



Elevation of secondary metabolites production through light-emitting diodes (LEDs) illumination in protocorm-like bodies (PLBs) of *Dendrobium* hybrid orchid rich in phytochemicals with therapeutic effects

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ABSTRACT

Gas-chromatography-mass-spectrometry revealed the presence of various bioactive compounds with anticancer properties in protocorm-like-body (PLB) cultures of a *Dendrobium* hybrid orchid (*Dendrobium* Enopi x *Dendrobium* Pink Lady). Pre-illumination of red fluorescent light lessened the stimulating effects of light-emitting diodes (LEDs) on secondary metabolites production among *in vitro* PLB cultures, possibly due to habituation. The highest flavonoid content of 16.79 $\mu\text{mol/g}$ of fresh weight (FW) was achieved under blue-red (1:1) LED for PLBs pre-treated with white LED for more than 3 subculture cycles. Phenolics content significantly reduced as PLBs pre-cultured under red fluorescent light for 2 subculture cycles were exposed to LED illuminations, where far red LED resulted in the lowest total phenolic content (18.85 $\mu\text{mol/g}$ FW). High intensity green LED (16.9 $\mu\text{mol/s}$) enhanced the accumulation of phenolics while amino acids such as L-leucine, glycine and proline exhibited no significant stimulating effect for secondary metabolites production.

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1. Introduction

Plants serve as an excellent natural source for the acquisition of bioactive compounds. These phytochemicals possess a wide array of biological activities and confer plants the ability to survive under adverse environmental conditions. Plant secondary metabolites have been widely utilized, particularly in pharmaceutical, for the production of antibiotics, immuno-modulators and antitumor drugs [1]. Secondary metabolites found within plants can be grouped into 5 major groups, namely non-ribosomal polypeptides, fatty acid-derived compounds and polyketides, terpenoids, steroids, alkaloids as well as enzyme cofactor [2]. Plants are the major producers for secondary metabolites which contribute 80 % of bioactive compounds production [3], along with the other organisms such as microbes and marine organisms.

Abbreviations: GCMS, Gas chromatography-mass spectrometry; PLB, Protocorm-like bodies; DDMP, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; LED, Light-emitting diode; GSH, Reduced glutathione; NF- κ B, Nuclear factor kappa B; PAL, Phenylalanine ammonia-lyase; ROS, Reactive oxygen species.

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The hybridization of two hybrid orchids, *Dendrobium* Enopi and *Dendrobium* Pink Lady, produces a new hybrid which is valued for the ornamental value it possesses. Aside from that, it is worthwhile to further explore the phytochemicals contained within this new hybrid, as *Dendrobium* itself is one of the largest genera in orchid family which has been proven to exhibit medicinal properties. Usage of many *Dendrobium* orchids as folk medicines can be backdated to more than 2 300 years ago [4]. In China, *Dendrobium* orchids are extensively cultivated mainly for their medicinal values and to meet the herbal medicine market demand [5]. *Dendrobium* orchid such as *Dendrobium* Nobile displays anti-aging and antimicrobial properties. It functions as analgesic [6] and has been used for treating menstrual pain and hyperglycemia [5]. Besides, several other *Dendrobium* orchids also exhibit various health benefits to human, for instances, *Dendrobium denneanum* which has been used as anti-inflammatory agent [7]; *Dendrobium huoshanense* which possesses hepatoprotective effects [8]; and the anti-diabetic effect exhibited by *Dendrobium officinale* [9].

In plant tissue culture, the source of illumination such as cool fluorescent white light often consists of full visible light spectrum (400–700 nm). However, plants might develop specific physiological and/or biochemical responses towards only a certain light spectrum of narrow range. Thereby, light emitting diode (LED) is

useful in this scenario as it allows the radiation of narrow light spectrum in order to meet the requirements of plants for their growth and development. The energy needed by electrons to overcome the semiconductor band gap within a LED determines the colour of the emitted light. This feature permits the adjustment of visible light spectrum to elicit growth and biochemical changes in plants.

Amino acids play a pivotal role as signalling molecules especially in stress-induced plant defense mechanisms. Moreover, amino acids are also essential for the biosynthesis of various secondary metabolites such as alkaloids, wherein amino acids serve as the building blocks and account for the biosynthesis of 12 000 known nitrogen-containing alkaloids [10]. Several other secondary metabolites, for instance, glucosinolates, are generated via biosynthetic pathways with amino acids as the precursors [11]. Hence, it is of great importance to explore the capacity of amino acids in elevating bioactive compounds accumulation.

Present study aims to identify the phytochemicals present within protocorm-like bodies (PLBs) of a new orchid hybrid which retains the potential to serve as an alternate source for the acquisition of bioactive compounds with medicinal values. LEDs are engaged in present study as the illumination system to elicit secondary metabolites production during PLBs proliferation stage. Amino acids are applied exogenously in conjunction with optimized LED irradiation to sustain the production of secondary metabolites.

2. Materials and methods

2.1. Source of PLB cultures

Thirty-day-old PLBs were aseptically excised into clumps and cultured with half-strength Murashige and Skoog (MS) solid media (pH 5.80) without hormone supplement. The cultures were maintained at $24^{\circ}\text{C} \pm 2$ and humidity of $51 \pm 2\%$ under illumination of cool white LED light ($17.0 \mu\text{mol/s}$) with photoperiod of 16 h for 30 days.

2.2. Extraction for bioactive compounds from PLBs

Thirty-day-old PLBs were weighed at 6 g and excised into shattered form. The extraction was started off by using 95 % ethanol as the extraction solvent [12]. PLBs were treated with 45 mL of 95 % ethanol and agitated at 120 rpm by using orbital shaker for 24 h at room temperature. The mixture was centrifuged at 120 rpm for 10 min, after which the supernatant will be collected. Ethanolic extract was left in 60°C water bath and allowed to completely evaporate prior to adding 45 mL of fresh 95 % ethanol. The steps from agitation and evaporation were repeated for subsequent 48 and 72 h. The extracted solution was subjected to gas chromatography-mass spectrometry (GCMS) for further analysis.

2.3. Gas chromatography-mass spectrometry (GCMS) analysis

The extracted compound mixture was subjected to $0.45 \mu\text{m}$ membrane filter prior to GCMS analysis. The mixture was injected into GCMS instrument (Agilent Technologies 7890A) and carried by inert helium gas with a flow rate of 1 mL/min. Sample was pressurized at 8.2 psi. Capillary column (30 m \times 250 μm) with a film thickness of 0.25 μm was engaged in the GCMS. Stationary phase engaged in GCMS was 5 %-phenyl-methylpolysiloxane (HP-5 ms). GCMS conditions were programmed as indicated in the work of Gomathi et al. [13] with slight modifications. The initial temperature was set at 60°C and held for 15 min, which increased

gradually at the rate of 6°C per minute up to 280°C and subsequently held for 5 min.

2.4. Effect of LEDs on PLBs pre-cultured with different conditions

Thirty-day-old *in vitro* PLBs maintained for one, two subculture cycles under red fluorescent light with intensity of $25.0 \mu\text{mol/s}$ (30 days per cycle) or more than 3 cycles under cool white LED illumination ($17.0 \mu\text{mol/s}$) were subjected under the illumination of 6 different LED spectra with similar intensity ($15\text{--}20 \mu\text{mol/s}$), namely cool white light (400–700 nm); far red (peak at 730 nm); green (white spectrum with increased green portion with peak at 530 nm); blue (peak at 440 nm); red (peak at 660 nm) and blue-red (peak at 440 and 660 nm respectively). PLBs were aseptically excised into clumps of 0.30 g and nourished with half-strength MS basal media (pH 5.80) supplemented with 2 % sucrose while 3.75 g/L of Gelrite was incorporated as the gelling agent. Three replicates were prepared for each treatment and maintained under different LEDs for 30 days with photoperiod of 16 h, after which the fresh PLBs were subjected to secondary metabolite analyses. All the cultures were maintained at temperature of $24 \pm 2^{\circ}\text{C}$ and humidity of $51 \pm 2\%$. Three biological replicates, each with three technical replicates were selected for total phenolic, total flavonoid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) analyses.

2.5. Effects of LEDs with varying intensities

Optimized pre-culture condition for PLBs secondary metabolites accumulation under different LED spectra was determined from previous experiment. PLBs exposed to cool white LED ($17.0 \mu\text{mol/s}$) for more than 3 subculture cycles were selected as the explants. Thirty-day-old PLBs were aseptically incised into clumps of 0.30 g and illuminated with 6 different LED lights with wavelengths identical with abovementioned spectra. Different intensity of each LED light: cool white light ($4.63 \mu\text{mol/s}$; $5.18 \mu\text{mol/s}$; $17.0 \mu\text{mol/s}$); far red ($1.11 \mu\text{mol/s}$; $9.12 \mu\text{mol/s}$; $20.8 \mu\text{mol/s}$); green ($0.77 \mu\text{mol/s}$; $6.15 \mu\text{mol/s}$; $16.9 \mu\text{mol/s}$); blue ($0.91 \mu\text{mol/s}$; $6.72 \mu\text{mol/s}$; $15.7 \mu\text{mol/s}$); red ($1.26 \mu\text{mol/s}$; $15.40 \mu\text{mol/s}$; $29.30 \mu\text{mol/s}$) and blue-red ($2.01 \mu\text{mol/s}$; $20.30 \mu\text{mol/s}$; $44.80 \mu\text{mol/s}$), were engaged as the illumination sources. Cultures were maintained at temperature of $24 \pm 2^{\circ}\text{C}$ and humidity of $51 \pm 2\%$.

2.6. Effect of amino acids on secondary metabolites accumulation

Thirty-day-old PLBs maintained under cool white LED illumination ($17.0 \mu\text{mol/s}$) for more than 3 subculture cycles were aseptically excised into clumps of 0.30 g and cultured with half-strength MS basal media. Different amino acid supplements which included L-leucine (25 and 50 mg/L), glycine (25, 50, 75 and 100 mg/L) and proline (25, 50, 75 and 100 mg/L) were incorporated into the media to study their respective effect on secondary metabolites accumulation as well as antioxidant capacity of PLB cultures. Media pH were adjusted to be within the range of 5.75–5.80 with 0.1 M of HCl or NaOH, prior to autoclaving under 1.05 kg/cm² and 121°C for 15 min (Tomy High Pressure Steam Sterilizer ES-315, Japan). All the cultures were illuminated with high intensity green LED ($16.9 \mu\text{mol/s}$) light, which has been optimized from previous experiment, with photoperiod of 16 h for 30 days before subjected to secondary metabolite analyses.

2.7. Secondary metabolites extraction

PLBs were weighed in 0.10 g and homogenized with 0.6 mL of 80 % (v/v) analytical reagent grade acetone by using pre-cooled mortar and pestle. The suspension was kept overnight in 1.5 mL centrifuge vial under darkness at 4°C [14]. The extract solution was

subjected to total flavonoid, total phenolic content and total antioxidant activity analyses.

2.8. Total phenolic content evaluation

A volume of 200 μL acetone extract was incorporated into 500 μL of 10 % (v/v) Folin-Ciocalteu's reagent and 500 μL of sterile distilled water. The mixture was vortexed to ensure the solutions were thoroughly mixed. Subsequently, the mixture was allowed to stand for 6 min prior to adding in 800 μL of 7.5 % (w/v) sodium carbonate [15]. The mixture was vortexed once again and incubated in dark for 30 min under room temperature before taking the absorbance reading at 765 nm by using UV-vis microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO). Total phenolic content ($\mu\text{mol/g}$ FW) was quantified by applying the equation obtained from the standard curve. Standard curve was established by engaging gallic acid serial dilutions (0–100 mg/L) to undergo the described protocols as the acetone extract at the absorbance of 765 nm.

2.9. Total flavonoid content evaluation

Acetone extract (100 μL) was incorporated into 100 μL of reagent solution containing 40 μL of 10 % aluminium chloride hexahydrate, 40 μL of 1 M potassium acetate, 600 μL HPLC grade methanol and 1 200 μL of sterile distilled water [16]. The mixture was vortexed to ensure the solutions were thoroughly mixed. Subsequently, the mixture was incubated in dark for 30 min under room temperature. The absorbance reading was taken at 415 nm by using UV-vis microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO). Total flavonoid content ($\mu\text{mol/g}$ FW) was quantified by applying the equation obtained from the standard curve. Standard curve was established by engaging quercetin serial dilutions (0–100 mg/L) to undergo the described protocols as the acetone extract at the absorbance of 415 nm.

2.10. Evaluation of total antioxidant activity through DPPH

Total antioxidant activity of the extract was evaluated according to pre-described assay [17] with slight modifications. A volume of 100 μL acetone extract was incorporated into 100 μL of 0.135 mM DPPH dissolved in 80 % (v/v) acetone. The mixture was incubated for 30 min in dark under room temperature to provide ample time for the extract to scavenge free radicals present within DPPH solution. Controls for DPPH assay were prepared by mixing 100 μL of 80 % acetone with 100 μL of 0.135 mM DPPH. Absorbance reading was taken at 517 nm by using UV-vis microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO) at every 2 min intervals up to the time where the absorbance readings reached a plateau. The absorbance reading recorded during the period where it reached stationary was applied into the following equation to ascertain the antioxidant capacity of the extract:

$$\text{DPPH inhibition percentage (\%)} = \frac{A_{\text{control}} - A_{\text{extract}}}{A_{\text{control}}} \times 100\%$$

2.11. Statistical analysis

The data collected was analyzed by using IBM Statistical Package for Social Sciences (SPSS) version 24 software. Experiments were conducted in a completely randomized design. Significant differences of secondary metabolites content as well as antioxidant activity between treatments involved in present study were determined by comparing the mean values of 9 replicates. One-way ANOVA was engaged in mean values

comparison at significance level of $p \leq 0.05$, while two-way ANOVA test was conducted to study the interaction effect between PLBs pre-culture conditions and LED treatments.

3. Results and discussion

3.1. Gas-chromatography-mass-spectrometry (GCMS) analysis

Total of 20 clear and narrow peaks were observed on chromatogram under GCMS profiling (Fig. 1). Compounds with more than 80 % matching percentage with GCMS library data were selected and further discussed (Table 1). Present study revealed the presence of several bioactive compounds within 30-day-old PLB cultures which come from different groups included phenolics, fatty acid, terpene, alkane, amide and esters. Mass spectrum of the selected bioactive compounds (mass/charge) with their respective molecular structures were analyzed by using mass-spectrometry for identification purposes as presented in Figs. 2 and 3.

Fatty acids such as n-hexadecanoic acid ($\text{C}_{16}\text{H}_{32}\text{O}_2$), 9,12-octadecadienoic acid ($\text{C}_{18}\text{H}_{32}\text{O}_2$), 6-octadecenoic acid ($\text{C}_{18}\text{H}_{34}\text{O}_2$) and pentadecanoic acid ($\text{C}_{15}\text{H}_{30}\text{O}_2$) with retention time of 39.234, 41.870, 42.007 and 42.289 min respectively, were identified in GCMS analysis (Table 1). The presence of flavonoid 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- ($\text{C}_6\text{H}_8\text{O}_4$) or DDMP in short, and phenol compound 2,4-bis(1,1-dimethylethyl) ($\text{C}_{14}\text{H}_{22}\text{O}$) were detected in 30-day-old PLB cultures. Besides, nitrogen-bearing phytochemicals such as hexadecanamide ($\text{C}_{16}\text{H}_{33}\text{NO}$) and octadecanamide ($\text{C}_{18}\text{H}_{37}\text{NO}$) of amide group as well as 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester ($\text{C}_{16}\text{H}_{22}\text{O}_4$) were identified in GCMS analysis. Terpenoid such as phytol which contains 20 carbons was also found to be present in the PLB cultures with GCMS retention time of 41.539 min.

Various fatty acids such as n-hexadecanoic acid, 9,12-octadecadienoic acid, 6-octadecenoic acid and pentadecanoic acid are present within PLB *in vitro* cultures in present study. All of which are bioactive compounds with great array of activities. Among these, n-hexadecanoic acid is proven to exhibit anti-inflammatory properties, by providing active bind sites which are compatible to phospholipase enzyme, thereby inhibiting their effect and prevents inflammation [18]. Studies have showed that n-hexadecanoic acid exhibits selective cytotoxicity towards leukemic cells of human [19]. On top of that, anti-inflammatory feature of linoleic acid (9,12-octadecadienoic acid) also helps in relieving rheumatoid arthritis and ocular surface inflammation [20]. Dietary intake of linoleic acid is associated with a decreasing rate of developing coronary heart disease as suggested by Farvid et al. [21]. Owing to its anti-inflammatory effect, linoleic acid has been suggested to possess hepatoprotective activity in rats [22]. The function of linoleic acid in inhibiting the action of enoyl-acyl protein reductase enzyme disrupts the synthesis of fatty acid and exhibits its antibacterial effect [23].

Petroselinic acid (6-octadecenoic acid) is an unsaturated fatty acid with double bond located in between carbon-6 and carbon-7, which is seen as a relatively rare octadecenoic acid in the group. Due to its distinct chemical structure, petroselinic acid permits the production of derivatives which are different from that of other oleic acids. It displays antimicrobial activity [24] and has been widely formulated in cosmetic products as moisturizer, skin care and anti-aging agent [25] by elevating collagen and decorin structural protein levels. Additionally, petroselinic acid functions in inhibiting the formation of arachidonic acid metabolites that serve as the precursors for inflammatory mediators [26]. Pentadecanoic acid is a saturated fatty acid with 15 carbon atoms which possesses antioxidant and antimicrobial properties [27]. This odd-chain fatty acid aids in reducing risk of developing type 2 diabetes and cardiovascular diseases. It has been reported that

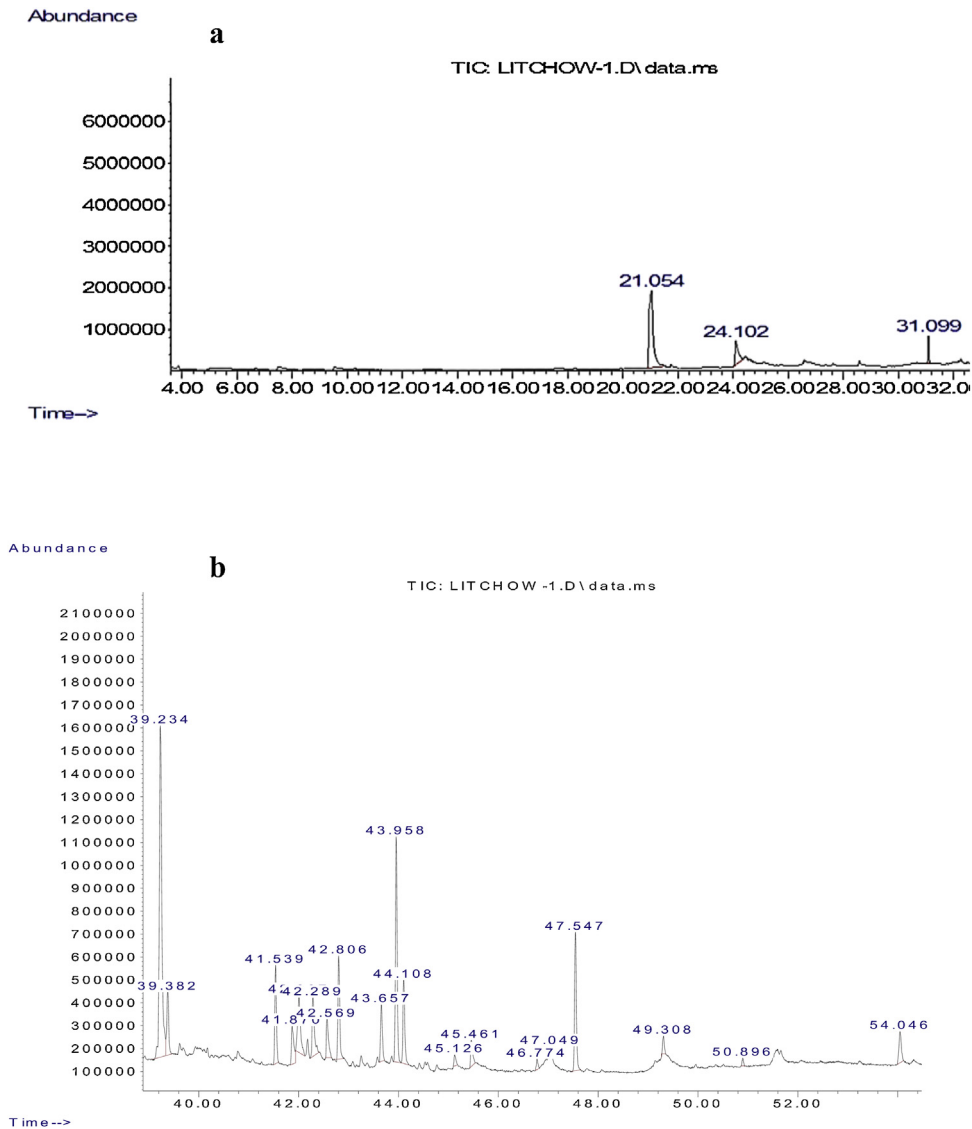


Fig. 1. GCMS chromatogram of 30-day-old *Dendrobium Enopi* x *Dendrobium Pink Lady* PLBs ethanolic extracts. Chromatogram profile of DDMP with retention time of 21.054 min and phenol, 2,4-bis(1,1-dimethylethyl)- with retention time of 31.099 min (a); Chromatogram profile of other phytochemicals present within PLB cultures (b).

pentadecanoic acid holds the capacity to undergo partial oxidation for succinyl-coenzyme A (succinyl-coA) production which further replenishes citric acid cycle, and promotes oxidative capacity of mitochondria [28]. Additionally, this compound has been used as lubricant and adhesive agents as well [29].

Phytol, a diterpene in its oxygenated form, is detected in present study. This acyclic diterpene alcohol is best known for the antibacterial properties it possesses. Phytol exerts its function as bactericide by inducing cell membrane damage, causing potassium ions leakage and further elevates ROS levels within bacteria. It serves as an effective bactericide against various bacteria such as *Staphylococcus aureus*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, and *Colletotrichum accutatum* [30]. Previous study found that phytol impairs DNA of *Pseudomonas aeruginosa* and inhibits the formation of reduced glutathione (GSH) which leads to oxidative stress [31].

DDMP belongs to flavonoids group and retains a strong antioxidant characteristic [32] which makes it an excellent free radicals scavenger. Being the major compound contributing to the anticancer effect of onions, DDMP has been confirmed to be able to induce human colon cancer cells death through apoptosis and ceases cancer cells growth [26]. DDMP expresses its anticancer

effect by inhibiting the activation of nuclear factor kappa B (NF- κ B), which is a transcription factor associated with various oncogenesis aspects. Apart than that, DDMP also exhibits anti-diabetic property by acting as α -glucosidase inhibitor [33], which in turn reduces postprandial hyperglycemia and prevents the breaking down of starch into its monomers, thereby delaying glucose assimilation.

Phenol-2,4-bis(1,1-dimethylethyl) is an antioxidant compound that alleviates photo-oxidative stress and acts as ultraviolet stabilizer. The antioxidant feature of phenol-2,4-bis(1,1-dimethylethyl) is beneficial in impeding oxidative stress caused by hydrogen peroxide accumulation. This antioxidant role of phenol-2,4-bis-(1,1-dimethylethyl) has been suggested to confer it the anti-neurodegenerative effect for neurological related diseases such as Alzheimer's disease, as this compound inhibits oxidative stress that is detrimental to neuronal cells [34]. In addition, this particular compound possesses antifungal properties and functions as an effective antifungal agent against various fungi such as *Curvularia lunata* [35], *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* [36]. Anticancer effects of phenol-2,4-bis(1,1-dimethylethyl) have been well-documented in the study of Malek et al. [37], where it is capable of inhibiting the growth of several cancer cells such as nasopharyngeal epidermoid, human

Table 1

Phytochemical compounds identified to be present within 30-day-old PLBs ethanolic extract followed by GCMS analysis.

Compounds	Retention Time (min)	Molecular Formula	Molecular Weight	Match Quality
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	21.054	C ₆ H ₈ O ₄	144.04	83
Benzofuran, 2,3-dihydro-	24.102	C ₈ H ₈ O	120.06	72
Phenol, 2,4-bis(1,1-dimethylethyl)-	31.099	C ₁₄ H ₂₂ O	206.17	96
n-Hexadecanoic acid	39.234	C ₁₆ H ₃₂ O ₂	256.24	86
2-methyl-2-methoxy-3-hydroxyindan-1-one-3-carboxylate	39.382	C ₁₃ H ₁₄ O ₅	250.08	50
Phytol	41.539	C ₂₀ H ₄₀ O	296.31	91
9,12-Octadecadienoic acid (Z,Z)	41.870	C ₁₈ H ₃₂ O ₂	280.24	99
6-Octadecenoic acid	42.007	C ₁₈ H ₃₄ O ₂	282.26	98
Pentadecanoic acid	42.289	C ₁₅ H ₃₀ O ₂	242.22	83
Hexadecanamide	42.569	C ₁₆ H ₃₃ NO	255.26	87
1,2,5,5,6,7-Hexamethylbicyclo[4.1.0]hept-2-en-4-one	42.806	C ₁₃ H ₂₀ O	192.15	46
1,4-Methano-1H-indene, octahydro-1,7a-dimethyl-4-(1-methylethenyl)-, [1S(1.alpha.,3a.beta.,4.alpha.,7a.beta.)]-	43.657	C ₁₅ H ₂₄	204.19	30
N-(7-Dimethylamino-4-methylcoumarin-3-yl)maleimide	43.958	C ₁₆ H ₁₄ N ₂ O ₄	298.10	72
Toluene, 4-chloro-2-fluoro-5-nitro-	44.108	C ₇ H ₅ ClFNO ₂	189.00	35
Octadecanamide	45.461	C ₁₈ H ₃₇ NO	283.29	97
13-Tetradecen-1-ol acetate	46.774	C ₁₆ H ₃₀ O ₂	254.22	89
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	47.547	C ₁₆ H ₂₂ O ₄	278.15	91
Cyclopentadecane	49.308	C ₁₅ H ₃₀	210.24	93
1H-Indole, 5-methyl-2-phenyl-	50.896	C ₁₅ H ₁₃ N	207.10	49
N-Methyl-1-adamantaneacetamide	54.046	C ₁₃ H ₂₁ NO	207.16	38

hormone-dependent breast, lung and cervical carcinoma cell lines. It ceases their growth by displaying cytotoxicity activities towards all the above-mentioned carcinoma cells. The acquisition of this compound from plants is natural and more preferable than their artificially synthesized analogues. Usage of plant-derived phenol-2,4-bis(1,1-dimethylethyl) as antimicrobial agents against phytopathogenic microbial is relatively safer and environmental friendly.

1,2-Benzenedicarboxylic acid, mono-(2-ethylhexyl) ester is a bioactive compound which has been proven to exhibit cytotoxicity towards various human cancer cell lines such as hepatocellular liver carcinoma and breast adenocarcinoma [38] while possesses little to none adverse effects on normal and healthy cell lines. The anticancer effect of 1, 2-benzenedicarboxylic acid, mono-(2-ethylhexyl) ester is attributed to its ability in inducing apoptosis of the cancer cells which in turn inhibits the cell growth. The accumulation of this compound has been reported in *Centratherum Punctatum* Cass. [39] and *Hertia cheirifolia* [40], both are medicinal plants with anti-inflammatory property.

The presence of other phytochemicals such as hexadecanamide, N-(7-dimethylamino-4-methylcoumarin-3-yl) maleimide (DACM), octadecanamide, 13-tetradecen-1-ol acetate and cyclopentadecane have been detected in the chromatography. Among the identified compounds, hexadecanamide (C₁₆H₃₃NO) is an amide which derives from hexadecanoic acid with anti-inflammatory effect and used as analgesic [41]. DACM (C₁₆H₁₄N₂O₄) is a fluorescent compound which exhibits its fluorescence only after undergoing chemical reaction with thiols under optimum conditions (pH 7). This compound has been employed as the staining agent in histochemical studies for the detection of sulfhydryl and disulfide groups [42].

Cytotoxic bioactive compounds exert their anticancer effects by interfering with different carcinogenesis stages such as inflammation, proliferation, invasion, angiogenesis and metastasis. Apoptosis is the occurrence of programmed cell death which is often accompanied by distinct morphological features and aided by various energy-dependent biochemical mechanisms [43]. This process is vital as it rapidly eliminates the dysfunctional cells in an irreversible manner and ensures normal cell turnover [44]. Failure to perform normal apoptosis is carcinogenic and also the leading cause of several diseases. For instances, neurodegenerative is an ischemic-related diseases and autoimmune disorders [43]. Hence,

the presence of phytochemicals in PLB *in vitro* cultures such as DDMP, phenol-2,4-bis(1,1-dimethylethyl) and 1,2-benzenedicarboxylic acid, mono-(2-ethylhexyl) ester, serves as an alternate source for the acquisition of anticancer compounds.

3.2. Effect of LEDs on PLBs pre-cultured with different conditions

Present study revealed that PLB pre-culture conditions were significantly associated with their subsequent exposure to LED treatments in influencing secondary metabolites accumulation as well as total antioxidant activity. The interaction between these two independent variables were significant ($p < 0.001$) for all of the mentioned parameters (Table 2), where F value of 45.934 is recorded for total flavonoid content ($F_{10,141} = 45.934$, $p < 0.001$); 17.422 for total phenolic content ($F_{10,141} = 17.422$, $p < 0.001$); 4.380 for DPPH inhibition rate in percentage ($F_{10,141} = 4.380$, $p < 0.001$).

In overall, PLBs pre-illuminated with cool-white LED exhibited significantly higher flavonoid content after being treated with different LED spectra (Fig. 4), as compared with the other two pre-culture conditions. Blue-red LED gave rise to exceptionally high flavonoids content among all the treatments with 16.79 $\mu\text{mol/g}$ FW of flavonoids was recorded. Flavonoids content accumulation declined as pre-culture conditions of PLBs differ.

The highest total phenolic content was induced by blue-red LED on PLBs pre-cultured with red fluorescent light for one cycle (Fig. 4) which recorded the accumulation of 110.54 $\mu\text{mol/g}$ FW. Yet there was no significant difference between these particular treatments with that of green LED on PLBs pre-cultured under cool-white LED for more than 3 subculture cycles, which accumulated 103.44 $\mu\text{mol/g}$ FW of total phenolic content. Among the PLBs pre-cultured under cool-white LED for more than three subculture cycles, white LED induced significantly low amount of total phenolic content with only 52.98 $\mu\text{mol/g}$ FW. Present study discovered that total phenolic accumulation capacity of PLBs reduced under LED treatments, after being pre-cultured for two subculture cycles under red fluorescent light. Induction rate of total phenolic was significantly underperformed for all the tested LED spectra as compared with other PLBs pre-culture conditions. The lowest total phenolic content was achieved under far red light treatment with 18.85 $\mu\text{mol/g}$ FW.

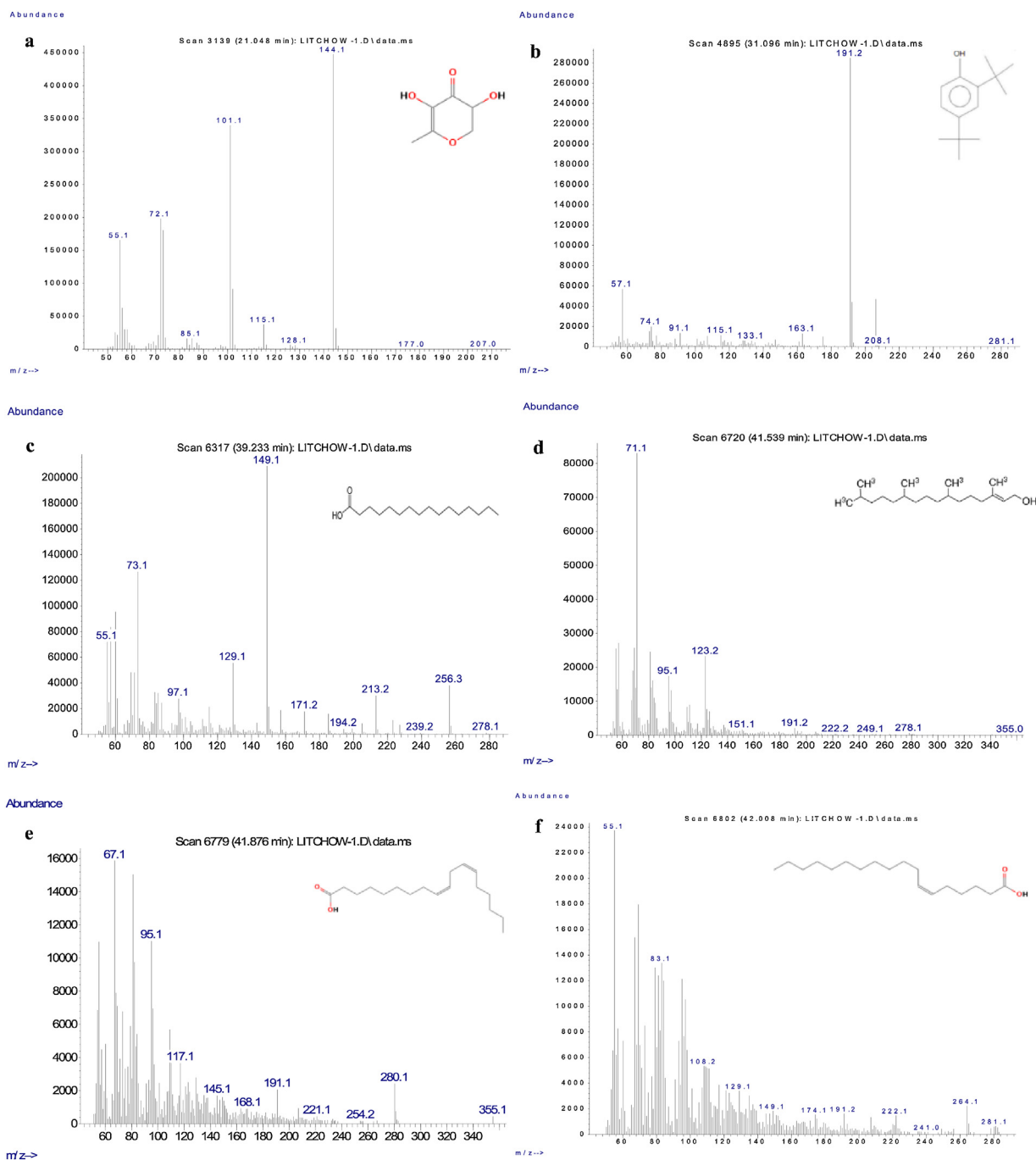


Fig. 2. Mass spectrum graphs of identified phytochemical compounds within ethanolic extract of 30-day-old PLBs and their respective chemical structure. 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (a); Phenol, 2,4-bis(1,1-dimethylethyl)- (b); n-Hexadecanoic acid (c); Phytol (d); 9,12-Octadecadienoic acid (Z,Z) (e); and 6-Octadecenoic acid (f).

Pre-culture conditions of PLB explants exhibited no obvious effect on total antioxidant activity for all the tested LED lights (Fig. 4). The highest total antioxidant activity was recorded within PLBs pre-cultured with red fluorescent light for two subculture cycles which further exposed to blue LED light with 75.68 % of DPPH inhibition rate.

The higher content of total flavonoids and phenolics for PLBs pre-treated with white LED prior to illuminating with various LED spectra could be attributed to the occurrence of habituation of PLBs towards light. Habituation arises whenever *in vitro* cultures exhibit growth and development responses towards certain exogenously supplied substance, in an independent manner [45]. Plant tissue

culture often involves the incorporation of plant growth regulators (PGRs) such as cytokinins and auxins, into the culture media to aid with the growth and development of *in vitro* cultures. However, upon the passage of time under *in vitro* conditions, cultures tend to develop habituation towards the exogenous PGRs. Callus cultures is more prone in exhibiting habituation, especially towards cytokinins and auxins [46]. As documented by Pischke et al. [47], callus cultures which often require cytokinin to promote proliferation and shoot organogenesis, possess the tendency to lose their dependence on cytokinin and become habituated over time, probably due to the over-expression of cytokinin receptor *CRE 1* gene. Hormone habituation has been well-documented in

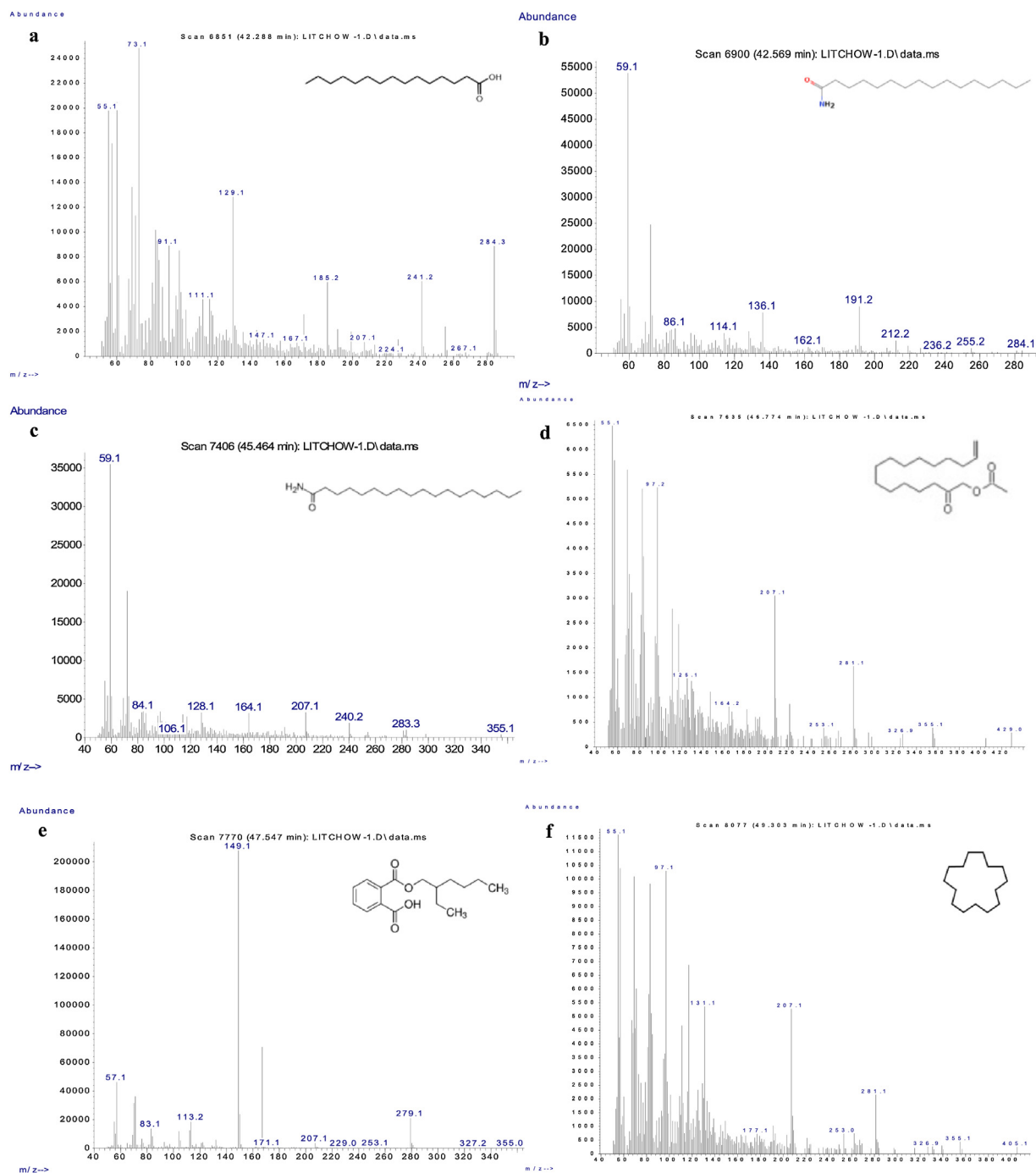


Fig. 3. Mass spectrum graphs of identified phytochemical compounds within ethanolic extract of 30-day-old PLBs and their respective chemical structure, Pentadecanoic acid (a); Hexadecanamide (b); Octadecanamide (c); 13-Tetradecen-1-ol acetate (d); 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (e); and Cyclopentadecane (f).

various previous studies. Yet, development of habituation of PLB cultures towards illumination source is equally noteworthy and needed to be studied in depth.

In present study, the lower content of total flavonoid evaluated under different LED treatments for PLBs pre-treated with red fluorescent light for one or two subculture cycles, is probably due to habituation towards the light source of the PLB stock cultures. Although having a relatively spiky light spectrum profile, red fluorescent light emits light with narrow spectra similar with LEDs. The cultivation of PLB stock cultures under this light source prior to LED treatments allows the cultures to develop defence mechanisms and confers them the ability to cope with the subsequent

LED treatments, which might no longer be perceived as a stress factor. Hence, the accumulation of flavonoids, which play a role in protecting plants against biotic and abiotic stresses, has been reduced. On the other hand, white LED-treated PLBs showed an upsurge of flavonoids content after exposure to different LED spectra, as compared with other pre-treatments. Flavonoids are essential for plants as UV stabilizer and scavenge the over-accumulated ROS induced by excessive UV radiation [48]. Besides, flavonoids also aid in plant defence mechanisms by acting as signalling molecules, allelopathic compounds, phytoalexins and detoxifying agents [49]. ROS present in excessive amount due to oxidative stress tends to elevate flavonoids content as well as their

Table 2
Two-way ANOVA test of between-subjects effects on the accumulation of total phenolic, total flavonoid and total antioxidant activity.

Source	Type III Sum of Squares	df	Mean Square	F value	Sig.
Total flavonoid					
LED	286.40	5	57.28	90.31	0.000*
Pre-culture conditions	2886.93	2	1443.46	2275.72	0.000*
LED x Pre-culture conditions	291.36	10	29.14	45.93	0.000*
Total phenolic					
LED	9981.21	5	1996.24	18.99	0.000*
Pre-culture conditions	98632.99	2	49316.49	469.14	0.000*
LED x Pre-culture conditions	18314.39	10	1831.44	17.42	0.000*
DPPH inhibition rate (%)					
LED	4751.23	5	950.25	4.30	0.001*
Pre-culture conditions	876.03	2	438.02	1.98	0.141
LED x Pre-culture conditions	9671.54	10	967.15	4.38	0.000*

Denoted asterisk (*) indicates significance at $p < 0.05$.

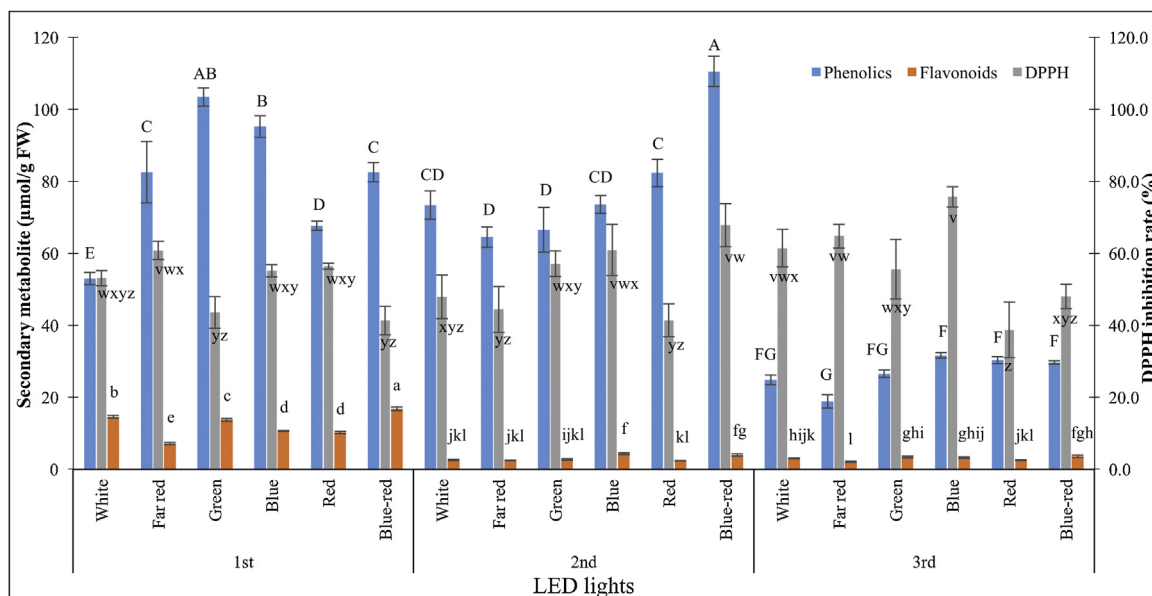


Fig. 4. Secondary metabolites accumulation of 30-day-old *Dendrobium Enopi* x *Dendrobium Pink Lady* PLBs pre-cultured under different light sources prior to illuminating under LED lights. Different letters indicate statistically significant differences ($p < 0.05$) using Duncan Multiple Range Test. (1st: > 3 subculture cycles under cool white LED; 2nd: 1 subculture cycle under red fluorescent light; 3rd: 2 subculture cycles under red fluorescent light).

distribution within plant. Flavonoids help in such a way that they rapidly eliminate excess ROS, owing to their antioxidant properties. In present study, the sudden change of illumination source from white LED with wide light spectrum (400–700 nm) to different LED lights with narrow light spectra, might have induced photo-oxidative and/or photo-inhibition stress and upraised flavonoids content.

Among the PLBs pre-treated with white LED, blue-red (1:1) LED induced the highest total flavonoid content. Red light plays a role in the combination spectrum of blue and red LEDs. The proportion of red light initiates photo-protective mechanisms of the cultures and promotes biosynthesis of antioxidant compounds [50], at the expense of photosynthetic efficiency. On the other hand, blue light portion in the blue-red LED triggers the accumulation of flavonoids through the activation of chalcone synthesis and dihydroflavonol-4-reductase gene expression [51]. Besides, blue light also aids in enhancing the activity of enzyme such as phenylalanine ammonia-lyase (PAL), which in turn stimulates the accumulation of secondary metabolites through phenyl-propanoid pathways [52]. Phytochromes which are sensitive towards red and far red lights, as well as phototropins in plants which response primarily towards blue light, are believed to play a role in determining the

biosynthesis of flavonoids. Chromophores present on photo-receptors confer cultures the ability to convert light signals into biochemical responses which might activate certain enzymes and interaction among proteins within the cultures [53]. Far red light often offsets the effects of red light in stimulating plant developmental responses while the ratio of far red and red light dictates various developmental activities and determines changes in terms of morphological, molecular as well as biochemical [54]. In present study, far red illumination alone without red light proportion resulted in the lowest flavonoids accumulation. It can be deduced that far red light retains adverse effect for flavonoids accumulation. Similar findings have also been reported by Bottomley et al. [55], where far red light offset the effect of red light which elevated the content of kaempferol derivatives in *Pisum sativum*.

The exceptionally low total phenolic content under white LED treatment for PLBs pre-treated with white LED, suggests that PLBs induce more of these phenolic compounds in order to counteract with the altered illumination sources. The engagement of LEDs as elicitors for secondary metabolites production has been demonstrated in the study conducted by Lian et al. [56]. Narrow light spectra generated by LEDs serve as the abiotic stress factor and

trigger the activation of plant defence mechanisms, which in turn leads to the accumulation of secondary metabolites [57]. High phenolics content under green LED could be due to the photo-damaging effect to photosynthetic pigments bring about by green spectrum [58]. The impairment of these essential pigments deprives one of the defence mechanisms against environmental stress, since photosynthetic pigments such as carotenoids, play a role in quenching reactive oxygen species (ROS) and confer plants the protection against photo-inhibition [59]. As a result, excessive ROS accumulation within the cultures activates the antioxidant gene defences, which subsequently stimulates the formation of antioxidants as well as the related enzymes [60]. Production of phenolic compounds is crucial to ensure the survivability of cultures especially under stressful conditions. The antioxidant properties of phenolics help in scavenging ROS which is detrimental to plants by preventing the oxidation of essential cellular components such as lipids, proteins and DNA [61]. Moreover, although green spectrum is not directly involved in plant growth and development, it has been proposed to contribute in reactions which are not directly exposed to light stimulus, for instance, antioxidants metabolism, since this particular spectrum can be transmitted with ease across plant tissues [50].

In present study, PLBs pre-treated with red fluorescent light for one subculture cycle is within the transition stage of habituation, whereby photo-oxidative stress induced by LED illumination is in the intermediate level. In order to compensate with the reduction of flavonoids, phenolics content remains high during this particular stage so as to mitigate plant oxidative stress by scavenging free radicals. The highest phenolics content under blue-red LED could be attributed to the blue light portion. Blue light exert its influence on the activities of PAL, by transforming *trans*-hydroxycinnamic acids, which inhibit PAL enzyme, to a less inhibitory form, *cis*-hydroxycinnamic acids, thereby ensuring the biosynthesis of phenolics [62]. In addition, red spectrum in blue-red LED also functions in activating phytochrome B, which further interacts with transcription factor such as vascular plant one-zinc-finger (VOZ) and mediates plant defence mechanisms [63]. Both phenolics and flavonoids of PLBs pre-treated with red fluorescent light for two subculture cycles, showed reduction after exposure to different LED spectra. This further supports the fact that PLB cultures exhibit habituation towards the narrow spectrum of red fluorescent light, wherefore

subsequent illumination of various LED spectra might no longer be perceived as abiotic stress factor.

3.3. Effects of LEDs with varying intensities

Comparison among far red, red and blue-red (1:1) LEDs showed the accumulation of flavonoids at its highest content under high intensity (44.80 $\mu\text{mol/s}$) blue-red LED light treatment, which recorded at 4.10 $\mu\text{mol/g FW}$ (Fig. 5). Generally, high LED intensity regardless of the spectra, stimulated highest flavonoids accumulation for each of the engaged LEDs. Mid-intensity blue-red (1:1) LED induced significantly higher total phenolic content of 113.68 $\mu\text{mol/g FW}$ as compared with the other light treatments (Fig. 5). It was noted that the intensity of 20.30 $\mu\text{mol/s}$ appeared to be the optimum intensity for phenolics induction under blue-red LED, as the phenolics content declined significantly as low or high intensity blue-red LED was employed as the lighting source. For red LED, there were no significant differences among all the tested intensities, where low, mid- and high intensity red LED correspondingly stimulated the accumulation of 91.77, 95.33 and 89.77 $\mu\text{mol/g FW}$ of phenolics content. Overall, PLBs treated with far red LED, exhibited higher antioxidant activity than the others. The highest antioxidant capacity was achieved under low intensity far red LED treatment, with a total of 18.26 % DPPH inhibition rate (Fig. 5), followed by 17.66 % and 17.05 % which correspondingly induced by high and mid-intensity far red LED.

High intensity green LED (16.9 $\mu\text{mol/s}$) illumination stimulated the accumulation of 3.52 $\mu\text{mol/g FW}$ of flavonoids content, which was the highest among all the tested treatments (Fig. 6). However it was found that there was no significant difference between this treatment with that of mid-intensity of green (6.15 $\mu\text{mol/s}$), white (5.18 $\mu\text{mol/s}$) and blue (6.72 $\mu\text{mol/s}$) LED which induced 3.09, 2.96 and 2.95 $\mu\text{mol/g FW}$ of flavonoids content respectively. It was noted that high intensity green LED prevailed over other light treatments as it stimulated significantly higher amount of total phenolic content. Total of 33.80 $\mu\text{mol/g FW}$ of total phenolic content which was the highest among the tested treatments was induced by this particular light treatment (Fig. 6). Generally, white LED of any tested intensities gave rise to a higher DPPH inhibition rate (%) than any other treatments (Fig. 6). The highest antioxidant activity, 78.99 % was achieved under low intensity white LED. This value was significantly higher than all the other engaged

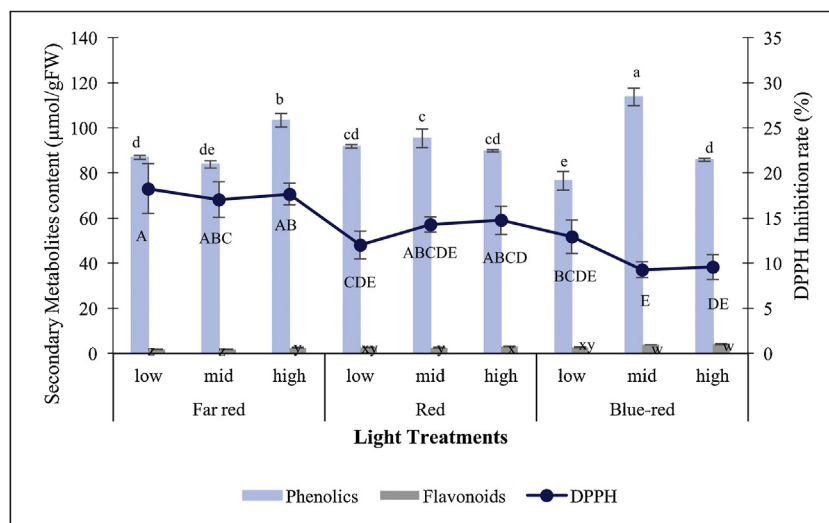


Fig. 5. Secondary metabolites accumulation of 30-day-old PLBs illuminated with far red, red and blue-red (1:1) LED spectrum of varying intensities. Different letters indicate statistically significant differences ($p < 0.05$) using Duncan Multiple Range Test.

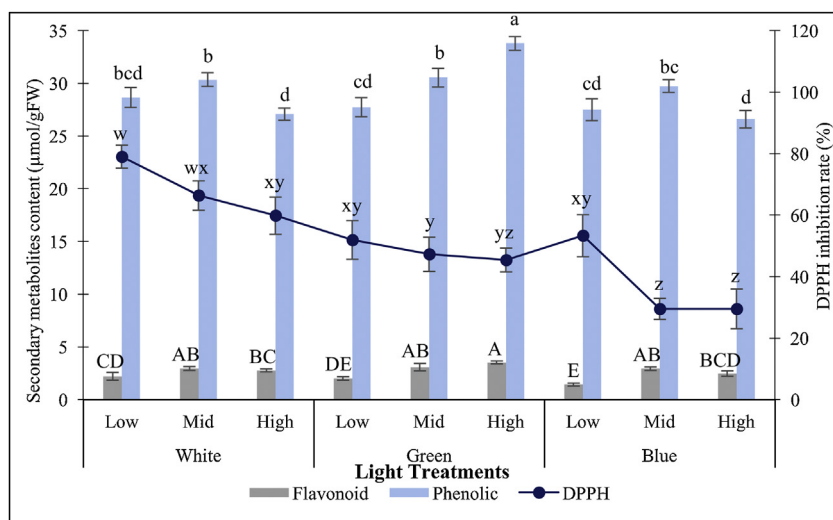


Fig. 6. Secondary metabolites accumulation of 30-day-old PLBs illuminated with white, green and blue LED spectrum of varying intensities. Different letters indicate statistically significant differences ($p < 0.05$) using Duncan Multiple Range Test.

treatments except for mid-intensity white LED, which recorded an antioxidant activity of 66.39 %. Among the same spectrum, low intensity of each of the spectra was found to induce the highest antioxidant activity.

In present study, high intensity green LED prevailed over mid-intensity blue-red (1:1) LED as it resulted in the accumulation of higher total phenolic content (Section 3.2.). Despite total flavonoid content was relatively lower under this light treatment, phenolics production was prioritized due to the discovery of the presence of an essential bioactive compound, phenol-2,4-bis(1,1-dimethyl-ethyl) within the PLBs. Thereby, this illumination was selected for subsequent experiment.

The contents of plant secondary metabolite vary among and within species. The concentrations fluctuate due to several abiotic factors such as light intensity as well as the wavelength [64]. Present study reveals that blue-red (1:1) LED of mid-intensity (20.30 $\mu\text{mol/s}$) induces high phenolics and flavonoids, suggesting that this intensity is appropriate for the production of secondary metabolites. Low intensity blue-red LED (2.01 $\mu\text{mol/s}$) might have limited the availability of light photons required for the utilization of phytochromes and phototropins for the conversion of light signals into phytochemicals accumulation. In contrast, high intensity blue-red LED (44.80 $\mu\text{mol/s}$) provides proper absorption spectrum for photosynthetic pigments. Hence PLB cultures might have prioritized their growth while limited carbon source restricts the production of secondary metabolites [65]. Primary growth always takes precedence over the production of secondary metabolites in plants [66]. Under optimal conditions for primary growth, the available carbon and nitrogen sources are often limited for the production of secondary metabolites, as most of these resources have been allocated to support plant primary growth.

In present study, far red LED stimulates relatively high total antioxidant activity within PLBs. This indicates that far red LED of any intensity, low (1.11 $\mu\text{mol/s}$), mid- (9.12 $\mu\text{mol/s}$) or high (20.8 $\mu\text{mol/s}$), induces photo-oxidative stress within PLB cultures and hence heightens their antioxidant capacity. The reduction of photosynthetic pigments under far red light could be the causal factor for the upsurge of PLBs antioxidant activity under this illumination. Alternative plant defence mechanisms such as non-enzymatic antioxidants which include ascorbic acid and glutathione might play a part in giving rise to a relatively higher antioxidant activity for PLBs under far red illumination, as they

play a protective role in impeding plant oxidative stress as ROS scavengers.

Green spectrum is often of non-essential for plant growth and development. However, it has been suggested to possess effects in plant photosynthesis as well as physiology responses towards environmental factors [67]. Present study reveals that increasing light intensity of spectrum consisting of green wavelength elevates phenolic and flavonoid contents. In guard cells, chlorophylls also function as photoreceptors that drive stomatal opening. The spectrum generated by green light possesses little effects on the action spectrum of guard cell chlorophylls, and hence limits stomatal opening [68]. Green spectrum with maximum peak of 540 nm reverses the action of blue light and inhibits stomatal opening [69]. While in a study done by Lurie [70], exposure of broad bean leaves towards green light induces only a slight opening of the stomata. Stomatal closure is often correlated with drought stress which stimulates the accumulation of secondary metabolites as a defence mechanism due to ROS overproduction. Thus, high intensity green light (16.9 $\mu\text{mol/s}$) illumination in present study might have elevated secondary metabolites of PLB cultures through these means. Furthermore, green light illumination helps in counteracting biotic as well as abiotic stress through the up-regulation of PAL and pathogenesis-related protein 1a genes [71]. The incorporation of green light portion in the blue-red LED spectrum promotes the net photosynthetic rates of lettuce [72]. In present study, green spectrum supplementation in white light could have enhanced the expression of light harvesting chlorophyll a/b-binding protein and PsbA genes, which encodes for D1 protein in photosystem II. Additionally, the further penetration of green light through plant tissues also aids in driving photosynthesis [73]. The promoted photosynthetic rates increase the availability of more carbon sources for the production of carbon-based secondary metabolites, for instance, phenolics.

Low intensity white LED (4.63 $\mu\text{mol/s}$) provides inadequate light source for the assimilation of PLBs, thereby inducing light stress. Low intensity white illumination exerts its influence in regulating several physiological metabolic processes such as photosynthesis along with antioxidant biosynthesis pathways [74]. Under this scenario, the exposure of PLB cultures to light stress might have altered the biochemical mechanisms by up-regulating the activities of certain antioxidants and/or substances with such effects, which collectively elevates the antioxidant capacity.

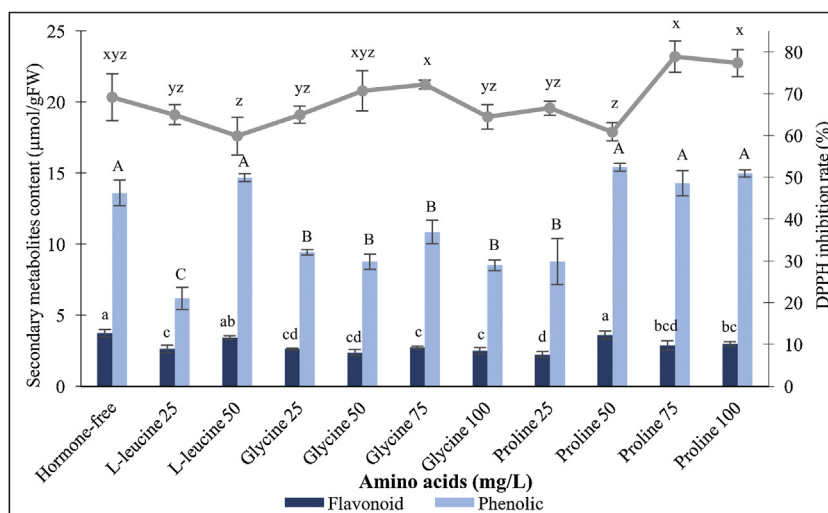


Fig. 7. Effect of different amino acid supplements on secondary metabolites accumulation of 30-day-old PLBs illuminated with high intensity green LED spectrum. Different letters indicate statistically significant differences ($p < 0.05$) using Duncan Multiple Range Test.

3.4. Effect of amino acids on secondary metabolites accumulation

Under high intensity green LED illumination, it was noticed that all the tested amino acids played no significant effects in elevating flavonoids content within PLBs (Fig. 7). The highest recorded total flavonoid content of $3.73 \mu\text{mol/g FW}$ was induced by half-strength MS basal medium without amino acid supplement. Generally, proline with concentrations higher than 50 mg/L generated higher total phenolic content within PLBs (Fig. 7) whereas glycine with concentrations ranged from 25 to 100 mg/L displayed no stimulating effect in enhancing phenolics content. It was found that 25 mg/L of proline induced the accumulation of $8.78 \mu\text{mol/g FW}$ of total phenolic content. Further increment of proline concentration to 50 , 75 and 100 mg/L significantly enhanced the phenolics content, with 15.41 , 14.30 and $14.98 \mu\text{mol/g FW}$ recorded for each concentration, respectively. Present study revealed that all the tested amino acids possess no prevailing effects on enhancing total antioxidant activity of the PLBs, as all the treatments exhibited no significant differences with that of the control treatment (Fig. 7) which stimulated 69.09% of DPPH inhibition rate.

Incorporation of high L-leucine concentration (50 mg/L) stimulates higher phenolics and flavonoids content than low concentration (25 mg/L) does. The assimilated exogenous L-leucine provides an essential source for the yielding of aldehydes. Degradation of L-leucine with the aid of various enzymes such as amino acid decarboxylase, amino acid deaminases and aldehyde synthase, plays a role in the biosynthesis pathways of plant secondary metabolites [75]. In addition, proline supplementation leads to high secondary metabolites production in present study. This can be attributed to the role of proline as signalling molecule during environmental stress conditions. Proline helps in restoring cellular redox balance and activates certain gene expression in plant defence mechanisms. Besides, proline biosynthesis cycle which is coupled with pentose phosphate pathway, generates the formation of erythrose-4-phosphate, a compound which serves as the precursor of shikimate pathway for biosynthesis of secondary metabolites [76]. The capacity of proline in inducing phenolics has been exhibited in the study conducted by Al-Jibouri et al. [77], where supplementation of proline promotes coumarin and eugenol content of *Verbascum thapsus* L. callus. The accumulation of proline in plants often occurs as a result of exposure to stressful conditions, which include drought, salinity, oxidative stress and UV irradiance [78]. In present study, the administration of

exogenously supplied proline acts as antioxidant molecule and aids in diminishing excessive ROS, thereby conferring the PLB cultures a relatively higher antioxidant capacity. The function of proline as antioxidant in quenching singlet oxygen radicals and prevents programmed cell death has been reported in previous study as well [79].

The low concentration of secondary metabolites evaluated within PLBs treated with glycine suggests that the cultures prioritize primary growth instead of secondary metabolites production in present study. The application of exogenous glycine tends to upraise the intake of macronutrients such as nitrogen, potassium and magnesium within plant [80], which subsequently support plant growth and development. This can be due to the affinity of glycine with nutrient elements present within the culture media. The formation of chelates facilitates the uptake and transportation of these nutrients [81].

Nevertheless, supplementary amino acids which include L-leucine, glycine and proline, possess no significant effect in enhancing secondary metabolites accumulation as well as antioxidant activity of PLB cultures, as in comparison with the control treatment. Results suggest that sole illumination of green LED is ample to serve the purpose of stimulating secondary metabolites production due to the capacities of green spectrum in stimulating several biochemical responses as aforementioned.

4. Conclusion

Bioactive compounds which included DDMP, phenol-2,4-bis(1,1-dimethylethyl) and 1,2-benzenedicarboxylic acid, mono-(2-ethylhexyl) ester that possess anticancer cancer properties have been detected in *in vitro* PLB cultures of *Dendrobium* *Enopi* x *Dendrobium* Pink Lady hybrid orchid. Present study demonstrated that PLB stock cultures pre-cultured under red fluorescent light for one or two subculture cycles showed a reduction in secondary metabolites production after exposure to various LED treatments. This suggests that PLB cultures exhibited habituation towards light after being exposed to illumination source of similar light profile for a prolonged period. At intensity of $16.9 \mu\text{mol/s}$, incorporation of green spectrum into white LED stimulated higher phenolics content, while the highest flavonoids content was achieved under mid- ($20.30 \mu\text{mol/s}$) and high intensity ($44.80 \mu\text{mol/s}$) blue-red (1:1) LED irradiation. Low intensity of both white ($4.63 \mu\text{mol/s}$) and far red ($1.11 \mu\text{mol/s}$) LEDs

enhanced total antioxidant activity of the cultures. Nonetheless, amino acid supplements such as L-leucine, glycine and proline (25–100 mg/L) exerted no satisfactory effects in promoting secondary metabolites accumulation.

Author contributions

LCY and SS designed and conducted the experiments, analyzed the data and wrote the manuscript. SS conceptualized and supervised the research. BLC supervised the research. LCY SS performed chemical analysis and validated the chemical data. LCY and SS critically revised and edited the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00497>.

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