Exogenous application of salicylic acid and gibberellic acid on biomass accumulation, antioxidant and anti-inflammatory secondary metabolites production in multiple shoot culture of Ajuga integrifolia Buch. Ham. ex D.Don

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1 Research Article for *Industrial Crops & Products*

2 Exogenous application of salicylic acid and gibberellic acid on biomass 3 accumulation, antioxidant and anti-inflammatory secondary metabolites 4 production in multiple shoot culture of *Ajuga integrifolia* Buch. Ham. ex 5 D.Don.

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18 # Abstract

19 *Ajuga integrifolia* Buch. Ham. ex D.Don is pharmacologically important plant, commonly
20 known as Kori Booti. Phytochemicals of this plant are used to treat variety of illnesses, ranging
21 from mild tooth ache to malaria and inflammatory diseases. In present study, exogenous role of
22 plant signaling molecules such as salicylic acid (SA) and gibberellic acid (GA) were investigated
23 for enhanced phytochemistry and biological activities in multiple shoot culture of *A. integrifolia*.
24 Shoots were exposed to various concentrations of SA and GA, and data was collected after 21
25 days. Significant increase in biomass (dry weight (DW): 17.9 g/L) was observed in response to
26 GA (5.0 mg/L) as compared with SA and control. High performance liquid chromatography was
27 employed to quantify eleven major secondary compounds. Results indicated that salicylic acid
28 promoted phytochemical production in shoot cultures whereas gibberellic acid down regulated
29 their biosynthesis. Salicylic acid (150 µM) was found optimum for maximum accumulation of
30 majority of compounds, while rosmarinic acid and caffeic acid levels were higher in response to
31 SA (75 µM) and SA (300 µM), respectively. *In vitro* antioxidant and anti-inflammatory activities
32 were significantly increased under SA (150 µM) treatment suggesting a direct correlation in
33 phytochemical biosynthesis and their respective biological activities.

34 **Keywords:** *Ajuga integrifolia;* Salicylic acid; shoot culture; Anti-inflammatory activity;
35 Phytochemicals; UPLC.

36

37

38 # 1. Introduction

39 Diseases are traced back since the origin of mankind on this planet. Humans are trying to combat
40 illnesses by exploitation of nature's primary resources such as microbes, animal and plants.
41 Plants have been a major source of effective substances to cure myriads of illnesses since
42 prehistoric times (Duraipandiyan et al., 2006). One of the many important medicinal plants
43 (MPs) is *Ajuga integrifolia*, commonly known as *Ajuga bracteosa* (Synonym) in Asian region,
44 belongs to family Lamiaceae. Both terminologies i.e. *bracteosa* and *integirfolia* are
45 interchangeable that are used to indicate same plant. *A. integrifolia* is a perennial herb that grows
46 up 5–50 cm tall and is usually found at high altitudes such as Himalayan region including
47 Pakistan, China and Afghanistan (Barkatullah et al., 2015; Jan et al., 2014). *A. integrifolia* plant
48 extracts have previously been studied for their enormous health benefit. Phytotoxic potential of
49 n-hexan mediated *A. integrifolia* extract showed herbicidal activity against Lemna minor
50 (Rehman et al., 2015) whereas anticancer activity of methanolic extracts was observed against
51 MCF-7 and Hep-2 cell lines (Pal et al., 2014). Multiple studies have demonstrated anti-
52 inflammatory and anti-plasmodial potential of *A. integrifolia* plant extracts by employing *in vivo*
53 mice models (Chandel and Bagai, 2010; Chandel and Bagai, 2011; Gautam et al., 2011;
54 Kaithwas et al., 2012).

55 Due to overexploitation of *Ajuga* plant on huge pharmaceutical demand, it is at the edge of
56 extinction as it has already been declared as endangered plant in Pakistan (Park et al., 2017; Rani
57 et al., 2017; Saeed et al., 2017). Use of biotechnological approaches such as *in vitro* cultures,
58 micro-propagation and organogenesis could really provide a suitable platform for conservation
59 of critically endangered plant (Park et al., 2017; Raghu et al., 2006; Shinwari, 2010).
60 Establishment of *in vitro* shoot organogenesis is considerably feasible method for conservation
61 of plants by producing whole plant in relatively short time irrespective to environmental
62 fluctuations (Gurel et al., 2011; Verma et al., 2011). Shoot culture of *A. integrifolia* was
63 previously established for optimum secondary metabolites production (Ali et al., 2018).

64 The present study was designed to determine the potential effects of salicylic acid and gibberellic
65 acid on enhanced production of pharmaceutically important secondary metabolites. The treated
66 shoot cultures were evaluated based on biomass accumulation (dry weight), phenolic and
67 flavonoids production, antioxidant, anti-inflammatory potential and special emphasize on

68 correlation of biological activities with secondary metabolites production upon treatment.
69 Quantification of shoot culture metabolites was performed using high performance liquid
70 chromatography (HPLC).

71
2. Materials and Methods

72
2.1. Explant Collection and Multiple Shoot Culture Establishment

73 *In vitro* shoot culture of *A. integrifolia* was carried out using leaf as explant source. Wild grown
74 plants were collected from Department of Biotechnology, Quaid-i-Azam University, Pakistan.
75 Leafs were separated from whole plants and surface sterilized for initiation of *in vitro* cultures.
76 Surface sterilization was done using 0.1% mercuric chloride solution for 40 sec, followed by 1
77 min treatment of 70% ethanol. Thereafter, plant leafs were washed three times with autoclaved
78 distilled water. Leaf sections (~0.5 cm^2) were further inoculated on MS media (Murashige and
79 Skoog, 1962) containing 3% w/v sucrose and 0.8% w/v agar as solidifying agent. To promote
80 shooting, MS media was additionally supplemented with 1.0 mg/L BAP (6-benzylaminopurine)
81 and pH of media was set at 5.6 – 5.7 prior to autoclave. The culture was grown in 100 mL
82 vessels under dark conditions at 25 ± 2°C for maximum biomass production and constantly
83 transferred to fresh media after 4 weeks.

84
2.2. Treatment of Salicylic Acid and Gibberellic Acid

85 Basal culture of *A. integrifolia* established under dark conditions was used as inoculum to check
86 the effects of salicylic acid and gibberellic acid. Briefly, 1.0 g of shoots inoculum (3-4 shoots)
87 was added in previously used MS hormonal media additionally supplemented with various
88 concentrations of SA (75 µM, 150 µM, 300 µM) and GA (1.0 mg/L, 2.5 mg/L, 5.0 mg/L).
89 Cultures without any treatment of SA or GA were considered as control. Cultures were placed
90 under florescent light photoperiod conditions (16 h light/ 8 h dark) at 25 ± 2°C for optimum
91 shoot growth. Data was recorded after 21 days for maximum production of biomass and
92 secondary metabolites.

93
2.3. Analytical Scheme

94 After harvesting at 21 day, cultures were dried over night at 45°C for estimation of Dry weight
95 (DW). Dried shoot samples were subjected to extraction procedure according to (Zahir et al.,
96 2014). Briefly, 100 mg dried sample was dissolved in methanol (500 µl) by vortexing (5 min)
97 and sonication (30 min) at room temperature. The procedure was repeated twice, followed by
98 centrifugation at 15,000 rpm for 12 min to remove supernatant. Pallet was discarded and
99 supernatant was collected and stored at 4°C in eppendorf tube.

100 ## 2.4. Phytochemical Estimation

101 ### 2.4.1. Phenolic and Flavonoids

102 (Singleton and Rossi, 1965) protocol was employed for total phenolic content (TPC) estimation
103 using Folin–Ciocalteu's reagent (FC) with minor adjustments. Briefly, extracted sample (20 µl)
104 from each treatment was thoroughly mixed with Na_2CO_3 (90 µl) and FC reagent solution (90 µl)
105 at room temperature in 96 welled microplate. Mixture was incubated for 5 min prior to taking
106 absorbance using microplate reader at 630 nm. Gallic acid was employed as standard and results
107 were expressed as gallic acid equivalent. Phenolic production was calculated by following
108 formula,

109 Total phenolic production (mg/L) = DW (g/L) ×TPC (mg/g)

110 Furthermore, total flavonoid contents (TFC) were calculated by (Ahmad et al., 2010) described
111 $AlCl_3$ colorimetric method. Sample extract (20 µl) was thoroughly mixed with potassium acetate
112 (10 µl) and aluminum chloride (10 µl) and dH_2O (160 µl) in 96 welled microplate. Microplate
113 reader was used to measure absorbance of mixture at 415 nm after 30 min incubation time.
114 Quercetin was employed as standard and results were expressed as quercetin equivalent.
115 Flavonoid production was calculated by following formula,

116 Total flavonoid production (mg/L) = DW (g/L) ×TFC (mg/g)

117 ### 2.4.2. Quantification of phytochemical via HPLC

118 The separation, identification and quantification of the main metabolites accumulated in *A.*
119 *integrifolia* cultures were performed by HPLC. Separation was performed using the previously
120 established method allowing a good separation of caffeic acid derivatives and flavonoids, as

121 described in (Nazir et al., 2019). The separation was achieved using an Hypersil PEP 300 C18
122 column (250 × 4.6 mm, 5 µM) with a guard column Alltech (10 × 4.1 mm) at 35°C, by a Varian
123 high-performance liquid chromatography system (Varian Prostar 230 pump, Varian Prostar 410
124 autosampler and Varian Prostar 335 Photodiode Array Detector), driven by Galaxie version
125 1.9.3.2 software. The detection of compounds was initially set at 320 nm. The mobile phase
126 consisted of a mixture of two solvents: A composed of HCOOH/ H_2O, pH = 2.1 and B composed
127 of CH_3OH (HPLC-grade solvents). During the run, the mobile phase composition varied
128 according to the following nonlinear gradient: 8% B (0 min), 12% B (11 min), 30% B (17 min),
129 33% B (28 min), 100% B (30−35 min), 8% B (36 min) with a flow rate of 1mL/min. An
130 equilibration time of 10 min was applied after each individual run. The quantification was based
131 on the retention times and UV spectra of authentic commercial (Sigma-Aldrich). All samples
132 were analyzed in triplicate.

133 ## 2.5. Antioxidant Potential of *A. integrifolia*

134 ### 2.5.1. DPPH Scavenging Activity

135 (Abbasi et al., 2010) protocol was used to determine free radical scavenging activity of shoot
136 extracts by employing DPPH reagent. Sample extract (20 µl) was thoroughly mixed with DPPH
137 solution (180 µl) in 96 welled microplate, followed by 60 min incubation period in dark. The
138 final concentrations of ascorbic acid (5, 10, 20, 40 µg/ml) and DPPH (180 µl) with DMSO (20
139 µl) were taken as negative control. Absorbance was taken at 517 nm using microplate reader and
140 results were calculated using following formula,

141 $$\text{Free radical scavenging activity (\%)} = 100 \times (1\text{-AE/AD})$$

142 Herein, AE: Absorbance value with sample in mixture, AD: Absorbance value without sample in
143 mixture.

144 ### 2.5.2. Ferric Reducing Antioxidant Power (FRAP)

145 (Benzie and Strain, 1996) method was employed to determine FRAP activity in shoot cultures of
146 *A. integrifolia*. Briefly, FRAP solution (190 µl) [composed of $FeCl_3.6H_2O$ (20 mM); acetate
147 buffer (300 mM, pH 3.6) and TPTZ (10 mM); ratio 1:10:1 (v/v/v)] was mixed with sample

extract (10 µl) from each treatment, followed by 15 min incubation time at room temperature. Absorbance was taken at 630 nm using microplate reader. Results were expressed as TEAC (Trolox C equivalent antioxidant capacity).

2.5.3. Antioxidant ABTS Assay

ABTS antioxidant activity of shoot cultures was measured according to (Tagliazucchi et al., 2010) method. Briefly, potassium persulphate (2.45 mM) was mixed with ABTS salt (7 mM, 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid)) in equal proportion and incubated in dark for 16 h to prepare ABTS solution. The absorbance was taken using microplate reader at 734 nm and adjusted to 0.7. Extract was mixed in solution at room temperature and incubated in dark for 15 min, followed by absorbance at 734 nm. Results were expressed as TEAC (Trolox C equivalent antioxidant capacity).

2.6. Anti-inflammatory Activity of *A. integrifolia*

2.6.1. COX-1 and COX-2 Inhibitory Activity

COX-1 (Ovine) and COX-2 (human) assay kit was used according to manufacturer instructions (701050, Cayman Chem. Co, Interchim, Montluçon, France) to determine inhibitory activity of shoot extracts against COX-1 and COX-2. Arachidonic acid (1.1 mM) and ibuprofen (10 µM) were used as substrate and positive control respectively. The kit was used to measure COXs peroxidase component. At 590 nm, Synergy II reader was used to check oxidized *N,N,N′,N′*-tetramethyl-p-phenylenediamine for 5 min in 96 well microplate.

2.6.2. 15-LOX Inhibitory Activity

Assay kit was used according to manufacturer instructions (760700, Cayman Chem. Co, Interchim, Montluçon, France) to determine inhibitory activity of shoot extracts against 15-LOX. Arachidonic acid (10 µM) and nordihydroguariaretic acid (100 µM) were used as substrate and positive control inhibitor respectively. During lipooxygenation reaction, the hydroperoxides concentration was measured by the kit using filtered soy 15-lipooxygenase standard in 10 mM Tris-HCl buffer at 7.4 pH. At 940 nm, absorbance was taken using Synergy II reader in 96 well microplate. After 5 min incubation of the inhibitor and enzyme, the absorbance was recorded

175 followed by incubation of 15 min after addition of the substrate and incubation for 5 min after
176 addition of chromogen.

177 **2.6.3. Secretory Phospholipase A2 (sPLA2) Inhibitory Activity**

178 Assay kit was used according to manufacturer instructions (10004883, Cayman Chem. Co,
179 Interchim, Montluçon, France) to determine inhibitory activity of shoot extracts against sPLA2.
180 Diheptanoyl thio-PC (1.44 mM) was employed as substrate whereas thiotheramide-PC (100 µM)
181 was considered, in this reaction, as positive control inhibitor. The cleavage of diheptanoyl thio-
182 PC ester releases free thiols which was measured by Synergy II reader (BioTek Instruments,
183 Colmar, France) at 420 nm in a 96-well microplate using DTNB (5-5′-dithio-bis-(2-nitrobenzoic
184 acid). Results were expressed as % inhibition by using following formula,

185 $$\% \text{ Inhibition} = [(\text{IA} - \text{Inhibitor})/\text{IA}] \times 100$$

186 Here, Inhibitor: denotes enzymatic activity in the presence of inhibitor; IA: 100% enzymatic
187 activity without inhibitor presence.

188 ## 2.7. Statistical Analysis

189 Whole experiment was conducted under controlled (temperature and light) conditions, performed
190 three times and repeated twice. Mean and standard deviations were calculated using Microsoft
191 Excel and graphs were prepared by Origin Pro 8.5.

192 # 3. Results and Discussion

193 ## 3.1. Biomass Production in *A. integrifolia* Shoot Culture

194 Growth pattern in response to various concentrations of salicylic acid and gibberellic acid in *A.*
195 *integrifolia* shoots was measured by calculating respective biomass accumulation i.e. dry weight.
196 Data was recorded after 21 days treatment and compared with control (non-treated).
197 Morphological changes in shoot formation were also noted (Fig. 1). Salicylic acid produced
198 relatively mature and compact shoots compared with gibberellic acid and control. Overall,
199 gibberellic acid stimulated biomass accumulation rather than salicylic acid. Higher biomass
200 (DW: 17.9 g/L) was accumulated in GA (5.0 mg/L) treated shoot cultures as compared with
201 control (DW: 11.6 g/L) (Table 1). Effect of gibberellic acid concentration was found to be in

202 direct relation with biomass production whereas biomass was decreased with increase in salicylic
203 acid concentration. Maximum dry weight was observed at lowest concentration of salicylic acid
204 cultures as previously been reported (Sivanandhan et al., 2013) in *Withania somnifera* liquid
205 shoot culture. Similar trend was reported in salicylic acid treated shoot cultures of two
206 *Hypericum* species (Coste et al., 2011). Gibberellic acid mediated signaling in plants for shoot
207 formation and leaf shape has extensively been studied in the past (Davière and Achard, 2013;
208 Pramanik et al., 2018). The rapid cell division and cell elongation in response to gibberellic acid
209 could be the reason behind immature shooting after 21 days and enhanced biomass accumulation
210 (Gupta and Chakrabarty, 2013) (Fig. 1&Table 1). On the other hand, growth promoting effect of
211 exogenous salicylic acid has been studied in maize, wheat and soybean (Gunes et al., 2007;
212 Gutiérrez-Coronado et al., 1998; Shakirova et al., 2003). Lower concentrations of SA promoted
213 shooting and rooting in wheat and soybean plants whereas higher concentrations i.e. 250 µM SA
214 showed negative effect on chamomile plantlets (Kováčik et al., 2009).

215 3.2. Phytochemical Production in *A. integrifolia* Shoot Cultures

216 Plants tend to produce variety of biomolecules as protection mechanism against environmental
217 stimuli either biotic or abiotic. These phytochemicals mainly consists of phenolics and
218 flavonoids which help plants in smooth growth and development (Ali et al., 2006; Tan et al.,
219 2004). Current study involves the investigation of phytochemical's accumulation in response to
220 various concentrations of salicylic acid and gibberellic acid in *in vitro* derived multiple shoot
221 cultures of *A. integrifolia*. Highest phenolic contents (TPC: 11.08 mg/g) were observed against
222 SA 150 µM shoot cultures. Overall, salicylic acid promoted phenolics biosynthesis compared
223 with gibberellic acid except GA (2.5 mg/L) which showed relatively favorable results (TPC:
224 10.55 mg/g) compared with control. Since phenolic production was calculated based on dry
225 weight and TPC values, phenolic production was found optimum (TPP: 173.26 mg/L) in
226 gibberellic acid (GA: 5.0 mg/L) treated shoot cultures which is almost 1.5 fold higher than
227 control (TPP: 118.87 mg/L) (Fig. 2a). Optimum flavonoid contents (TFC: 1.85 mg/g) were noted
228 in SA 300 µM supplemented shoot cultures compared with control (TFC: 1.34 mg/g). Flavonoid
229 biosynthesis was observed to be dose dependent i.e. higher SA or GA concentration, higher
230 would be the flavonoid value. Similarly, flavonoid production (TFP: 28.5 mg/L) was found
231 optimum in GA (5.0 mg/L) shoot cultures compared with rest of treatments (Fig. 2b). Previously,

232 (Pérez-Tortosa et al., 2012) studied the effects of salicylic acid on enhanced production of
233 rosmarinic acid and antioxidant activity in shoots of *Thymus membranaceus*. Similarly, (Coste et
234 al., 2011) reported significant increase in hyppericin and pseudohypericin production in
235 salicyclic acid treated shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*.

236 3.3. Antioxidant Evaluation in Multiple Shoot Culture of *A. integrifolia*

237 Plants produce cellular DNA damaging reactive oxygen species (ROS) such as H_2O_2, superoxide
238 and free radicals, when they endure environmental fluctuations or pathogenic attack (Moran et
239 al., 1994). Such stressed conditions could prevent plant's normal growth and development
240 processes through oxidative damage mechanism (Alexieva et al., 2001; Yu and Anderson, 1997).
241 To cope up with stress, plants produce large variety of secondary metabolites such as phenolics,
242 flavonoids, terpenoids etc. These plant biochemicals play vital role in mitigation of ROS by
243 acting as natural defense barrier (Ashry and Mohamed, 2011; Mittler, 2002; Samuolienė et al.,
244 2010). In current study, various *in vitro* based antioxidant assays were studied to investigate and
245 evaluate antioxidant capacity of *A. integrifolia* shoot extracts. These assays include DPPH (2,2-
246 diphényl-1-picrylhydrazyle), FRAP (ferric reducing antioxidant power) and ABTS (2,2-azino-
247 bis-3-ethylbenzothiazoline-6-sulphonic acid). FRAP and ABTS works based on ET (electron
248 transfer) and HAT (hydrogen atom transfer) mechanism, respectively whereas DPPH is mixed
249 type assay, based on both mechanisms. Maximum DPPH activity (89.3%) was recorded in shoot
250 culture of *A. integrifolia* treated with salicylic acid (SA 150 μM) followed by SA 300 μM (87%)
251 compared with control (83.9%). Induction of gibberellic acid (GA) in shoot culture media
252 significantly reduced DPPH activity as compared with SA and control (Fig. 3a). Similarly, SA
253 150 μM treated shoot cultures showed optimum FRAP (532 μM) activity compared with rest of
254 treatments (Fig. 3b) whereas ABTS activity was found higher (435.5 μM) in SA 300 μM treated
255 shoots. Gibberellic acid negatively regulated antioxidant activities i.e. FRAP and ABTS in shoot
256 culture of *A. integrifolia*. (López-Orenes et al., 2013) studied the effects of exogenously applied
257 salicylic acid on antioxidant potential in shoots of *Cistus heterophyllus.* Results indicated that
258 lower concentration of salicylic acid was found effective in phenolics metabolism and
259 antioxidant activity, by triggering indigenous salicylic acid production in *C. heterophyllus*
260 shoots. Results of our study revealed a positive correlation in phytochemical biosynthesis and
261 antioxidant activity as previously studied (Pérez-Tortosa et al., 2012) in shoot cultures of *Thymus*

membranaceus. Higher level of phenolic contents in response to SA treatment could possibly be the reason of optimum antioxidant activities in *A. integrifolia* shoot culture. Similar dependent correlation of phytochemical and *in vitro* antioxidant activities have been observed in past studies (Khan et al., 2018; Shah et al., 2019; Ullah et al., 2019a; Ullah et al., 2019b).

3.4. Anti-Inflammatory Potential of *A. integrifolia* Multiple Shoot Culture

Anti-inflammatory activity is mainly evaluated by means of % inhibition of different enzymes responsible for inflammation process including cyclooxygenases (COX-1, COX-2), 15-lipooxygenase (15-LOX) and secretory phospholipase A2 (sPLA2) (Mohale et al., 2014). These enzymes are the key targets of non-steroidal anti-inflammatory drugs to reduce inflammation in response to harmful stimuli. Plants ability to inhibit these enzymes through phytochemicals, mainly flavonoids, shows their therapeutic role in development of alternative medicines (Tungmunnithum et al., 2018). Anti-inflammatory activity of *A. integrifolia* shoot extracts was measured using *in vitro* % inhibition assays of these enzymes. Optimum inhibitory activity was recorded against sPLA2 (39.51 ± 1.04%), COX-1 (34.44 ± 0.93%), followed by 15-LOX (34.07 ± 0.37%) and COX-2 (23.40 ± 1.01%) in shoot extracts treated with salicylic acid (SA 150 µM). Overall, salicylic acid showed stimulatory effect on anti-inflammatory activity in *A. integrifolia* shoots whereas gibberellic acid down regulated its activity compared with control. Anti-inflammatory activity results in terms of % inhibition are shown in Table 2. Comparative analysis of anti-inflammatory activities and phytochemical production in response to SA and GA suggested that these phytochemicals play vital role in executing enzymatic inhibition that are responsible for inflammation in the body (Guo et al., 2008; Lim et al., 2009; Szopa et al., 2018). Anti-inflammatory potential of *A. integrifolia* has previously been studied using *in vitro* and *in vivo* rat models (Hsieh et al., 2011; Kayani et al., 2016; Singh et al., 2012). Gautam et al (Gautam et al., 2011) also reported COX-1 and COX-2 inhibition activity of *A. integrifolia* using whole plant ethanol extract. This is the first study, evaluating enhanced anti-inflammatory activities in *in vitro* derived *A. integrifolia* shoot culture in response to SA and GA.

3.5. Phytochemical Quantification of *A. integrifolia*

For quantitative analysis of individual compounds present in shoots of *A. integrifolia*, an effective HPLC method was employed. These phytochemicals actively form plant's defense

291 response against harsh environmental conditions to ensure smooth development (Balasundram et
292 al., 2006; Ullah et al., 2019a). In current study, 11 compounds were quantified in response to
293 salicylic acid and gibberellic acid treated shoot cultures. Overall, salicylic acid produced
294 optimum results whereas gibberellic acid down regulated phytochemicals production compared
295 with control. Optimum levels of chlorogenic acid (285.27 µg/g), apigenin (490 µg/g), luteolin
296 (493.61 µg/g), quercetin (542.3 µg/g), aucubin (187.09 µg/g), harpagoside (146.06 µg/g),
297 catalpol (91.67 µg/g) and 8-O-acetyl harpagoside (161.3 µg/g) were observed in shoot cultures in
298 response to SA 150 µM. On the contrary, higher levels of rosmarinic acid (569.8 µg/g) and
299 caffeic acid (332.9 µg/g) were accumulated against SA 75 µM and SA 300 µM treatments,
300 respectively (Table 3). Maximum production of harpagide (375.2 µg/g) was noted in untreated
301 (control) shoot cultures of *A. integrifolia*. Stimulatory effects of salicylic acid have previously
302 been reported in different *in vitro* derived cultures (Gadzovska et al., 2013; Mendoza et al., 2018;
303 Nadeem et al., 2019). (Largia et al., 2015) studied the enhanced bacoside A production in
304 salicylic acid treated *in vitro* derived shoot cultures of *Bacopa monnieri* L. Similar results were
305 reported by (López-Orenes et al., 2013) and (Pérez-Tortosa et al., 2012) in *Cistus heterophyllus*
306 and *Thymus membranaceus* shoot cultures against salicylic acid treatment. Negative effect of
307 gibberellic acid has been reported by Corbin et al (Corbin et al., 2013) in cell culture of *Linum*
308 *usitatissimum* L. Gibberellic acid significantly down regulated *LuPLR1* gene expression which is
309 responsible of biosynthesis of anti-cancerous lignan in flex. Phytochemicals, quantified in
310 current study, have huge pharmaceutical importance. They paly vital role as anticancer agents
311 through induction of apoptosis, oncogenes suppression and interruption of intracellular
312 communication (Mohammad Nabavi et al., 2015; White et al., 1989).

313 **4. Conclusion**

314 In current study, stimulatory effects of various concentrations of salicylic acid and gibberellic
315 acid on biomass and phytochemical synthesis were investigated in *in vitro* derived multiple shoot
316 culture of *Ajuga integrifolia*. Gibberellic acid showed maximum biomass accumulation by
317 stimulating shoot growth compared with salicylic acid and control. Salicylic acid proved to be
318 effect for total phenolic and flavonoid contents whereas phytochemical production was
319 significantly high in case of gibberellic acid treatment. *In vitro* based antioxidant and anti-
320 inflammatory activity were also found higher under the influence of salicylic acid which showed

321 positive correlation with plant secondary compounds. A significant increase in these 322 pharmaceutically important compounds was observed by quantification of high performance 323 liquid chromatography.

324 Conflict of Interest

325 The authors declare no conflict of interest.

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490

491 # Figures & Tables:

492

493 **Table 1.** Dry weight production in response to salicylic acid (SA) and gibberellic acid (GA) in multiple
494 shoot culture of *A. integrifolia***.**

Treatment	Concentration	dry weight (g/L)
Control	BAP 1.0 mg/L	11.66 ± 0.853
	SA 75 µM	14.21 ± 0.638
Salicylic Acid	SA 150 µM	14.90 ± 1.043
	SA 300 µM	13.66 ± 3.951
	GA 1.0 mg/L	15.25 ± 2.448
Gibberellic acid	GA 2.5 mg/L	16.27 ± 0.962
	GA 5.0 mg/L	17.97 ± 1.749

495
496 BAP: 6-benzylaminopurine

497

498

499

500 **Table 2.** Anti-inflammatory potential of *A. integrifolia* shoot cultures grown under different SA and GA
501 treatments.

Treatment	% Inhibition			
	COX-1	COX-2	15-LOX	$sPLA_2$
Control	32.37 ± 1.361	21.38 ± 0.997	29.20 ± 2.055	34.67 ± 0.443
SA 75 µM	31.10 ± 1.449	20.13 ± 1.204	32.35 ± 1.943	37.14 ± 1.394
SA 150 µM	34.44 ± 0.932	23.40 ± 1.015	34.07 ± 0.372	39.51 ± 1.044
SA 300 µM	31.77 ± 1.667	20.79 ± 1.743	33.58 ± 1.743	38.49 ± 1.493
GA 1.0 mg/L	17.83 ± 1.302	17.17 ± 0.846	23.59 ± 0.844	29.51 ± 1.943
GA 2.5 mg/L	16.22 ± 1.843	15.60 ± 1.563	22.71 ± 2.511	28.08 ± 1.338
GA 5.0 mg/L	32.37 ± 0.984	17.40 ± 1.337	22.68 ± 0.442	28.27 ± 1.437

502
503 SA: Salicylic Acid, GA: Gibberellic Acid, COX-1: Cyclooxygenase-1, COX-2: Cyclooxygenase-2, 15-LOX: 15-
504 Lipooxygenase, $sPLA_2$: Secretory Phospholipase A2.

Table 3. HPLC based quantification of valuable metabolites in shoot culture of *A. integrifolia*.

Compounds	Treatments						
	BAP	Salicylic Acid (µM)			Gibberellic Acid (mg/L)		
	Control	75	150	300	1.0	2.5	5.0
Chlorogenic Acid	232.5 ± 3.05	249.2 ± 1.41	285.2 ± 1.93	278.8 ± 4.11	222.3 ± 3.59	221.1 ± 2.83	239.5 ± 0.55
Caffeic Acid	196.2 ± 6.04	269.4 ± 3.95	316.2 ± 4.22	332.9 ± 2.01	193.8 ± 1.87	230.2 ± 8.55	185.4 ± 2.79
Rosmarinic Acid	399.9 ± 3.05	569.8 ± 1.94	519.1 ± 6.09	546.6 ± 5.93	409.4 ± 3.56	355.6 ± 6.83	328.8 ± 9.64
Apigenin	455.6 ± 3.95	460.1 ± 2.04	490.1 ± 4.31	488.5 ± 1.99	436.1 ± 1.05	431.3 ± 3.41	439.6 ± 4.22
Luteolin	463.9 ± 1.91	461.7 ± 3.63	493.6 ± 5.49	486.8 ± 9.81	465.7 ± 5.26	431.3 ± 7.33	436.1 ± 3.07
Quercetin	515.6 ± 2.53	512.6 ± 4.79	542.3 ± 1.37	493.6 ± 6.61	465.7 ± 9.05	438.1 ± 3.43	444.8 ± 5.19
Harpagide	375.2 ± 1.45	358.4 ± 6.67	356.9 ± 3.87	366.4 ± 8.23	213.6 ± 2.73	174.3 ± 5.74	251.3 ± 4.92
Aucubin	153.3 ± 7.65	165.8 ± 2.84	187.1 ± 6.57	175.2 ± 1.84	64.5 ± 9.04	76.5 ± 4.39	88.1 ± 1.74
Harpagoside	129.5 ± 5.54	112.1 ± 8.51	146.1 ± 1.84	117.4 ± 7.48	81.9 ± 3.82	71.2 ± 5.44	57.8 ± 1.63
Catalpol	74.9 ± 3.10	73.5 ± 7.45	91.6 ± 2.29	65.8 ± 4.84	39.6 ± 8.85	46.2 ± 3.48	29.4 ± 9.57
8-*O*-Acetyl Harpagoside	153.3 ± 2.04	141.6 ± 4.51	161.3 ± 1.23	144.9 ± 5.43	88.4 ± 8.74	75.6 ± 3.66	67.9 ± 6.73

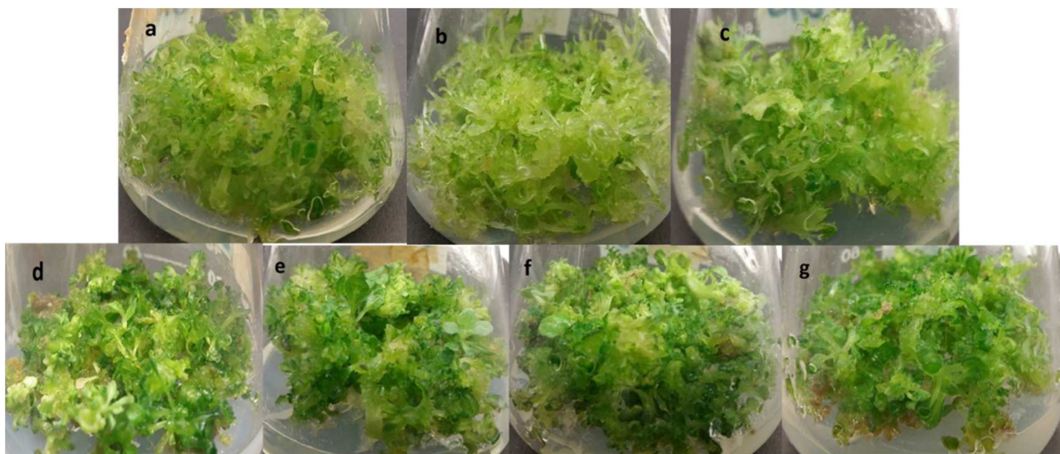

Fig. 1. Effect of gibberellic acid (a: 1.0 mg/L, b: 2.5 mg/L, c: 5.0 mg/L) and salicylic acid (d: control, e: 75 µM, f: 150 µM, g: 300 µM) on biomass production in multiple shoot culture of *A. integrifolia*.

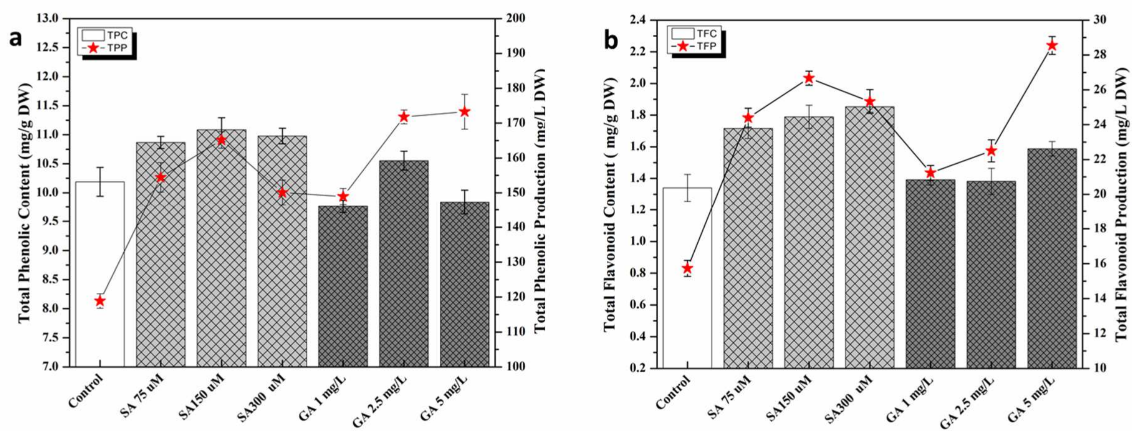

Fig. 2. Influence of SA and GA on **a)** Total phenolic content (TPC), Total phenolic production (TPP) **b)** Total flavonoid content (TFC), Total flavonoid production (TFP) in multiple shoot culture of *A. integrifolia*.

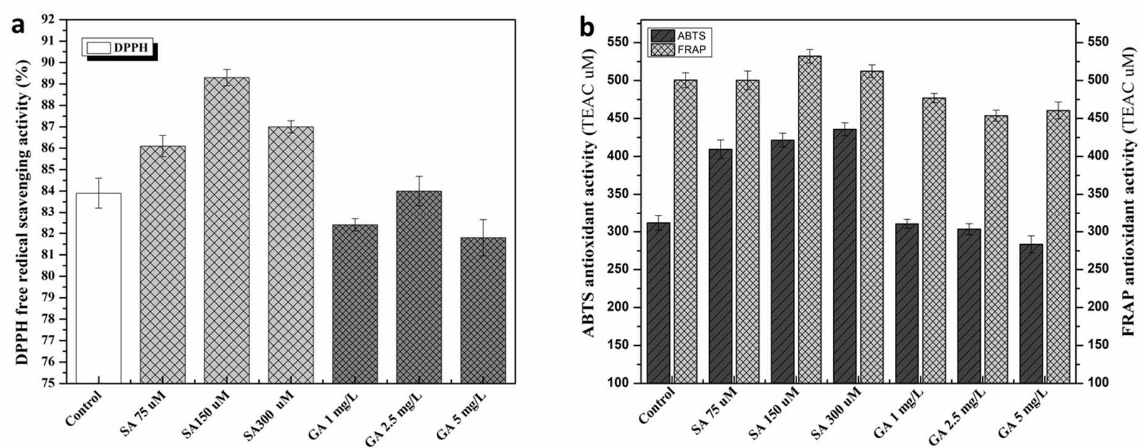

Fig. 3. *In Vitro* antioxidant activities in shoot culture of *A. integrifolia* in response to salicylic acid and gibberellic acid treatment **a)** DPPH activity, **b)** ABTS and FRAP activity. Values represent means ± standard errors from triplicates.