe actif et le parasite *in vivo* (Athanasiadou et Kyriazakis, 2004) ou alors les conditions

de milieu (pH, température,…) peuvent modifier la structure, donc l'activité des substances actives *in vitro*. De plus, ce sont des extraits qui sont testés *in vitro*, donc une forte concentration en principes actifs, ce qui n'est pas toujours le cas si la plante entière est administrée *in vivo*.

Une solution serait d'adapter les tests *in vitro* aux conditions *in vivo* afin de s'assurer que l'utilisation du principe actif soit la meilleure possible. De nouvelles méthodologies sont développées dans ce sens. Par exemple un essai *in vitro* sur l'efficacité des métabolites secondaires dans le jus de rumen a été mis au point (Molan et al., 2000). Les tests *in vivo* doivent quant à eux se rapprocher au maximum des différentes conditions d'utilisation. Une des limites des tests *in vivo* appliqués expérimentalement lors de ces travaux est qu'ils ont été réalisés en hors sol, en mono-infestation, alors qu'au pâturage l'animal s'infeste régulièrement en ingérant l'herbe et il est au contact de plusieurs types de parasites.

Bien que le hors sol constitue un système d'élevage, Il conviendrait de développer des essais expérimentaux *in vivo* sur pâturage, afin d'étudier d'autres conditions d'élevage. Parmi les nombreuses questions que l'on pourrait se poser au pâturage on peut citer : quelles sont la fréquence et la quantité de ressources à propriété anthelminthique à faire consommer aux animaux au pâturage pour que le traitement soit efficace contre la re-contamination.

2. Des résultats à consolider

2.1. Approfondissement des tests *in vivo*

Le choix des plantes, pour nos travaux, était basé sur la présence de principes pharmacologiques potentiellement actifs sur les parasites (études bibliographiques) : alcaloïdes, tanins condensés, acides aminés non protéiques. Pour la plupart des plantes testées, les résultats montrent que les substances ciblées pourraient effectivement être celles responsables de l'effet nématicide sur *H. contortus*. Par contre, chez le bananier *(Musa x paradisiaca)*, les résultats montrent que les principes actifs suspectés (tanins condensés) ne sont pas ceux responsables de l'activité nématicide. En effet, le dosage des tanins condensés (TC) révèle un très faible niveau de concentration.

Nous avons observé, lors du screening phytochimique, un résultat fortement positif pour la présence, dans les deux organes tige et feuille de la plante, de saponosides et autres triterpènes et stérols (article 4); et de flavonoïdes dans la tige. Il est donc nécessaire d'étudier les principes actifs responsables de l'activité nématicide du bananier, en testant ces classes de molécules directement *in vitro,* d'autant plus qu'un phénomène de synergie n'est pas à exclure. Par ailleurs, la feuille de bananier a une meilleure valeur nutritionnelle que la tige (article 6). Il apparaît donc intéressant de tester l'effet de la feuille seule *in vivo* afin de valoriser à la fois les propriétés nématicide et nutritionnelle du bananier.
L'essai *in vivo* sur les feuilles de manioc a bien montré que l'effet nématicide est dû pour partie à des TC (article 7). Il reste alors à établir la dose de TC effectivement efficace. En plus d'être réalisée *in vitro*, cette mesure doit également l'être *in vivo,* ce qui permettrait d'établir la valeur seuil pour l'effet antinutritionnel.

Les hypothèses quant à la nature des principes actifs impliqués sont basées sur des études *in vitro* appuyées par la phytochimie, mais les essais *in vivo* doivent être répétés pour les deux plantes (bananier et manioc) directement avec les classes de molécules, afin d'une part de s'assurer de leur implication dans l'activité (surtout pour le bananier); mais également de mieux étudier leur pharmacologie et leur pharmacodynamique.

D'autre part, dans les essais *in vivo* (article 6 et 7), il est observé un effet sur le développement des œufs en L3 dans les fèces, ce résultat déjà retrouvé dans la littérature (Nguyen et al., 2005) confirme également les résultats des tests *in vitro.* Il faut maintenant déterminer si la substance active agit dans les fèces après avoir traversé tout le tractus gastro-intestinal, ou bien si elle a agit en amont au niveau du vers femelle et de quelle manière. Cela peut être mis en évidence en mettant en culture, hors fèces, les œufs issus des animaux alimentés avec la plante bioactive. De même, un effet sur l'installation des L3 a été observé avec le bananier, cette donnée peut être évaluée, par exemple, en testant la plante sur la larve dégainée.

De plus, à l'occasion de nos travaux, nous avons pu voir qu'un autre aspect méthodologique devait être corrigé. Nous avons observé un effet sur la production d'œufs et l'avons interprété comme étant une réduction de la fécondité des vers femelles. Ce dernier paramètre mérite d'être précisé et est à interpréter avec précaution.

En effet, certains auteurs mesurent la fécondité en comptant le nombre d'œufs *in utero* (Terefe et al., 2005) et d'autres l'estiment avec le rapport : nombre d'œufs dans les fèces / charge parasitaire (Stear and Bishop, 1999). La première méthode est une mesure effectuée à un instant t et ne tient pas compte du renouvellement des œufs chez le ver femelle, ce qui peut fausser l'interprétation du résultat. Nous avons utilisé la première méthode alors que la deuxième semble être la plus appropriée. Un autre moyen serait de développer, comme pour *Teladorsagia circumcincta*, une méthode d'estimation indirecte en mesurant la longueur des vers femelles, corrélée à la fécondité (Stear and Bishop, 1999).

2.2. Extraction, évaluation quantitative, test des métabolites secondaires

L'étude de la ressource brute et/ou de familles de molécules comme réalisée dans le travail de thèse a certes, l'intérêt de permettre l'évaluation anthelminthique, mais il n'éclaire pas suffisamment sur les mécanismes mis en place. Il conviendrait donc, comme annoncé précédemment, de prolonger les travaux engagés par des tests impliquant les sous-familles ou groupes de molécules suspectées comme actives. Ce travail doit, à terme, impliquer des approches de phytochimie.

La plupart des travaux sur l'usage anthelminthique des plantes posent l'hypothèse de l'implication de certaines substances dans l'activité nématicide. Ces travaux s'appuient sur le fait que la plante soit connue pour sa forte concentration en des principes actifs particuliers. Cependant leur concentration dans la ressource végétale n'est pas toujours évaluée.

Il est important de réaliser cette mesure car elle constitue un indicateur de la valeur anthelminthique et/ou antinutritionnelle de la plante. C'est la raison pour laquelle nous avons procédé au dosage des TC pour les ressources végétales utilisées *in vivo*. Il faut donc caractériser et quantifier les substances actives présentes dans les plantes, ces étapes sont réalisables grâce à l'outil phytochimique. Cet outil a été utilisé dans nos travaux mais peu valorisé.

En effet, afin de caractériser les substances actives dans les plantes, nous avons utilisé la chromatographie sur couche mince (CCM), le screening phytochimique, les dosages spectrophotométriques et la chromatographie liquide haute pression (HPLC), (articles 2, 3, 4 et 5). Le screening phytochimique doit être réalisé dans un premier temps, afin d'avoir une vue d'ensemble des classes chimiques représentées dans la plante. Même s'il ne s'agit que d'une étape qualitative, le screening présente l'intérêt d'aider à prévoir les substances à viser, sachant que la plupart du temps, ce sont les classes prépondérantes qui constituent les substances efficaces. Cette étape n'a pas été utilisée pour toutes les ressources, car nos bases de recherches étaient fondées sur la littérature. Cependant nos résultats négatifs concernant les TC sur le bananier nous ont amenés à réaliser un screening phytochimique (article 4). Ce résultat nous a permis d'approfondir nos connaissances sur la plante, mais il conduit également à dire qu'il est important de passer par cette étape préliminaire. Une application sur les autres plantes étudiées est en cours à l'URZ.

Une fois la substance visée, il est ensuite important de passer à la mesure quantitative. En effet, c'est cette valeur qui sera utilisée pour appuyer l'activité et la suite des tests *in vitro* et *in vivo*. Parmi les principes actifs, nous avons pu quantifier que TC et polyphénols totaux (articles 4, 5, 6 et 7). Lors de la mise en place de ce dosage (méthode vanilline-HCl) nous avons pu mettre en évidence la nécessité d'avoir comme standard, les TC purifiés de la plante à doser. En effet, dans notre cas, l'utilisation du standard Quebracho généralement préconisé (Hagerman and Butler, 1989), sous-estime la mesure car chaque plante renferme des TC particuliers. L'HPLC nous a servi pour la différentiation et l'identification de ces TC (article 5).

Il nous faut donc maintenant séparer les classes phytochimiques, voire, purifier les groupes intra classes (en utilisant l'HPLC ou la chromatographie sur colonne ouverte), afin de procéder ensuite à des tests *in vitro* sur les stades concernés, d'une part, mais également à leur dosage précis (par des méthodes chromatographiques, colorimétriques, …). En effet, ces substances purifiées pour chaque plante individuellement, pourront être utilisées comme standards.

La connaissance de la classe chimique à étudier permet également d'adapter les conditions des essais (aussi bien *in vivo* et *in vitro*) à la substance, afin notamment, de ne pas altérer son efficacité si elle est fragile (traitement préalable de la plante, conditions d'utilisation, sensibilité à la température, etc…). L'identification des classes chimiques permet aussi de procéder par la suite à leur dosage dans les différents organes végétaux, afin d'étudier les valeurs seuils de concentration et la partie de la plante à administrer. Par la suite, le dosage associé au test *in vitro* constitue un moyen de prévision de la composition de la ration, voire d'établissement d'une formulation, dans le cas d'une distribution directe du fourrage. Ce process permet de généraliser l'attribution d'une efficacité à la présence d'un métabolite secondaire donné, permettant de déterminer la « valeur nématicide » de la ressource végétale.

Enfin, les méthodes d'évaluation de l'activité nématicide des plantes sont variées, et il existe des lacunes méthodologiques dans les différentes approches utilisées ce qui conduit à des débats quant à la cohérence de leur activité et donc leur potentiel d'utilisation pour le contrôle des parasites (Athanasiadou et al., 2007). D'où la nécessité d'avoir des méthodes standardisées pour le screening de l'activité nématicide des ressources végétales (entières ou extraits).

3. Aliment, médicament et nutricament, quelles stratégies ?

3.1. Contraintes des nutricaments

Un alicament ou nutricament, peut être défini comme un aliment (ou partie d'aliment) naturel qui possède des propriétés thérapeutiques car enrichi en un ou plusieurs principes actifs.

Parmi les plantes recensées, certaines peuvent démontrer un potentiel en tant que nutricament. En effet, les plantes peuvent avoir outre leurs qualités anthelminthiques, un potentiel nutritionnel non négligeable. C'est le cas des feuilles de manioc ou de bananier.

Il est nécessaire d'en tenir compte dans la formulation de l'aliment s'il s'agit de développer un nutricament. Dans ce cas, il faut faire la part des choses entre l'effet anthelminthique souhaité et l'effet nutritionnel, car certains métabolites secondaires peuvent constituer des facteurs antinutritionnels lorsque leur concentration atteint un certain seuil. C'est le cas des tanins condensés et des saponosides, que l'on retrouve en plus grande quantité respectivement dans les feuilles de manioc et chez le bananier, mais également des composés alcaloïdes, terpènes, lactones, glycosides et autres phénoliques.

Par exemple, la consommation excessive de tanins condensés est associée à une réduction de l'ingestion et de la digestibilité, un disfonctionnement du métabolisme du rumen et une toxicité des muqueuses (Kumar et Singh, 1984; Nguyen et al., 2005; Silanikove et al., 1996).

Consommées en grandes quantités, la plupart des saponines entraînent une baisse de l'ingestion et causent des déficiences nutritionnelles, une hémolyse et dans des cas extrêmes, la mort de l'animal (Athanasiadou et Kyriazakis, 2004; Milgate et Roberts, 1995).

La consommation excessive de glycosides cyanogéniques, de terpènes et d'alcaloïdes peut entraîner des dégâts neurologiques (Bruneton, 1999). D'autres composés comme les cystéine-protéinases sont hautement toxiques en dépit de leur très bon effet anthelminthiques (De Amorin et al., 1999).

Ainsi, lors de la formulation de la ration alimentaire, ces contraintes doivent être prises en compte afin d'allier le meilleur effet nématicide possible à la plus basse concentration en facteurs antinutritionnels. D'après l'étude bibliographique réalisée dans le cadre de nos travaux, les plantes disponibles pour un usage en tant que nutricament sont peu nombreuses. Il semble donc que l'usage des plantes en tant que médicament seul, soit prédominant. La question de la transformation de la ressource végétale en médicament est donc posée.

Quoi qu'il en soit, il est nécessaire de procéder à des tests *in vitro* et cliniques afin de déterminer le degré de toxicité (DL 50) ainsi que les effets secondaires des substances impliquées.

3.2. Molécules à intérêts multiples

Certaines molécules d'intérêt anthelminthique peuvent avoir d'autres usages en production animale. Nos travaux *in vivo* ont porté sur deux plantes contenant chacune, en quantité non négligeable, des métabolites secondaires rentrant dans cette catégorie : les saponines et les tanins condensés.

Les saponines (ou saponosides sous leur forme hétéroside) sont connues pour leurs propriétés défaunantes (réduction des de la population de protozoaires du rumen).

Cette action sur les protozoaires permet, d'une part d'améliorer l'utilisation de l'azote par l'animal, et d'autre part de réduire la production de méthane (Makkar et al., 2007; Wina et al., 2005). Indirectement la présence de saponosides dans l'aliment pourrait contribuer à la l'amélioration de la valeur environnementale des élevages.

Les tanins condensés ont une action protectrice sur les protéines dans le rumen, les rendant disponibles post-rumen. Le niveau de production est ainsi amélioré par une meilleure utilisation de la matière azotée et l'émission de méthane s'en trouve réduite (Animut et al., 2008; Beauchemin et al., 2007; Khang et Wiktorsson, 2000; Ramirez-Restrepo et Barry, 2005). De plus, la concentration en azote dans les urines se trouve diminuée et davantage présente dans les fèces sous une forme plus stable. Il en résulte donc ici aussi, une amélioration de la valeur environnementale, avec une moindre déperdition de l'azote dans la nature. Des études *in vitro* ont également montré que les tanins condensés réduisaient la production de scatole et d'indole dans le rumen, composés exerçant un effet négatif sur la qualité et la flaveur de la viande (Schreurs et al., 2007). Enfin, les tanins font partie des polyphénols qui sont connus pour leurs propriétés antioxydantes (inhibition de la peroxydation des lipides par les peroxygenases).

V. CONCLUSION ET PERSPECTIVES

Les ressources végétales représentent une réelle alternative aux anthelminthiques de synthèse. L'inventaire bibliographique réalisé lors de cette étude a permis de recenser 194 plantes appartenant à 74 familles botaniques; et potentiellement utilisables en santé animale en zone Caraïbe. Les plantes à usage anthelminthique testées *in vitro* sur le parasite *Haemonchus contortus* ont toutes montré un effet nématicide, en particulier sur le développement larvaire de L1 à L3. Les ressources nouvellement testées sur *Haemonchus contortus* sont : *Annona muricata, Rauvolfia viridis, Tabernaemontana citrifolia L., Neurolena lobata, Cucurbita moschata, Lantana camara, Senna alata, Crescentia cujete L.et Bidens pilosa.*

Les études *in vitro* plus approfondies, associées à la phytochimie, ont permis de suspecter les familles phytochimiques impliquées dans l'activité sur les stades parasitaires. Les classes chimiques des terpénoïdes (saponosides, cucurbitacine B), des composés acides aminés (acide aminé cucurbitine, protéine cucurmosine) et des phénoliques (flavonoïdes, tanins condensés) semblent affecter les stades de développement larvaire. Les alcaloïdes indoliques agissent, quant à eux sur le développement larvaire, la migration des L3 et le stade adulte du parasite. Le stade adulte est également affecté par certains flavonoïdes et terpénoïdes.

Les essais *in vivo* ont permis de globaliser les effets en conditions réelles, montrant que les substances agissent bien sur le développement du parasite. Le développement des œufs issus d'animaux ayant ingéré la plante (*Manihot esculenta, Musa x paradisiaca*) est inhibé. Cependant, l'installation des vers n'est pas affectée par la substance et les animaux ne présentent aucuns effets secondaires, aux conditions et doses administrées.

La complémentarité des essais *in vitro* et *in vivo* a été étayée lors de ces travaux de thèse. En effet, concernant les deux ressources *Manihot esculenta Crantz*et *Musa x paradisiaca,* les effets sont dirigés dans le même sens, ce qui permettra de poursuivre les travaux *in vitro*. Ces ressources peuvent donc être utilisées comme plantes modèles pour la détermination des valeurs seuils de concentration à administrer *in vivo,* sur la base des travaux initiés *in vivo*. Des essais complémentaires sont à conduire au pâturage pour mieux comprendre les modalités d'action des plantes sur les différents stades de développement des nématodes pathogènes des ruminants.

Il est par ailleurs connu que, la mâturité, l'organe, l'espèce de la plante, la nature du sol, le climat sont autant de facteurs qui influencent la concentration en substance active. Il est donc nécessaire d'avoir des méthodes de caractérisation standardisées et rapides pour évaluer la plante. Concernant les classes phytochimiques actives, une étude qualitative, associée à une étude quantitative et à des tests *in vitro* sur permettrait, non seulement de déterminer les substances présentes, déterminer les valeurs de concentrations seuils pour préparer les rations, afin d'allier si possible, l'effet anthelminthique à l'effet nutritionnel. Ces travaux de thèse montrent l'intérêt d'allier le savoir phytochimique au savoir zootechnique, notamment pour développer la notion de nutricament.

Les ressources végétales à propriétés anthelminthiques peuvent donc s'insérer à plusieurs niveaux dans des systèmes de contrôle intégré contre les SGI : par une action directe sur la mortalité des vers adultes, sur la fécondité des vers femelles ou sur le développement de œufs en larves infestantes, entrainant une diminution des populations infestantes sur les pâturages et donc une diminution de la réinfestation des animaux, renforçant ainsi les mesures de gestion des pâturages. Une action indirecte peut également intervenir via la nutrition, qui renforce les défenses de l'hôte, ce qui constitue un complément aux mesures de sélection génétique.

La solution pour le contrôle des strongyloses gastro-intestinales en limitant l'usage des anthelminthiques chimiques, semble être la lutte intégrée avec une approche multicritères dans laquelle la phytothérapie prendrait une part. Pour cela, il est nécessaire de standardiser les méthodes de dosages sur les plantes antiparasitaires chez les animaux (niveaux anthelminthique, nutritionnel et antinutritionnel), afin d'avoir une approche globale prenant en compte: le potentiel antiparasitaire, le potentiel nutritionnel (pouvant avoir un impact sur le potentiel immunomodulateur de la plante), ainsi que le critère économique des ressources végétales, pour les intégrer au mieux dans les systèmes de production animale dans un but d'amélioration du niveau de production tout en diminuant les intrants agricoles.

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NOM DE L'AUTEUR : MARIE-MAGDELEINE Carine **ANNEE :** 2009

TITRE DE LA THESE : ***Etude de ressources végétales tropicales pour un usage anthelminthique en élevage de ruminants***

Résumé : Le parasitisme gastro-intestinal (GI) est un problème majeur de santé et de bien-être des ruminants d'élevage. Il affecte la santé animale et a un impact négatif sur la productivité globale des troupeaux. Depuis les années 1960, la chimioprophylaxie est le principal moyen de lutte. Cependant, l'émergence de résistances des strongles aux anthelminthiques de synthèse est devenue un phénomène répandu dans le monde entier. Ces facteurs ont conduit la recherche à s'interroger sur des solutions alternatives pour lutter contre le parasitisme GI des ruminants. Ce travail de thèse s'inscrit dans la programmation scientifique de l'unité INRA-URZ sur l'évaluation multicritères des ressources végétales et la lutte intégrée contre le parasitisme GI des ruminants. La lutte intégrée combine plusieurs méthodes de lutte parmi lesquelles figure la phytothérapie. L'objectif de ce travail de thèse est d'étudier l'activité nématicide de certaines ressources végétales contre le strongle digestif *Haemonchus contortus*; et secondairement de caractériser la classe chimique responsable de l'activité puis d'expliquer le mode d'action. **Dans une première partie**, un recensement bibliographique des ressources végétales disponibles aux Antilles-Guyane pour un usage vétérinaire a été réalisé. Cela a permis de constater que ces régions disposent d'un véritable arsenal végétal pour un large panel de pathologies. Un screening *in vitro* sur l'éclosion des œufs et le développement larvaire du modèle *H. contortus* a alors été réalisé sur 14 plantes, qui ont toutes montré un effet anthelminthique sur au moins un stade. Le niveau d'efficacité varie entre 20% et 55% sur l'éclosion des œufs; et de 26% à 98% pour le développement larvaire. **Dans une deuxième partie**, les 4 plantes: *Cucurbita moschata, Tabernaemontana citrifolia, Musa x paradisiaca et Manihot esculenta,* ont été sélectionnées par rapport à la diversité en métabolites secondaires d'intérêt qu'elles contiennent : tanins condensés, flavonoïdes, alcaloïdes, composés aminés, terpénoïdes; pour réaliser des essais complémentaires *in vitro*. Les essais in vitro ciblaient les 4 stades clés: éclosion, développement de L1 à L2, migration des larves L3 et motilité des vers adultes. Ces plantes ont toutes montré un effet nématicide avec des variations en fonction des stades, mettant en évidence la spécificité des molécules relativement aux stades parasitaires. Ainsi, les alcaloïdes indoliques agissent sur les 4 stades, les flavonoïdes et terpénoïdes affectent le stade adulte, tandis que les tanins condensés, composés aminés, flavonoïdes et terpénoïdes ont tous un impact sur le développement larvaire. **Dans une troisième partie**, les deux modèles bananier (*Musa x paradisiaca) et* feuilles de manioc *(Manihot esculenta)* ont été évalués *in vivo*. En plus de leur potentiel nématicide et nutritionnel, ces deux ressources ont l'intérêt de permettre la valorisation de co-produits de productions végétales agricoles. L'implication des tanins condensés du manioc a été confirmée par l'utilisation du PEG comme inhibiteur spécifique. Des effets sur l'installation des larves infestantes (uniquement pour le manioc), sur la fécondité des vers femelles et sur le développement larvaire ont été observés (pour les deux plantes).

En conclusion, ces travaux montrent d'une part, que les ressources végétales représentent une réelle alternative aux anthelminthiques de synthèse; et d'autre part, l'intérêt d'allier le savoir phytochimique au savoir zootechnique, notamment pour développer la notion de nutricament. La solution pour le contrôle des strongles GI en limitant l'usage des anthelminthiques chimiques semble être la lutte intégrée, avec une approche multicritères dans laquelle la phytothérapie prendrait une part. Pour cela, il est nécessaire de standardiser la recherche sur les plantes antiparasitaires (méthodes *in vitro*, valeurs seuil pour les principes actifs antinutrutionnels). Une approche globale prenant en compte non seulement le potentiel antiparasitaire, le potentiel immunomodulateur et nutritionnel mais aussi le critère économique, permettrait d'intégrer au mieux les ressources végétales dans les systèmes de production animale, dans un but d'amélioration du niveau de production tout en diminuant les consommables agricoles.

AUTHOR NAME : MARIE-MAGDELEINE Carine **YEAR :** 2009

TITLE OF THE THESIS : ***Study of tropical plant resources for anthelmintic use in ruminant livestock***

Abstract: Gastrointestinal parasitism (GI) is a major health and welfare problem of farmed ruminants. It affects animal health and has a negative impact on overall productivity of herds. Since the 1960s, chemotherapy is the principal means of nematode control. However, the emergence of resistance of strongyles to chemical anthelmintics has become a widespread phenomenon in the world. These factors led the search to consider alternative solutions to fight against GI parasites of ruminants. This thesis is part of the scientific program of the INRA-URZ unit on multicriteria evaluation of plant resources and integrated management against GI parasites of ruminants. Integrated management combines several control methods including herbal medicines. The objective of this thesis is to investigate the nematicidal activity of some plant resources against the *Haemonchus contortus* digestive strongyles, and secondly to characterize the chemical class responsible for the activity and then to explain the mode of action. **In a first part**, a literature survey of plant resources available in the Antilles-Guyane for health use has been made. This has shown that these regions have a veritable arsenal of plants for a wide range of pathologies. A screening for *in vitro* egg hatching and larval development with the model *H. contortus* was then performed on 14 plants, all showed anthelmintic effect on at least one stage. The efficiency varies between 20% and 55% on the hatching of eggs, and 26% to 98% for larval development. **In a second part**, the 4 plants: *Cucurbita moschata, Tabernaemontana citrifolia, Musa x paradisiaca and Manihot esculenta,* were selected in relation to diversity in secondary metabolites of interest that they contain: condensed tannins, flavonoids, alkaloids, amino compounds, terpenoids, to carry out *in vitro* tests. The *in vitro* targeting the 4 key developmental stages: egg hatching, development of L1 to L2, L3 larval migration and motility of adult worms. These plants have all shown a nematicidal action with variations depending on the stage, highlighting the specificity of the molecules with respect to parasitic stages. Thus, indole alkaloids act on the 4 stages, flavonoids and terpenoids affect the adult stage, while condensed tannins, amino compounds, flavonoids and terpenoids have an impact on the larval development. **In a third part**, both models banana (*Musa x paradisiaca*) and cassava leaves (*Manihot esculenta*) were evaluated *in vivo*. In addition to their potential nutritional and nematicidal effects, these two resources have the interest to allow recovery of co-products from agricultural crops. The involvement of condensed tannins from cassava was confirmed by the use of PEG as a specific inhibitor. Effects on the installation of infective larvae (only for cassava), on the fertility of female worms and on the larval development were observed (both plants). In conclusion, these studies show first, that plant resources are a real alternative to synthetic anthelmintics, and secondly, the interest to combine phytochemical knowledge with zootechnical knowledge, particularly in order to develop the concept of nutriceuticals. The solution for the control of GI strongyles by limiting the use of anthelminthic chemicals seems to be integrated management, with a multi-criteria approach in which the herbal take a share. For this it is necessary to standardize research on antiparasitic plants (*in vitro* methods, threshold values for the active antinutrutional factors). A comprehensive approach taking into account not only the antiparasitc potential, the immunomodulator potential and nutrition but also the economic criterion, would incorporate the best plant resources in livestock production systems in order to improve the level of production while reducing agricultural inputs.