Monochromatic lights-induced trends in antioxidant and antidiabetic polyphenol accumulation in in vitro callus cultures of Lepidium sativum L.

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Abstract

Lepidium sativum L. is an important edible, herbaceous plant with huge medicinal value as cardio-protective, hepatoprotective and antitumor agent. This study was designed and performed to investigate biosynthesis of plant's active ingredients in callus cultures of *L. sativum* in response to the exposure of multi spectral lights. Optimum biomass accumulation (15.36 g/L DW), total phenolic and flavonoid contents (TPC; 47.43 mg/g; TFC; 9.41 mg/g) were recorded in calli placed under white light (24h) compared to rest of the treatments. Antioxidant enzymatic activities i.e. superoxide dismutase and peroxidase were found optimum in cultures exposed to green light (SOD; 0.054 nM/min/mg FW, POD; 0.501 nM/min/mg FW). Phytochemical analysis further confirmed the potential influence of white light exposure on enhanced production of plant's metabolites. Significant enhancement level of major metabolic compounds such as chlorogenic acid (7.20 mg/g DW), quercetin (22.08 mg/g DW), kaempferol (7.77 mg/g DW) and minor compounds including ferulic acid, sinapic acid, protocatechuic acid, vanillic acid and caffeic acid were recorded in white light compared to control (photoperiod), whereas blue light increased the *p*-coumaric acid accumulation. Moreover, callus cultures of this plant under white light (24h) showed highest *in vitro* based anti-diabetic and antioxidant activities compared to other conditions. Finding of our current study revealed that multi spectral lights are proved to be an effective strategy for enhancing metabolic quantity of antioxidant and anti-diabetic bioactive compounds in callus cultures of *L. sativum* L.

Keywords: Polyphenols, *Lepidium sativum*, Monochromatic light quality, Chlorogenic acid, Quercetin, Kaempferol

1. Introduction

For centuries, medicinal plants have been recognized for their wide pharmaceutical and therapeutic applications. Almost every culture has documented their use either in the form of root, seeds and leaves to fight against many diseases [1]. Medicinal plants are rich source of phytochemicals, having wide range of anti-microbial, anti-cancerous and anti-tumor applications in medical field [2, 3].

Lepidium sativum L., a herbaceous medicinal plant commonly known as garden cress, belongs to family *Brassicaceae* with average height of 30 to 50 cm. *L. sativum* is also an edible plant, especially its seeds provide enormous health promoting benefits [4-6]. Biological activities including antimicrobial, anticancer, bronchodilator and allelopathic activity have previously been reported from aqueous extract of this plant [7, 8]. Phytochemical profiling revealed the existence of important polyphenolic (phenolic acids and flavonoids) constituents including sinapic acid and its derivative, caffeic acid, quercetin, kaempferol, *p*-coumaric acid, and other phenolic compounds as well as glucosinolates that could play vital role in plant defense and antioxidant potential [9, 10]. *L. sativum* seeds contains pale yellow, semi-dry oil 20-25%, while main fatty acid in it are linolenic acid, comprises 32-34 % [11]. Oil is identified to contain several important compounds like palmitic, oleic, linoleic, stearic, benzyl cyanide, sterols, behenic, sitosterol. *L. sativum* also carries natural anti-oxidant, and vitamin E and carotenoid which guard the oil from rancidity. Seven imidazole alkaloids, in which five lepidine (B, C, D, E and F) are dimeric and there are two monomeric semi lepidinoside A and B alkaloid were previously testified in seeds [12].

Secondary metabolites have previously been studied for their anticancer potential. Aqueous extracts of leaves and seeds of *L. sativum* were investigated, and the results pointed out that the extract can fight against HEp2 cells and MCF-7 human breast cancer cell lines [13]. Multiple studies have been conducted on mouse model for hepatoprotective [14], antihypertensive and diuretic activity [15], cardio-protective action [16] and hypoglycemic activity [17] of *L. sativum* extracts. The type 2 diabetes mellitus occurs mainly because the body cannot take up and use insulin effectively that leads to hyperglycemic condition [18]. There are two key enzymes, α-glucosidase and α-amylase, involve in proper carbohydrate digestion [19]. Inhibition of these enzymes can delay the starch digestion process, thus controlling the glycemic index in the body

[20]. Different inhibitors have been used in clinical practices which sometimes cause gastrointestinal abnormalities [21]. Plant's active ingredients have potential to act as natural inhibitors with minimum side effects [22]. Antioxidant term usually applies to sum up huge class of compounds that act as reducing agents by giving up electrons by employing various modes of actions [23]. There are two major mechanistic approaches through which antioxidants inactivate free radicals i.e. ET (electron transfer) and HAT (hydrogen atom transfer) [24]. Different *in vitro* assays to determine antioxidant potential of an individual compound have been established and perfected in past few years. These assays provide extensive information on mechanistic approach involved in antioxidant potential of compounds. Excess of free radicals in the body can generate a cascade of reactions that later play a role in development of cancer and other neurodegenerative diseases. Advanced glycation end product (AGEs) formation in response to free radical oxidative stress is perfect example of its deleterious actions which results in cardiovascular disease and Alzheimer's disease development due to protein dysfunction [25].

Plant phytochemicals responsible for such medical applications have a huge demand in pharmaceutical industry. Development of these pharmaceutical products is difficult due to low availability and high variability of phyto-content in their natural environment and not to mention the lack of appropriate extraction procedures [26]. *In vitro* cultures establishment provides unlimited availability of plant phytochemicals with easy harvesting, irrespective to ever changing environmental conditions [27, 28].

For industrial scale manufacture of these active secondary metabolites, multiple strategies in last few decades have been utilized. For *in vitro* derived cultures, elicitation has shown promising results for enhanced production of phyto-content [29, 30]. Elicitors primarily activate physiological cascades, resulting in stimulation of plant defense mechanism and production of phytochemicals [31, 32]. Light is a considerable factor in optimum regulation of multiple growth related pathways. Intensity and wavelength variations may influence phytochemical accumulation [33, 34]. Light of multiple spectra significantly changes plant morphology when applied in a controlled environment as previously been studied on various plant species [35, 36].

The primary focus of current study is the establishment of *in vitro* callus culture of *L. sativum* for sustainable production of commercially suitable anticancer, antioxidant and anti-diabetic phytochemicals and to explore the potential influence of different monochromatic lights under

controlled conditions on morphology and biochemical characteristics of *L. sativum* callus culture.

2. Material and Methods

2.1. *In vitro* germination of plant

Seeds of *L. sativum* were taken from Department of Biotechnology, Plant Cell Culture Laboratory (PCCL), Quaid-i-Azam University Islamabad, Pakistan. Viability of *L. sativum* seeds was examined by employing water float test and the seeds were further subjected to surface sterilization using mercuric chloride (0.1 %) solution for 30 seconds, followed by 40 seconds of treatment with ethanol (70%). To eliminate contamination, sterilized seeds were further thoroughly washed 3 times using sterilized autoclaved distilled water. Murashige and Skoog medium (MS0) [37] was used to inoculate sterilized seeds containing agar (0.8% w/v) as solidifying agent and sucrose as source of carbon (3%). Media with adjusted pH 5.6 – 5.7 were autoclaved for 20 min at 121°C temperature prior to inoculation and flasks were under fixed photoperiod cycle (16/8 light/dark) in growth room at optimum temperature of 25±2°C.

2.2. Callus culture establishment from *in vitro* derived plantlets

In vitro derived plantlets (21 days old) were used as source of explants. Leaf and stem (0.5 cm^2, 1.0 cm) were excised from plantlets and inoculated in MS media along with multiple concentrations of thidiazuron (TDZ) and α-naphthalene acetic acid (NAA) alone and in combination (Supplementary Table 1). Explants (4-5) of both stem and leaf were cultured in each flask. The whole experiment was performed in triplicates and placed in photoperiod cycle (16h light/8h dark) at 25 ± 1°C temperature. Data on callus initiation days and callus induction frequency was collected on weekly basis. Callus was sub-cultured on respective hormonal fresh media after every 4 weeks. Harvesting of callus was done after 4^{th} week of 2^{nd} subculture and data on fresh weight (FW) and dry weight (DW) production was obtained respectively.

2.3. Monochromatic Lights Treatment on callus culture

Different source of monochromatic lights were employed as elicitor on explant derived callus including Red Light (24 h, wavelength 660 nm), Green Light (24 h, wavelength 510 nm), Blue Light (24 h, wavelength 460 nm), Yellow Light (24 h, wavelength 570 nm), Dark (24 h), White

Light (24 h, wavelength 400-700 nm) and photoperiod cycle (16/8 h light/dark). Based on higher biomass accumulation, stem derived callus (1g) was used to inoculate 30ml MS media flask supplemented with optimized hormonal concentration and placed under different sources of monochromatic light of intensity of 40-50 µMol $m^{-2}s^{-1}$ as previously described, recorded using Lux meter (SU10, Jeio-tech). Experiment was performed in triplicates and harvested after 28 days of inoculation for determination of FW, DW and phytochemical analysis.

2.4. Analytical Scheme

2.4.1. Sample Extraction

For evaluation of phenolic, flavonoid content and DPPH activity of calli samples, extraction procedure was done according to Zahir et al [38] with some changes. Briefly, 100mg callus powder (dried at 60°C for 24h) was thoroughly mixed with 500µl of methanol (1:5), vortexed (10 min) and sonicated (30min). Whole procedure for phytochemical extraction was repeated twice and centrifuged (15000rpm, 10min). Supernatant of each sample was removed and stored (4 °C) for further analysis.

In order to determine peroxidase (POD) as well as superoxide dismutase (SOD) activities, fresh samples of calli were subjected to extraction according to Nayyar and Gupta [39] with minor adjustments. Briefly, fresh calli samples (100 mg) were mixed with 1 ml KH_2PO_4 buffer (50 mM, pH7.0) containing 1% PVP. Homogenization of sample mixture was performed and centrifuged at 15000 rpm for half an hour. After centrifugation of sample, supernatant was stored (4 °C) for further analysis.

2.4.2 Estimation of secondary metabolites contents

For determination of total phenolic contents (TPC), Singleton and Rossi [40] protocol was used by employing Folin-Ciocalteu reagent with minor modifications. Methanol extracted sample (20 µL) was mixed in FC reagent (90 µL) and sodium carbonate (90 µL) in reaction mixture. Absorbance of mixture was taken at 630nm wavelength by micro-plate reader (Thermo Scientific Multiskan GO). TPC were expressed in equivalent of standard (gallic acid, GAE/g).

Aluminum chloride colorimetric method as previously performed by [41] was employed in order to estimate total flavonoid content (TFC). Aluminum chloride and potassium acetate both added

(10 µL each) in reaction mixture along with 20 µL of methanol extracted sample and raised the total volume up to 200 µL with distilled water. Absorbance of reaction mixture was taken after 30 min incubation at 415 nm using micro-plate reader. TFC were expressed in equivalent of standard (quercetin, QE/g).

2.4.3. DPPH Scavenging Activity

Antioxidant potential of callus extracts was determined using DPPH reagent as studies by Abbasi et al. [42]. The reaction mixture included sample extract (20 µl) and DPPH solution (180 µl), incubated for approximately 60 min in dark, followed by absorbance measurement at 517nm. The final concentrations of ascorbic acid (5, 10, 20 and 40 µg/ml) and DPPH (180 µl) with DMSO (20 µl) were considered as negative control. Following formula was used to calculate FRSA activity,

% DPPH activity = 100 × (1-AE/AD)

Here AE used to express mixture absorbance with sample addition and AD expresses the DPPH absorbance without sample addition.

2.4.4. Peroxidase (POD) and Superoxide Dismutase (SOD) Activity

For estimation of antioxidant enzyme activity, extract from fresh samples was used. Lagrimini [43] method was employed with slight adjustments for POD activity. The reaction mixture was prepared for each sample using 20 µl calli extract (fresh), 0.1 ml distilled H_2O, 20 µl hydrogen peroxide (27.5 mM; 10x), 20 µl guaiacol (100 mM, 10×) and 40 µl KH_2PO_4 buffer (50 mM; pH 7). Same reagent amounts were employed as control without adding any extract of sample. Microplate reader was used to perform sample's absorbance at wavelength of 470 nm and the enzymatic activity expressed by employing following formula:

A = ELC

A used to express absorbance of sample, C = presence of enzyme concentration expressed in terms of nM/min/mg FW), L= Length of wall (0.25 cm) and E is used to express Extinction coefficient (6.39 $mM^{-1}cm^{-1}$).

Protocol proposed by Giannopolitis and Ries [44] was used to perform SOD activity. Mixture was prepared by adding EDTA (20µl, 1 mM), methionine (20µl, 130 mM), calli extract (60µl, fresh), 2µl riboflavin (0.02 mM), phosphate buffer (78µl, 50 mM, pH 7), and 20µl NBT (0.75 mM). Absorbance at 660nm wavelength was measured by micro-plate reader after 7 min of incubation under fluorescent light.

2.4.5. High Performance Liquid Chromatography Quantification

Metabolites quantification was done by HPLC using HPLC grade solvents and standards (Sigma Aldrich. Separation was performed on Hypersil PEP 300 C18, 250 x 4.6 mm, 5 µm particle size equipped with a guard column Alltech, 10 x 4.1 mm was utilized at 35 °C and Varian high-performance liquid chromatography system (equipped with Varian Prostar 230 pump Meta chem Degasit, Varian Prostar 410 autosampler and Varian Prostar 335 Photodiode Array Detector (PAD) and driven by Galaxie version 1.9.3.2 software was used in detection of compounds at 280 and 320 nm wavelength. The mobile phase of HPLC system was using two solvents (A: acetonitrite and B: acidified formic acid (0.1%v/v) ultrapure water). Mobile phase composition range varied during the 60 min run from 5:95 to 100:0 (solvent A: B v/v) according to linear gradient with 0.8ml/min flow rate. Re-equilibration time of 10 min was applied after each individual run. Quantification was done based on retention time compare to commercial reference standards (Sigma Aldrich). Examination of given samples was done three times and outcomes were denoted as mg/g dry weight.

2.5. Ferric Reducing Antioxidant Power (FRAP) Assay

To evaluate FRAP potential of callus cultures, protocol proposed by Benzie and Strain [45] was utilized. Briefly, extracted samples (10 µl) were mixed with of FRAP (190 µl) solution [composed of acetate buffer (300 mM, pH 3.6); $FeCl_3.6H_2O$ (20 mM) and TPTZ (10 mM); ratio 10:1:1 (v/v/v)]. Reaction mixtures were then stored at 25 ± 1 °C for approximately fifteen min. Absorption was measured with Microplate Reader (BioTek ELX800, Bio-Tek Instruments) at 630 nm. Antioxidant capacity of samples was expressed in terms of TEAC (Trolox C equivalent antioxidant capacity) and whole procedure was repeated three times.

2.6. Antioxidant ABTS assay

The method of Tagliazucchi et al. [46] was used to evaluate ABTS antioxidant potential. Briefly, ABTS (2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) salt (7mM) was thoroughly mixed with 2.45mM of potassium persulphate in equal proportion to prepare ABTS solution and the solution was placed in dark for 16h. The absorbance was recorded at 734 nm and adjusted to 0.7 and then mixed with the extracts and placed in dark at 25 ± 1°C for 15 min. Microplate reader (ELX800, BioTek Instruments) was used to record absorbance of mixture at 734nm. Antioxidant capacity of samples was expressed in terms of TEAC and whole procedure was repeated three times.

2.7. Anti-AGEs Formation Activity

The inhibitory capacity of formation of AGEs (advanced glycation end products) was determined as described by Kaewseejan and Siriamornpun [47].. Extracts were prepared at a concentration of 50µg/ml in DMSO mixed with 20 mg/ml BSA (Sigma Aldrich) and 0.5 M glucose (Sigma Aldrich) solution, both were prepared in phosphate buffer and 1 ml of 0.1 M phosphate buffer containing 0.02 % (w/v) sodium azide at pH 7.4. The amount of fluorescent AGE formed was measured after 5 days incubation of reaction mixture in dark at 37°C by using a fluorescent spectrometer (Bio rad Versa Fluor) with an excitation wavelength set at 330 nm and emission wavelength set at 410 nm. For each sample extract, anti-AGEs formation was showed as % inhibition with respect to the relative control (same volume addition of DMSO).

2.8. α-glucosidase inhibition

The α-glucosidase was partially purified from rat intestinal acetone powder (Sigma). Protocol used for immobilization of α-glucosidase was previously described by hano et al. [48] on CNBr-activated Sepharose 4B. To determine immobilized enzymatic activity, chromogenic method [48] was performed using polyethylene filter (0.45 µm) end-capped column. In brief, the assay was performed using intestinal fluid (1mL) consisting of 4-nitrophenyl-α-D-glucopyranoside (5mM, 4NPG; Sigma). The reaction mixture was stopped after 30 min incubation time at 37°C by column filtration and further adding sodium carbonate (1M) solution. The activity of enzymes was determined against blank by measuring the increased absorbance value at 405nm wavelength. Percentage inhibition was determined by calculating difference in absorbance value in presence as well as in absence of extracts. Experiment was repeated four times.

2.9. α-amylase inhibition

The α-amylase from porcine pancreas was purchased from Sigma. A chromogenic method as previously described [48] was used to measure the activities of soluble a-amylase. Briefly, enzyme was prepared in phosphate buffer (0.1 M, pH6.8) with 1u/mL concentration and thoroughly mixed in 5mM of 4-nitrophenyl-α-D-maltopentaoside (4NPM; Sigma). Furthermore, the reaction mixture was incubated in presence and absence of the extract at 37°C for 30 min. Sodium carbonate (1M) solution was used in same volume to stop the reaction and the activity of enzymes was determined against blank by measuring the increased absorbance value at 405nm wavelength. Percentage inhibition was determined by calculating difference in absorbance value in presence as well as in absence of extracts. Experiment was repeated four times. .

2.5. Statistical analysis

All the experiments were conducted in synchronized way in triplicates. Mean value and standard error for each experiment was calculated and graphs were generated using Origin software (8.5). Data were shown as mean ± SE. One-way analysis of variance (ANOVA) with significant difference $P < 0.05$ was used to compare the means of different treatments.

3. Result and Discussion

3.1. Hormonal treatment for callogenesis:

Callus induction from both stem and leaf explants was carried out for an optimal accumulation of secondary metabolites and biomass in response to various concentrations of plant growth regulators (PGRs), applied alone and/or in combination. Callus induction was observed against all treatments of PGRs from both explants (Supplementary Table 1). Callus induction frequency largely varied with respect to type of explant and concentrations of PGR's used. Highest callus induction frequency (100%) was observed at NAA (3.0, 4.0, 5.0 mg/L) and TDZ + NAA (2.5 + 1.0, 5.0 + 1.0 mg/L) in case of leaf and stem explants. Results indicated that TDZ showed lower callus induction potential when applied alone compared to its combination with NAA. Similar observation was reported by Younas et al. [49] for *Silybum marianum* L. callus culture in which TDZ along with NAA showed more callogenesis potential compared to TDZ alone. A positive trend was observed between NAA concentrations and callus induction frequency whereas in case

of TDZ and TDZ+NAA, higher concentrations inhibited the callogenesis in both leaf and stem explants (Supplementary Table 1). This could likely be due to suppression of endogenously produced hormones [50, 51]. Morphological variations were also recorded in present study which indicated that compact callus was produced for TDZ and TDZ+NAA unlike callus produced against NAA alone presented as friable (Fig. 2 a,b,c). Overall, stem explant was observed more responsive towards callogenesis as compared to leaf explant as previously studied by Anjum et al [52] for *Linum usitatissimum* L.

3.2. Trend in Biomass Accumulation:

Different PGRs were employed on leaf and stem explants from *L. sativum* for optimum production of biomass in *in vitro* callus culture. Optimum biomass (FW; 106.88 g/L, DW; 9.233 g/L) accumulation was observed with 2.5 mg/L TDZ + 1.0 mg/L NAA for stem explant (Fig. 1b). However, leaf explant showed relatively different response by accumulating high biomass (FW; 74.333 g/L, DW; 7.69 g/L) at 4.0 mg/L TDZ (Fig. 1a). This differential response could be due to biochemical and physiological potential of different explant type and tissues. Multiple factors influences on callus formation including plant genotype, growth conditions, explant type and optimum PGR concentration [51]. Higher concentrations of TDZ and NAA, when applied alone, promoted callus biomass in both explant types. Our results are similar to limited study of Abdellatef et al. [53] which employed various concentrations of 2, 4- D, TDZ and NAA to evaluate *in vitro* callus formation potential of *L. sativum* from different explants. Similar trend of biomass production was observed for TDZ in *Artemisia absinthium L.* callus culture [54]. However, no previous study is available on combined effect of TDZ and NAA on *L. sativum*. In case of stem explant, TDZ + NAA significantly lower the biomass production at higher and lower concentration except optimum level (Fig. 1b). This suggest that auxins and cytokinins in combination at optimum level, produces synergistic effect for callus formation as studied in previous reports [55-57]. Furthermore, hormonal optimized callus culture was placed under different spectral lights to investigate the potential effect on biomass accumulation. Optimum biomass production (FW; 166.28 g/L, DW; 15.36 g/L) was recorded in cultures placed in continuous light (24h) followed by yellow light (FW; 146.32 g/L; DW; 12.87 g/L) as compared to control (Fig. 1c). Cultures placed under dark conditions produced lowest biomass (FW; 110.16 g/L, DW; 8.7 g/L) compared to rest of lights. Maximum biomass production in cultures exposed

to continuous white light could possibly be due to more energy level than other lights, playing vital role in plant's physiological and chemical processes. Tariq et al. [58] previously concluded growth enhancing effect of white light compared to other lights in *A. absinthium* callus culture. Similar results of high biomass accumulation in cell culture under white light were previously reported [59, 60]. Influence of different spectral lights varies significantly with plant species, intensity and quality of applied light [61]. Morphological changes were also recorded as cultures grown under dark conditions appeared to be pale yellow unlike those grown under continuous white light, blue and yellow (Fig. 2d-i).

3.3. Accumulation of Phenolic and Flavonoid Contents

Plant secondary metabolites play vital role in defense against environmental stress and regulation of plant growth and development [62]. Optimum level of these metabolites (phenolics and flavonoids) in response to different hormones, explant source and spectral lights was also investigated in this study. Overall, TDZ in combination with NAA produced the best results in case of both leaf and stem derived callus cultures compare to their sole treatments. Highest TPC value (stem; 36.47 mg/g, leaf; 34.10 mg/g) were recorded in callus cultures at TDZ (2.5 mg/L) + NAA (1.0 mg/L) (Supplementary Fig. 1a). Optimum total phenolic production (TPP 232.6 mg/L) was observed at TDZ (1.0 mg/L) + NAA (1.0 mg/L) in case of leaf derived cultures whereas stem derived cultures showed optimum TPP (336.83 mg/L) at TDZ (2.5 mg/L) + NAA (1.0 mg/L) (Supplementary Fig. 1b). Similarly, optimum TFC (7.47 mg/g) level was recorded in stem derived culture at TDZ (1.0 mg/L) + NAA (1.0 mg/L) whereas maximum TFP level (52.53 mg/L) was recorded at TDZ (2.5 mg/L) + NAA (1.0 mg/L) in stem derived culture (Supplementary Fig. 1c, d). Overall, a positive correlation of secondary metabolites production and callus biomass accumulation was found which is in accordance with previous study of Ali and Abbasi [63].

Based on highest biomass accumulation, total phenolic and flavonoids production, stem derived callus culture of *L. sativum* at TDZ (2.5 mg/L) + NAA (1.0 mg/L) hormonal concentration was used to determine potential influence of multi spectral lights on secondary metabolites. Light is considered a very effective abiotic elicitor that can stimulate biosynthesis of plant's precious metabolic compounds [64]. Various callus cultures have been tested in the past for enhanced metabolites biosynthesis upon exposure to multi spectral lights [65, 66]. Cultures grown under

white light (24h) showed maximum TPC (47.43 mg/g), followed by cultures grown in dark (44.05 mg/g) and blue light (40.54 mg/g) and similar trend was observed in TFC accumulation under influence of all lights (Fig. 3a). Total phenolic and flavonoid production (TPP, TFP) were found in positive correlation with biomass accumulation of cultures grown under different lights (Fig. 3b). Different studies have been reported on beneficial influence of white light in *in-vitro* derived cultures. Maximum level of anthocyanin in hairy root cultures was reported under white light in *Echinacea purpurea* [67, 68]. Khan et al. [69] reported enhanced production of anticancer compounds under exposure of white light in callus culture of *Fagonia indica*. Adventitious root cultures of *Withania somnifera* L. produced optimum results when placed under white light [60]. Contrarily, blue and red lights have also been reported to have significant influence in biosynthesis of plant metabolic compounds in different studies [49, 64, 70]. Effects of monochromatic lights have previously been tested for enhanced metabolites synthesis in *in vitro* cultures of various plant species [71-74]. Such anomalies suggest that influence of light intensity and quality on active ingredients of plant can vary from plant's species to species.

3.4. Free radical scavenging activity

Plants under stress conditions tend to produce large quantities of reactive oxygen species (ROS) that has harmful effect on growth and development of cells by damaging the DNA [75-77]. Plants are naturally equipped with defense mechanism to mitigate harmful effects of ROS by utilizing antioxidant enzymes [78]. Natural occurrence of phenolics, flavonoids, carotenoids and terpenoids in plants act as antioxidant compounds by providing protection against oxidative damages [79, 80]. In current study, optimum scavenging free radical activity (92.1 %) was observed in stem derived callus culture in response to TDZ (2.5 mg/L) + NAA (1 mg/L), followed by 91.9 % and 91.5 % at TDZ (0.1 mg/L) + NAA (1 mg/L) and TDZ (5.0 mg/L) + NAA (1.0 mg/L) respectively whereas maximum scavenging activity in leaf derived callus culture (90.5%) was noted at TDZ (5.0 mg/L) + NAA (1.0 mg/L) (Supplementary Fig. 2). The lowest antioxidant activity for both leaf (61.5 %) and stem (72.5 %) derived cultures was recorded at NAA (0.1 mg/L). Scavenging activity of hormonally optimized calli was also recorded under different spectral lights after 28 days exposure. Maximum antioxidant activity (95.1%) was observed under white light (24h), followed by dark (94.2%) and blue light (93.2%) whereas yellow light had a least effect on antioxidant activity (85.2%) (Fig. 4). A positive

correlation was observed between enhanced phytochemicals biosynthesis (phenolic and flavonoids) and high free radical scavenging activity under white light (24h). A similar correlation was observed in a study of Khan et al. [81] which implies that scavenging activity of callus culture is directly proportional to amount of phytochemical produced. Current results are in accordance with different studies [59, 68] stating the importance of light on biosynthesis of phenolic compounds and their antioxidant activity.

3.5. Lights effect on peroxidase and superoxide dismutase activity

Potential influence of different lights on POD and SOD activities was also investigated in callus culture of *L. sativum* at optimized hormonal concentration (TDZ: 2.5 mg/L + NAA: 1.0 mg/L). Maximum POD enzyme activity (0.501 nM/min/mg FW) was observed in green light, followed by control (0.46 nM/min/mg FW) and white light (0.43 nM/min/mg FW) respectively whereas blue light significantly reduced the POD enzymatic activity (0.308 nM/min/mg FW) compared to other lights (Fig. 5). Similarly, highest SOD activity was also observed in green light followed by continuous white light (Fig. 6). These antioxidant enzymatic activities play vital role in sustaining cell viability during metabolic reactions by protecting them from free radicals toxicity [82] and spectral lights have a significant role in regulation of these enzymatic activities. Hina et al. [64] studied the influence of different lights on SOD and POD activities on callus culture of *Prunella vulgaris* in which highest antioxidant enzymatic activities were observed under yellow light. A study conducted by Tariq et al. [58] on callus culture of *A. absinthium* in which red light showed prominent impact on POD enzymatic activities. Different spectral lights also influence micronutrients like Zn or Mn in plant cells that act as inorganic co-factor for antioxidant enzymes [83].

3.6. Phytochemical Analysis

Plant phytochemicals play vital role in plant defense, survival and growth regulation and are widely distributed among plant species [62]. Application of an elicitor such as light can enhance biosynthesis of these precious metabolites which possess huge pharmaceutical value. These metabolites can be easily quantified using high performance liquid chromatography (HPLC). Current study includes quantitative analysis of 9 phenolic compounds from *L. sativum* callus cultures grown under different spectral light. Higher level of total secondary metabolites was

observed in white light (39.19 mg/g), followed by dark (35.14 mg/g) compared to photoperiod (control) (26.1 mg/g) whereas yellow light (23.64 mg/g) had negative effect on secondary metabolites. Major metabolic constituents quantified from the callus cultures were chlorogenic acid, quercetin and kaempferol. Maximum level of quercetin (22.08 mg/g DW) was observed under white light (24h) exposure, followed by dark (19.73 mg/g DW) and blue light (16.74 mg/g DW). Similar trend was observed in kaempferol biosynthesis (white light (24h); 7.77 mg/g DW, dark; 6.94 mg/g DW; blue light; 5.76 mg/g DW). Yellow light significantly down regulated the biosynthesis of metabolites as compared to control (Table 1.). Likewise, minor metabolic compounds such as caffeic acid, ferulic acid, vanillic acic, sinapic acid and protocatechuic acid were found higher under white light (0.988 mg/g DW, 0.665 mg/g DW, 0.181 mg/g DW, 0.077 mg/g DW and 0.055 mg/g DW respectively) whereas *p*-coumaric acid was recorded higher in blue light treated cultures (0.220 mg/g DW). Our results suggest the potential role of white light application for enhanced biosynthesis of plant's precious metabolites. These compounds have huge market value in pharmaceutical industry. Different studies reported their vital role as neuroprotective and anticancer agent by down regulating the oxidative stress and oncogenes, apoptosis and cell cycle arrest [84-86]. Polyphenolic compounds tend to show enhanced anticancer activity by interacting with cytochrome p450 and essential enzymes such as protein kinases and polymerases [87, 88].

3.7. Effect of multispectral lights on antioxidant and anti-diabetic potential of *L. sativum* callus cultures

Antioxidant activity of *L. sativum* callus cultures placed under different monochromatic lights was evaluated by callus extract using FRAP, ABTS assay and inhibition of vesperlysine-like AGEs and pentosidine-like AGEs formation. Results of FRAP antioxidant assay suggested that white light grown cultures produced optimum antioxidant activity (629.78 uM), followed by blue light (576.41 uM) and dark (552.36 uM) (Fig.7.a). Similar trend was recorded for ABTS assay which showed highest activity in white light (401.44 uM), followed by dark (377.78 uM) (Fig.7.b). The antioxidant potential of both assays was expressed in TEAC (Trolox C equivalent antioxidant capacity). Results indicate a positive correlation between antioxidant potential and phenolic contents of callus cultures when exposed multispectral lights. Enhanced production of phenolic compounds could possibly be the reason behind significant increase in antioxidant

activities of extract samples under white light. Direct correlation of antioxidant potential and secondary metabolites has previously been documented in different studies. [89, 90]. Furthermore, inhibition of formation of AGEs (pentosidine- and vesperlysine-like) was recorded in treatment of callus extracts of *L. sativum*. Significant increase in inhibition activity was shown by white light treated cultures (65.83 %, 56.47 %) against pentosidine and vesperlysine like AGEs formation respectively (Fig.7.c). Minimum activity was recorded in cultures treated with yellow light for both type of anti-AGEs formation. Trend in polyphenolic accumulation and anti-AGEs formation in response to various lights indicate the therapeutic potential of these secondary metabolites to protect the human body. Formation of AGEs is usually observed in eye lens of diabetic people, erythrocytes and in plasma [91]. Therefore, the application of these therapeutic metabolites as inhibitors can prove to be useful in fight against complicated diseases.

Anti-diabetic potential in lights treated callus extracts of *L. sativum* was evaluated by measuring immobilized α-glucosidase and solubilized α-amylase inhibitory activities. Results indicated that callus extracts were more effective in α-amylase inhibition compared to membrane bound α-glucosidase (Fig.8). Extract of white light treated callus showed maximum α-amylase inhibitory activity (67.55 %), followed by dark (66.42 %) compared to control (41.63%). Highest inhibitory activity of α-glucosidase was recorded in dark (55.20 %), followed by white light (54.55%) whereas yellow light showed lowest anti-diabetic potential (28.41%). Finding of this assay revealed that anti-diabetic potential in *L. sativum* is largely due to its inhibition ability toward pancreatic α-amylase rather than α-glucosidase as previously studied by Hano et al. [48] in determining the anti-diabetic activity of flax seeds. Eddouks et al. [17] previously studied the *L. sativum* seed's extract on diabetic rats for possible hypoglycemic effects. This is the first study determining the effect of lights on enhanced anti-diabetic activity in *L. sativum* callus extracts. In general, we cannot exclude the fact that phytochemicals (other than the one's quantified in current study) could play a role for observed biological activities.

4. Conclusion

In current study, optimized callus culture establishment of *L. sativum* was achieved using various concentrations of TDZ and NAA alone and in combination for sustainable production of industrially important secondary metabolites. Further, we conclude that light an excellent abiotic elicitor for increased biosynthesis of plant metabolites. Among treatment of different

monochromatic lights, white light (24h) enhanced the level of phenolic profile and antioxidant potential of plant. White light significantly increased caffeic acid, chlorogenic acid, quercetin and kaempferol level after 28 days of exposure. Blue light had a potential effect on *p*-coumaric acid compared to rest of lights. This study revealed the potential applications of multi spectral lights on sustainable production of metabolites from *in vitro* derived callus cultures of *L. sativum*.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1: Effect of multi spectral lights on accumulation of polyphenolic metabolites ((mg/g DW)) in callus culture of *Lepidium sativum*.

Marker Compounds (mg/g DW)	Different Lights Treatment						
	Control	Red	Green	Blue	Dark	White Light	Yellow
caffeic acid	0.641 ± 0.01	0.684 ± 0.04	0.726 ± 0.065	0.74 ± 0.03	0.880 ± 0.06	**0.988 ± 0.024**	0.578 ± 0.03
ferulic acid	0.435 ± 0.009	0.463 ± 0.03	0.492 ± 0.021	0.50 ± 0.051	0.593 ± 0.07	**0.665 ± 0.044**	0.393 ± 0.011
vanillic acid	0.135 ± 0.004	0.131 ± 0.005	0.153 ± 0.0033	0.140 ± 0.028	0.169 ± 0.016	**0.181 ±0.0075**	0.120 ± 0.077
p-coumaric acid	0.137 ± 0.006	0.162 ± 0.007	0.208 ± 0.005	**0.220 ± 0.047**	0.170 ± 0.063	0.168 ± 0.0054	0.130 ± 0.086
sinapic acid	0.064 ± 0.0018	0.061 ± 0.005	0.071 ± 0.0093	0.073 ± 0.0065	0.074 ± 0.0034	**0.077 ± 0.0038**	0.059 ± 0.0033
protocatechuic acid	0.049 ± 0.0064	0.046 ± 0.002	0.053 ± 0.0019	0.050 ± 0.0044	0.054 ± 0.0065	**0.055 ± 0.0025**	0.045 ± 0.006
chlorogenic acid	4.99 ± 0.855	5.20 ± 0.822	5.56 ± 1.209	5.61 ± 0.99	6.52 ± 1.66	**7.20 ± 1.555**	4.55 ± 0.66
Quercetin	14.55 ± 1.776	15.45 ± 2.05	16.41 ± 1.999	16.74 ± 2.44	19.73 ± 3.055	**22.08 ± 1.877**	13.15 ± 1.363
Kaempferol	5.10 ± 1.055	5.42 ± 0.79	5.76 ± 0.622	5.89 ± 0.883	6.94 ± 1.22	**7.77 ± 1.22**	4.61 ± 0.92
Total	26.11	27.63	29.45	29.96	35.14	**39.19**	23.64

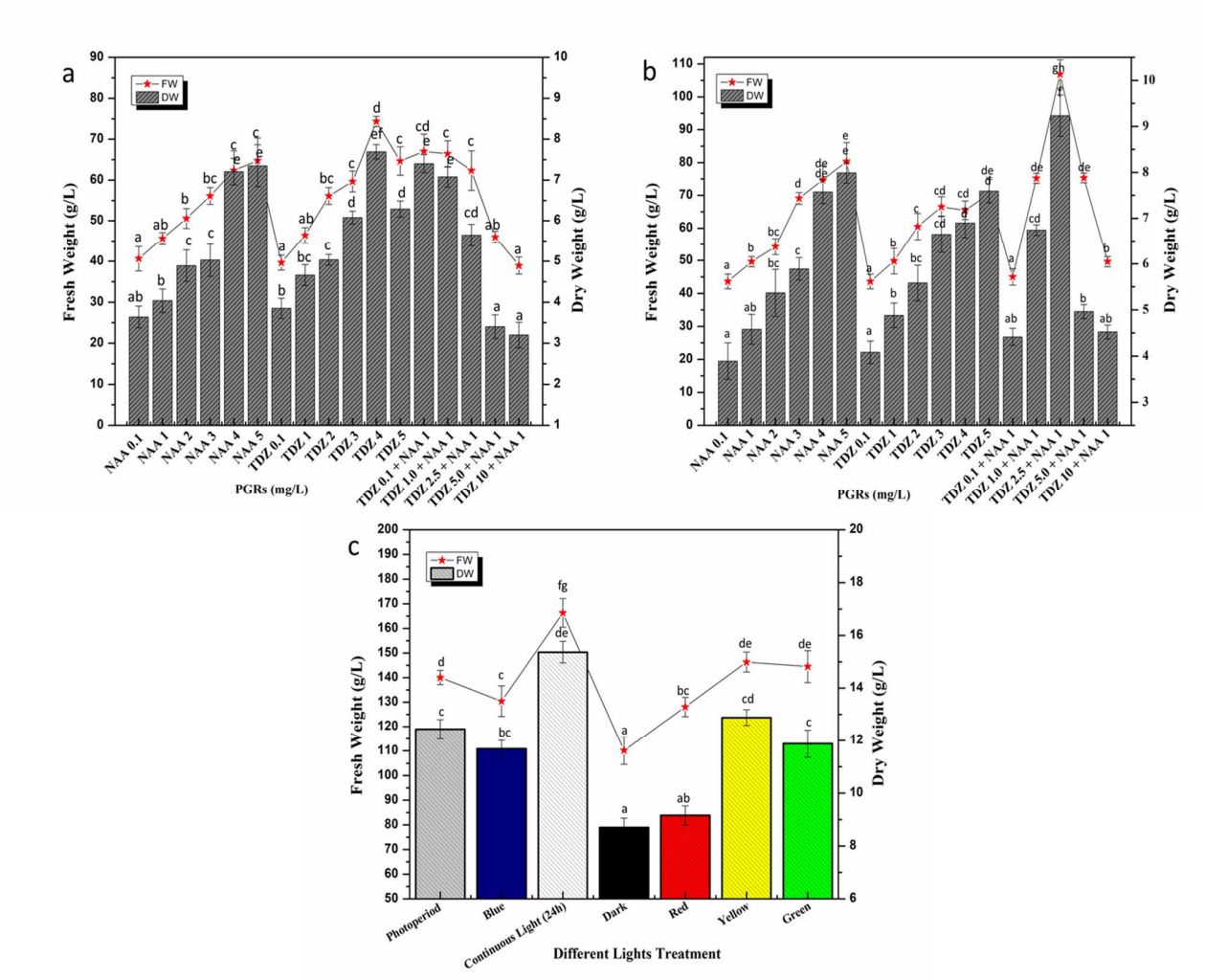

Fig. 1 a) Fresh and dry callus biomass from leaf explant **b)** Fresh and dry callus biomass from stem explant culture on MS medium supplemented with NAA and TDZ alone and in combination **c)** Fresh and dry callus biomass under different monochromatic lights. Values are means of triplicates with the standard deviation. Columns with similar alphabets are not significantly different ($P < 0.05$).

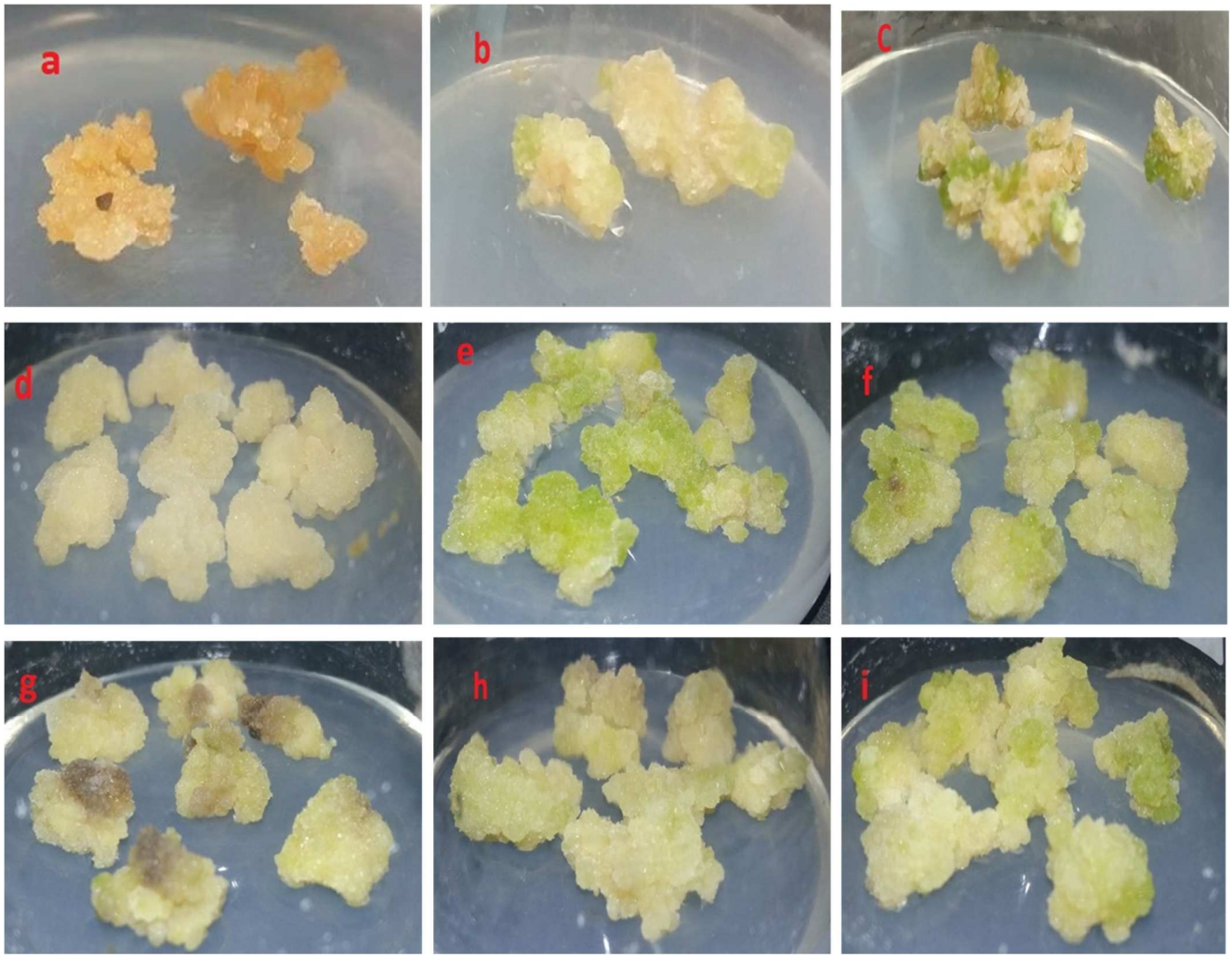

Fig 2. Effect of PGRs and different monochromatic lights on Calli biomass and morphology from stem explant after 4 weeks of culture **(a)** NAA 5mg/l **(b)** TDZ 4mg/l **(c)** TDZ + NAA (2.5mg/l, 1mg/l) **(d)** Dark **(e)** Continuous light (**f**) Blue (**g**) Red (**h**) Green (**i**) Yellow

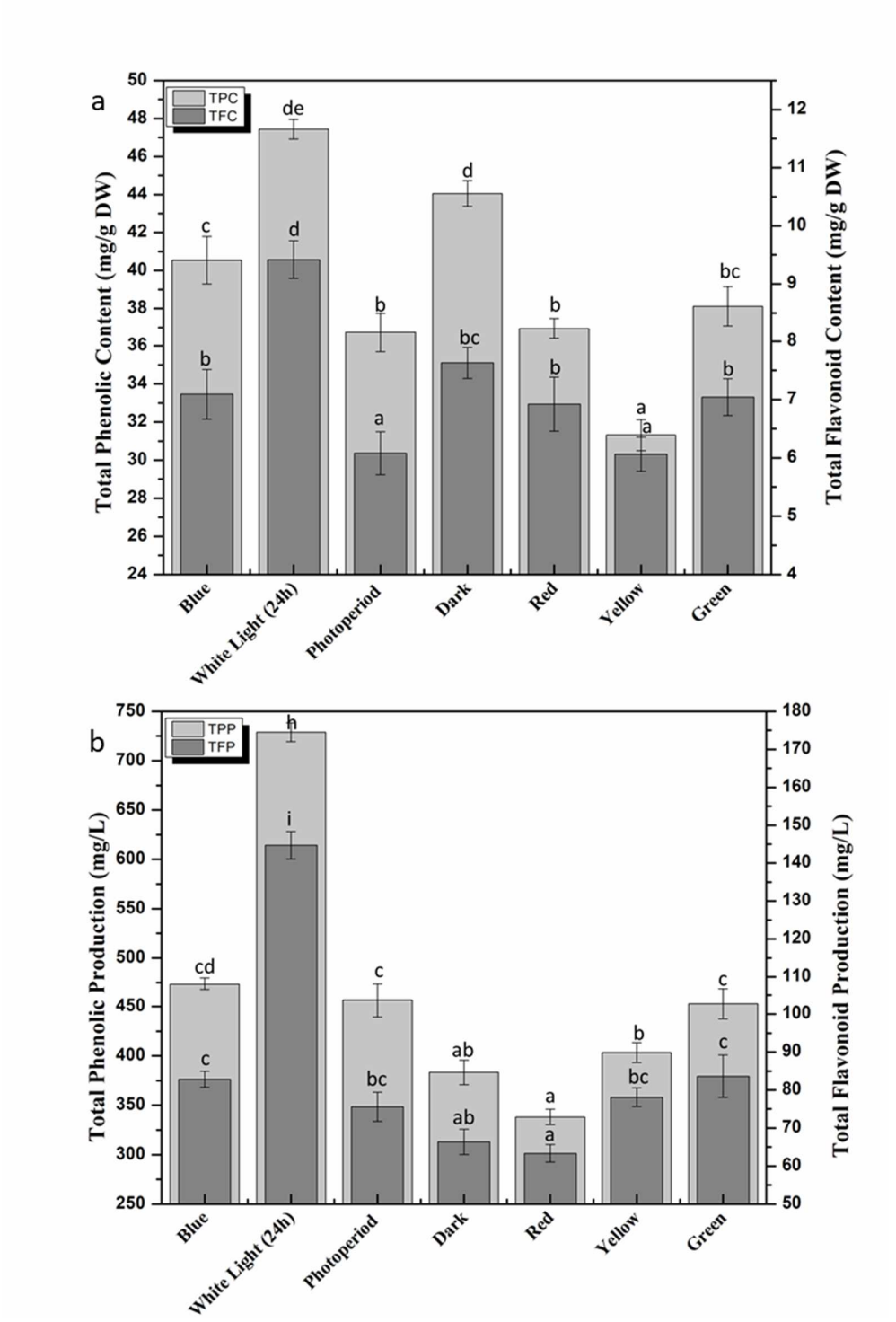

Fig. 3 Effect of different monochromatic lights on **a)** Total Phenolic Content (TPC, mg/g DW) and Total Flavonoid Content (TFC, mg/g DW) **b)** Total Phenolic Production (TPP, mg/L) and Total Flavonoid Production (TFP, mg/L) at optimized hormonal conditions. Values represent means ± standard errors from triplicates. Columns with similar alphabets are not significantly different ($P < 0.05$).

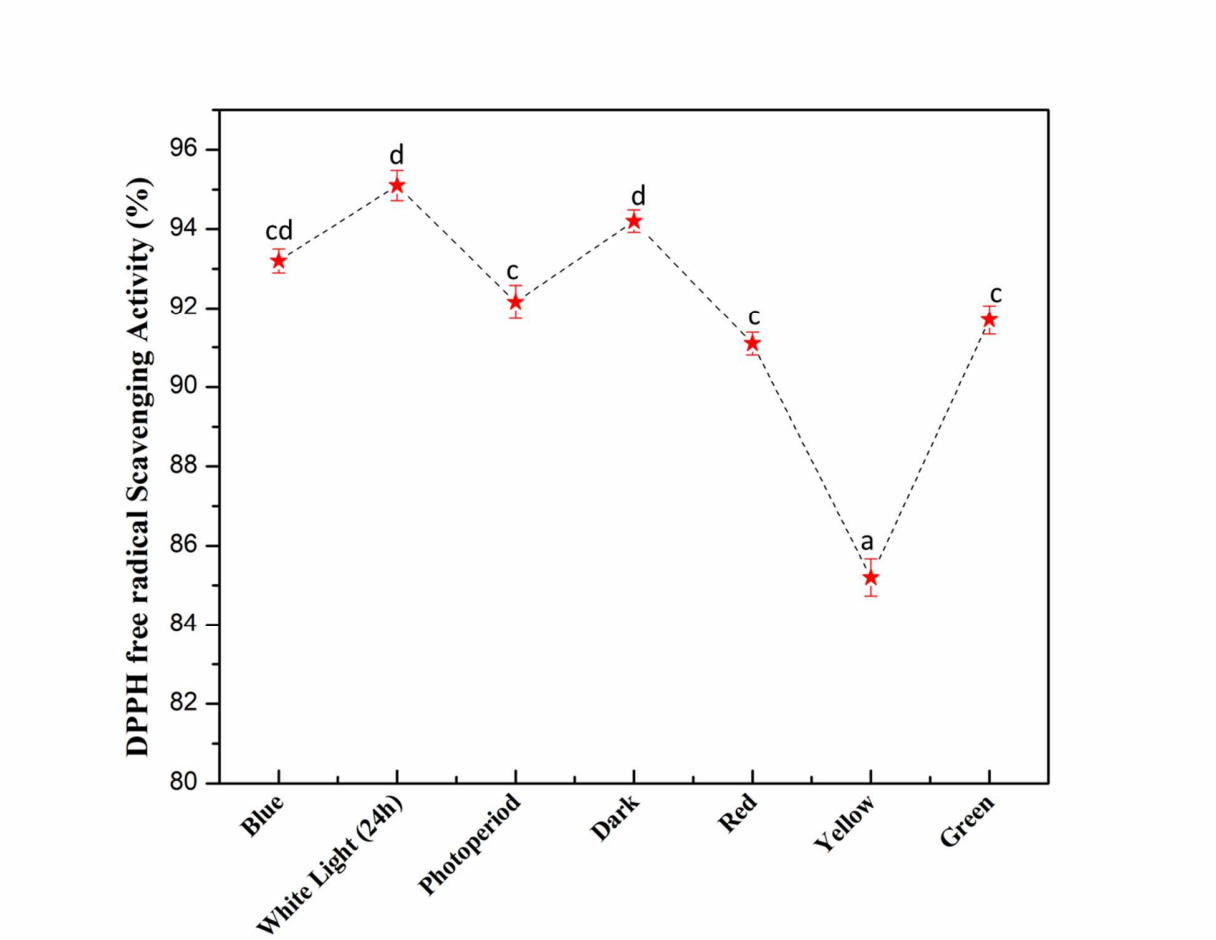

Fig. 4 DPPH Free radical scavenging Activity (%) in response to Monochromatic lights treatment at optimized hormonal concentration. Values represent means ± standard errors from triplicates. Lines with similar alphabets are not significantly different ($P < 0.05$).

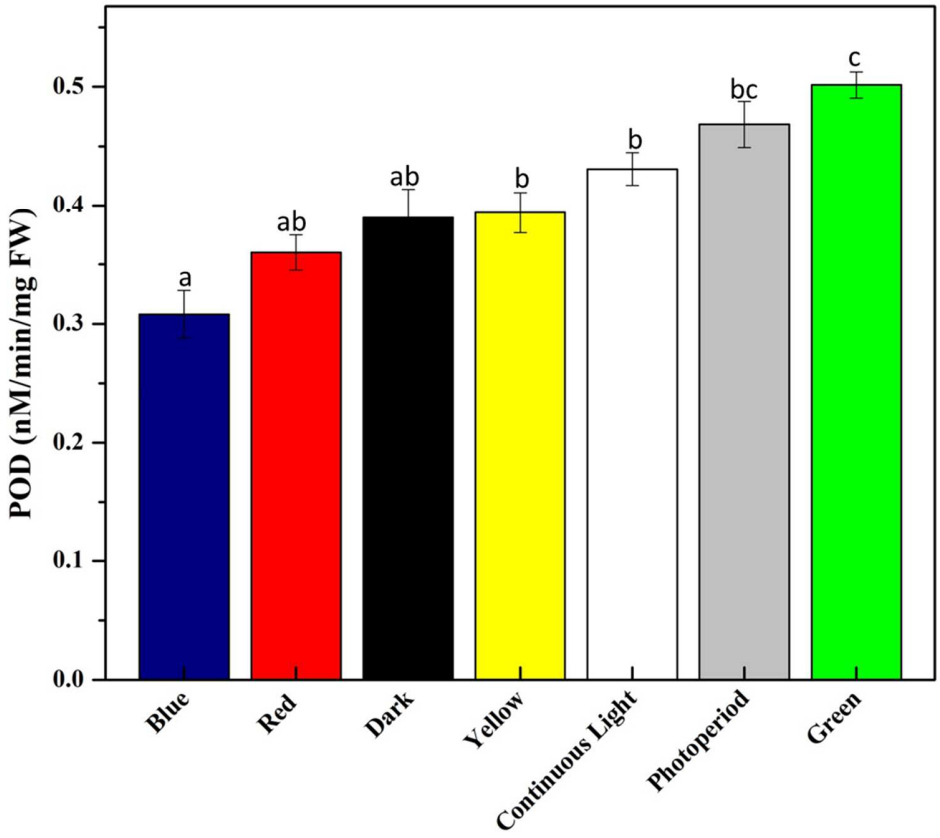

Fig. **5** Influence of different lights treatment on peroxidase (POD) activity of callus samples. Values represent means ± standard errors from triplicates. Columns with similar alphabets are not significantly different ($P < 0.05$).

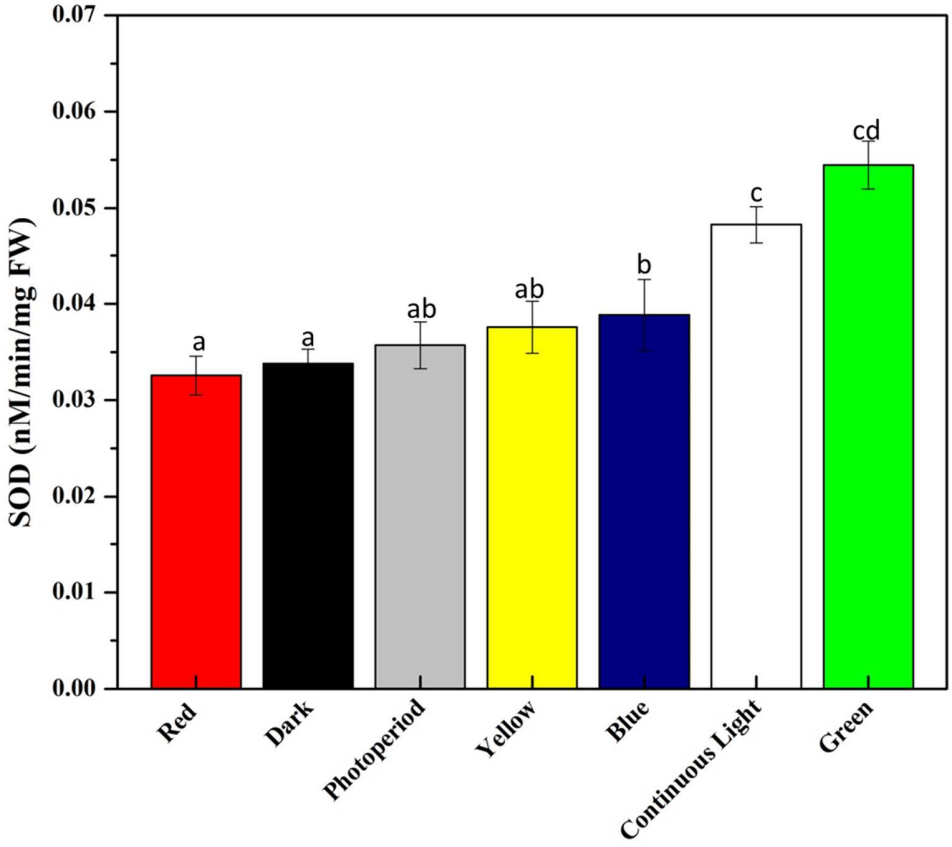

Fig. **6** Influence of different lights treatment on superoxide dismutase (SOD) activity of callus samples. Values represent means ± standard errors from triplicates. Columns with similar alphabets are not significantly different ($P < 0.05$).

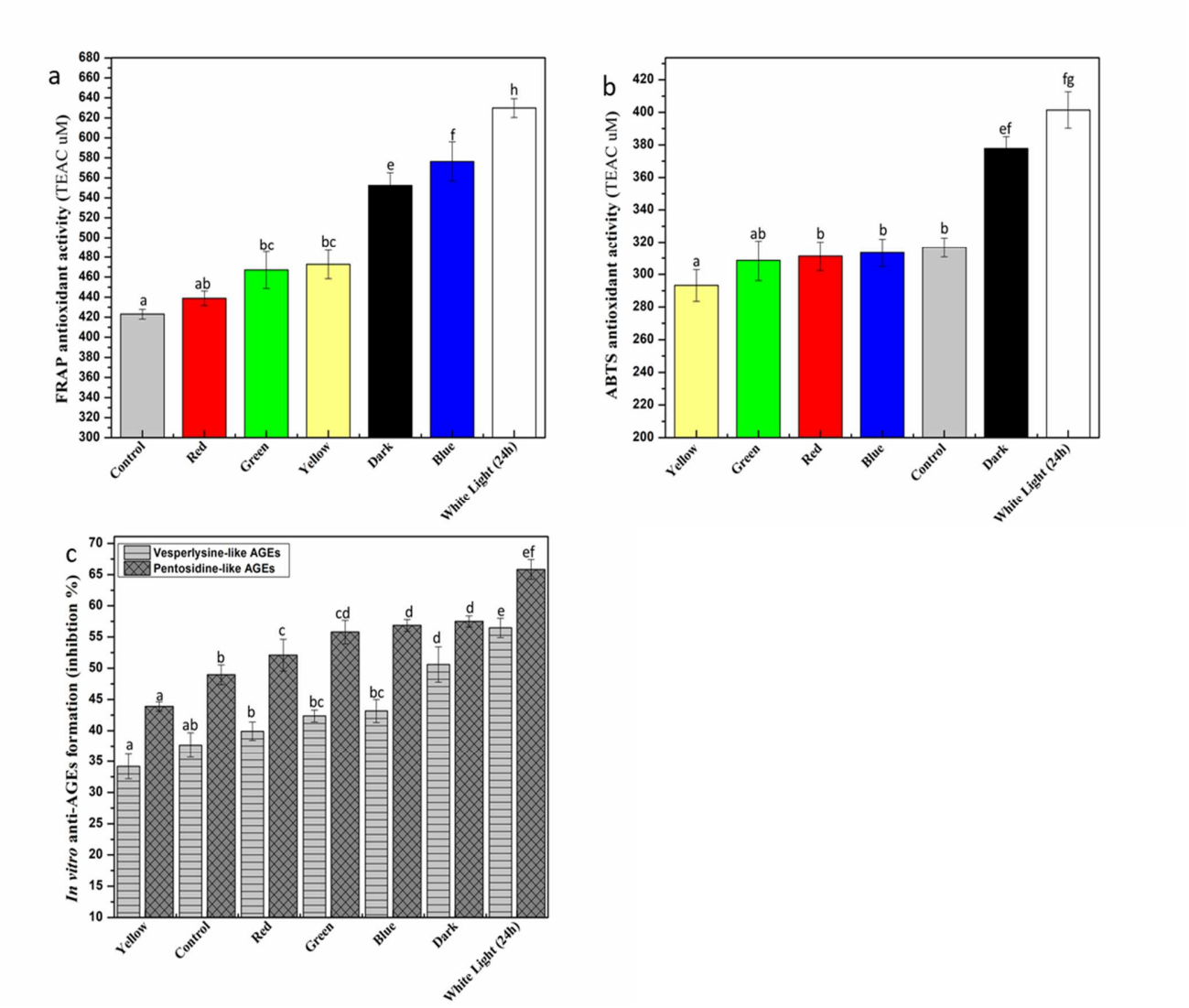

Fig.7. *In vitro* antioxidant (**a, b**) and anti-AGEs formation (**c**) potential of stem derived callus culture place under multispectral lights. (TEAC: Trolox C equivalent antioxidant activity, expressed in µM). Columns with similar alphabets are not significantly different ($P < 0.05$).

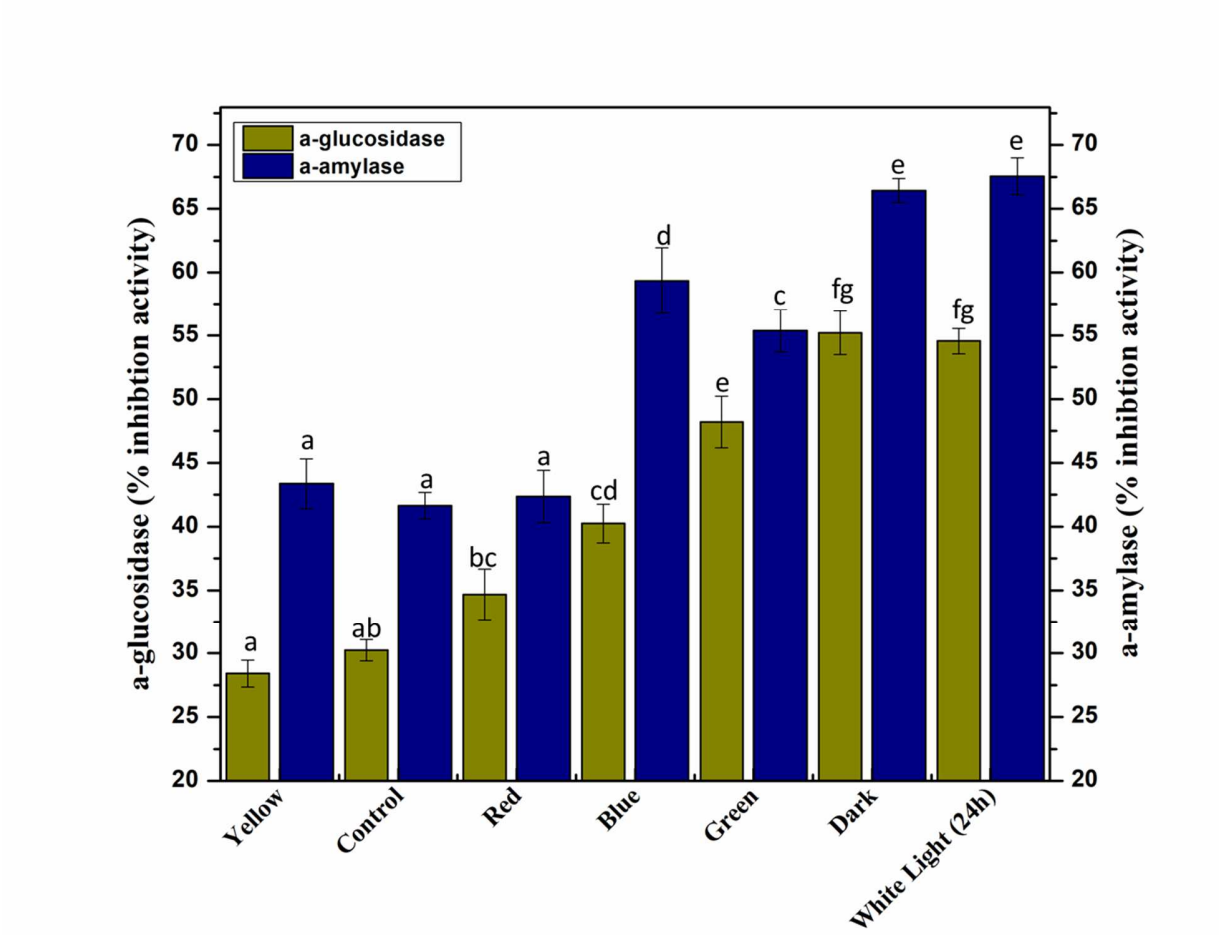

Fig.8. *In-vitro* anti-diabetic potential of *L. sativum* callus extracts in response to multispectral lights. Columns with similar alphabets are not significantly different ($P < 0.05$).