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Research Article

Synergistic Effect of Multi regulatory Molecule "Melatonin" and UV-C on the Production of Invaluable Secondary Metabolites in Callus Culture of Kasni (Cichorium intybus)

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ABSTRACT

One of the lucratively significant medicinal plant species, Cichorium intybus has a wide range of therapeutic applications. This use has been related to the presence of several pharmacologically significant phytochemicals. This medicinal plant has been used, aimed at in vitro research for the enhanced accumulation of essential chemicals synthesized by plants, nevertheless, the combined action of melatonin and UV-C as an elicitor, persists to be profoundly explored. The contemporary study has assessed the individual, as well as synergistic effects in melatonin and UV-C, derived callus cultures of Cichorium intybus. Melatonin and UV-C both serve crucial roles in the biochemical and physiological processes that occur in plant cells. The cultures that were treated with melatonin (3mg/L) and UV-C (30mins) individually, had the maximum fresh weights (FW: 331.66 g/L; 269 g/L) and dry weights (DW: 14.25 g/L; 16.0 g/L), respectively. Whereas, in the case of combined treatment, Mel (3mg/L) + UV-C (30mins) displayed the highest (FW: 164.25g/L; DW: 14g/L). Optimal total phenolic content (14.36 µgGAE/mg DW), total phenolic production (201.4 µgGAE/mg DW) total flavonoid content (57.6 µgQAE/mg DW), total flavonoid production (806.4 µgQE/mg DW) and free radical scavenging activity (92.1 %) was noticed in Mel (3mg/L) + UV-C (30mins) derived callus cultures. The HPLC findings revealed that improved accumulation of medicinally significant metabolites was documented in callus culture treated with a combination of melatonin 3mg/L + UV-C 10 mins i.e. (10.993 µg/mg DW) which is more than three folds higher as compared to control (3.41 µg/mg DW). Cinnamic acid was found higher (3.878 µg/mg DW) in cultures treated with a combination of melatonin 3mg/L + UV-C 10 mins which is nearly six folds higher than control (0.643 µg/mg DW). Majority of secondary metabolites e.g. vanillic-acid (0.07 µg/mg DW), rutin (0.446 µg/mg DW), gallic-acid (0.725 µg/mg DW), catechin (0.915 µg/mg DW), gentisic-acid (0.186 µg/mg DW), cinnamic acid (1.95 µg/mg DW), luteolin (1.12 µg/mg DW) and myricetin (0.52 µg/mg DW) showed higher accumulation at melatonin 3 mg/L. Catechins are well known to scavenge free radicals and chelate redox-active metals and showed the highest accumulation (1.17 µg/mg DW) at UV-C 10 mins. The finding of this research illustrates how melatonin and UV-C impact the production of biomass and phytochemical synthesis in C. intybus-derived callus culture.

Keywords: Cichorium intybus, UV-C, Melatonin, Phenolics, Flavonoids, TRP, TAC, Callus culture

Abbreviations: BAP: 6-Benzylaminopurine; DPPH: 2, 2-Diphenyl-1-picrylhydrazyl; DW: Dry weight; FW: Fresh weight; NAA: α-Naphthalene acetic acid; TFC: Total flavonoid content; TPC: Total phenolic content; TAC: Total antioxidant capacity; TRP: Total reducing potential

1. Introduction

Therapeutic plants have been the basis of conventional medicine for millennia and are considered a possible source of biologically active compounds with elevated medicinal potential. According to an estimate, there are more than 1300 medicinal plants utilized in Europe, out of which 90% are garnered from wild resources; while in the United States, about 118 out of 150 prescription drugs are founded on natural sources. Additionally, nearly 80% of individuals in emergent nations are entirely dependent on plant-derived drugs in order to meet primary healthcare demands and 25% of licensed drugs in advanced countries are gleaned from therapeutic plant species found in wild. Pharmaceutical companies are focusing on specific molecules from medicinal plants with unique metabolic roles, using cutting-edge technological methodologies. Cichorium intybus, also known as chicory, is one such remarkable medicinal species. It is a woody perennial herb that belongs to the Asteraceae (dandelion) family[1]. This plant is tall, upright and has a robust taproot, reaching a height of 40-110 cm[2]. This plant, which was once only found in Europe and northern Africa, is becoming increasingly common in moderate and semi-arid areas like South Africa globally[3].

It's a propitious herb that's high in carbohydrates, proteins and mineral elements and it is employed in place of conventional medicines, food, coffee and feed. Cichorium plants are well-known for their therapeutic and medicinal capabilities. Anti-bacterial, laxative, diuretic, anti-bilious and anti-pyretic qualities are known to exist in all sections of the plant, particularly the roots and leaves[4].

In conventional Indian medicine, Chicory has been utilized to treat diarrhea, fever, spleen and liver enlargement, gout, jaundice, AIDS, insomnia, splenitis, tachycardia, gallstones, gastroenteritis, sinus problems, dysmenorrhea, cancer, bruises and rheumatism. It can also be taken as a tonic. It is prized in China for its liver and digestive system tonic properties. Chicory (particularly the flower) has long been used as a folk remedy in Germany for a variety of diseases. The roasted roots are used as a caffeine-free coffee replacement and are less well recognized as ruminant grazed fodder[5].

C. intybus has a robust commercial prospective due to the great quantities of fructooligosaccharides (FOS), commonly known as inulin, in its roots, which can be employed as a substitute for fat and sugar. Cellulose, proteins, fatty acids, pectin, choline, vitamins, alkaloids, benzoisochromenes, amino acids, minerals sucrose, polyphenols, cellulose, flavonoids, anthocyanins, carotenoids, tannins, caffeic acid derivates, coumarins, sesquiterpene lactones, are among the other phytoconstituents found in C. intybus. Chicory is known to possess antidiabetic, nematicidal, antihepatotoxic cardioprotective, antiallergic, antihepatotoxic, antineoplastic, anti-inflammatory, prebiotic, immunostimulatory and antioxidant effects, according to therapeutic studies[6].

Chlorogenic acids, one of the several chemicals found in chicory, have a wide range of bioactivities. Esterification of caffeic acid (3,4-dihydroxy-trans-cinnamate) with hydroxyl sites present on the quinic acid ring in the plant generates chlorogenic acid (3-O-caffeoylquinic acid and 5-O-caffeoylquinic acid)[7].

Cichorium intybus is well recognized for its abundance. Plants in their natural environment, on the other hand, display batch-to-batch variance in their secondary metabolite production as a consequence of the adaptations they have developed to cope with abiotic and biotic stress. The discrepancy is one of the main reasons for the inconvenience in isolating and purifying metabolites from plants, which makes it a tedious and less productive process[8].

Researchers in the field of biotechnology, are working to establish methods for medicinal plant production and increased secondary metabolite yield. Medicinal plants are overexploited in their natural habitat, posing a serious threat to the survival of these species. A high proportion of disease casualties also increases the likelihood of plant resource depletion[9].

Because of their high conspicuousness, these drug-producing species must unavoidably be mass-produced at a quick rate. When compared to field matured plant material, cell culture are most commonly employed for potentially increased synthesis of medicinally significant chemicals[10].

When compared to natural production systems, in-vitro culture provides stable manufacturing schemes that are free of environmental constraints, produces uniform quality metabolites that are almost certainly agrochemical-free and ensures abundant concentrations of objective compounds with increased biomass. Callus cultures yield less interfering chemicals than field-grown plants, allowing for more effective biomolecule isolation[11].

Over the years, several strategies have been used to elevate the manufacture of economically significant secondary metabolites on a large scale. Elicitation has proven to be an effective approach for increasing phyto-contents in in-vitro cells to date[12]. Something that can commence plant defense mechanism by producing secondary metabolites, comes under the heading of "elicitor". Elicitors are often divided into two groups: biotic and abiotic[13].

Multiple physiological events are induced by elictors, which eventually prime to the initiation of a cascade of responses, which includes the expression of genes involved in defense, the creation of reactive oxygen species and ultimately the accumulation of vital phytochemicals. Nevertheless, elicitation is greatly influenced by various factors e.g. type, dose, cultural factors and specificity of a particular elicitor[14].

Melatonin is a mammalian indoleamine neurohormone that is produced in the pineal gland of mammals. It is deemed a well-conserved and extensively distributed chemical. According to a study, it aided to assuage stress triggered by environmental and chemicals[15]. There are around 5700 reports regarding the existence of melatonin in varied species of flora and fauna. Melatonin is found in relatively high concentrations in the flowers and leaves of a plethora of medicinal plants and is employed in the treatment of neurological disorders[16].

Melatonin and serotonin ratios are considered to influence a variety of processes i.e. circadian rhythms, light/dark responses, plant morphogenesis in vitro and seasonality. Melatonin is also thought to regulate physiological processes such as free radical detoxification, environmental responses and diurnal responses[16].

The use of ultraviolet (UV) light as an elicitor has acquired a lot of interest because of its powerful and special effects on therapeutic phytochemicals in a variety of medicinal plants[17]. UV-C (190-280 nm), which is used in a variety of fruits, medicinal plants and vegetables, is the most operative type of

UV irradiation for increasing plant metabolites production. UV light causes stress in plants, which stimulates their defensive mechanism and causes them to produce phytoalexin. Plant factories produce these substances as more defensive reactions, such as precious secondary metabolites, defensive antioxidative enzymes and specific changes in the cell wall, to deal with UV-C triggered oxidative damage through the production of reactive oxygen species that act as scavengers[18].

Elicitation procedures are occasionally coupled to improve their efficacy. Melatonin and various light regimes, for example, have increased the antioxidant and anti-inflammatory properties, along with the silymarin production, in S. marianum-derived callus[19]. The interaction of UV-C and melatonin amplified the targeted phenylpropanoid secondary metabolites and antioxidant profile of Ocimum basilcum L. callus in another investigation[20].

There is currently no previous study on Cichorium intybus elicitation by the synergetic impact of melatonin and UV-C radiations. As a result, the current research will look into the effects of melatonin and UV-C radiation on callus cultures of this species. In addition, several in vitro cell free assays under melatonin and UV-C stress were used to examine the antioxidative profile and HPLC was used to quantify the production of these chemicals. As a result, this is the first study to demonstrate the interaction of melatonin and UV-C radiations on the accumulation of pharmacologically important phytochemicals in Cichorium intybus in vitro culture.

2. Materials and Methods

2.1. Seed germination and callogenesis

The water float method was used to test the viability of Cichorium intybus seeds. Seedlings of in vitro cultivated Cichorium intybus plantlets were used to obtain leaf-derived explants. The National Agricultural Research Center (NARC) provided seeds (National Agricultural Research Center, Islamabad, Pakistan). Seeds were placed in sterilized vials containing solid MS medium supplemented with a carbon source (sucrose: 3 percent) and a gelling agent (agar: 0.8 percent) at a pH of 5.6–5.7 before autoclaving after external sterilization with distilled water (3 times), followed by 3 min ethanol (70 percent) and 1 min sodium hypochlorite treatment. All vials were kept in the growth chamber for germination under the same light cycle (16/8 h light/dark) and temperature (25 ± 2 ◦C). Callus cultures were established by employing leaf explants (0.5 cm^2) from 28-day-old in vitro-derived plantlets grown on MS media supplemented with NAA (1 mg/L) and BAP (2 mg/L), as previously optimized by. For callogenesis, the vials were moved to the growth room. Callus was subcultured every four weeks to obtain a good-sized bulk of callus for elicitor treatment.

2.2. Preparation of elicitor

Melatonin (1mM) was made by dissolving it in distilled water and stirring it continuously for 1 hour to ensure appropriate mixing.

2.3 Elicitors treatment on callus culture

2.3.1 UV-C Treatment: The impact of UV-C stress on the growth of callus culture of *Cichorium intybus* was assessed by relating it to control. A lamp of UV-C with radiation intensity of 3 W/m2 (254 nm; Spectro line, model ZQJ-2) and a wavelength of 254 nm was employed. After inoculation on MS media, the callus culture was subjected from a distance of 15 cm to UV-C radiations for varied exposure periods. Calli were exposed to UV-C for six different periods (10, 20, 30, 40, 50 and 60 min). The UV-C lamp was stabilized before exposure. The complete experiment was retained in the growth room for about four weeks. The control Calli was not exposed to UV light. After four weeks, the callus was harvested to assess biomass production and accumulation of therapeutic phytochemicals.

2.3.2 Melatonin Treatment: The impact of melatonin on callus culture of *Cichorium intybus* was assessed by shifting fresh callus (0.5 g) from previously subcultured callus onto MS media, which was optimized with hormones (1NAA mg/L & BAP: 2 mg/L) and various melatonin concentrations (0.1, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L). The control group consisted of media that had been depleted of melatonin. The experiment was retained in the growth chamber for four weeks. The calli were extracted for additional study, after 28 days of inoculation.

2.3.3 Combined Melatonin + UV-C Treatment: Findings related to optimization of callus culture on modified UV-C exposure time indicated maximum growth responses on UV-C (30 mins), in comparison to the rest of the UV-C treatments. Whereas, among various concentrations of melatonin, optimum growth response was seen on 3.0 mg/L. To inspect the combined effects of both elicitors, an optimized media (NAA: 1 mg/L & BAP: 2 mg/L) with melatonin (3 mg/L) as an elicitor was inoculated with callus (0.5 g), keeping this value constant. Calli were inoculated and subjected to UV-C for 10, 20, 30, 40, 50 and 60 minutes. A callus that received neither melatonin nor UV-C stress was employed as a control group. The entire experiment was repeated thrice before being harvested for further phytochemical examination after four weeks.

2.3.4 Preparation of Sample Extracts: After four weeks, callus exposed to elicitor treatment was harvested on Whatman filter paper and allowed for an hour to eliminate excess water content. All callus cultures that had been treated had their fresh weight assessed. After a two-day incubation period at 40°C, the dry weight was measured again. Using a motor and pestle, dry calli was pounded into a fine powder. Protocol for extract preparation proposed by[28] was followed.

2.3.5 Evaluation of Total phenolic content (TPC): The 96-well plate was used to conduct the assay. 20 µl aliquot from the 4 mg/ml stock solution of each extract was added to the corresponding well and then 90 µl of the FC reagent was added. After an incubation period of 30 mins at 37 °C, 90 µl of sodium bicarbonate was added to each well on the plate, respectively. The absorbance of test extracts was measured at 630 nm utilizing a microplate reader. Gallic acid was employed as the positive control in two-fold serial (2.5, 5, 10, 20, 40 g/ml) and methanol as a negative control to generate a calibration curve. The assay's outcomes were given as µg gallic acid equivalent (GAE)/mg of the extract.

2.3.6 Evaluation of Total flavonoid content (TFC): A 96-well plate was employed for the assay's performance. 20 µl aliquot from the test (4mg/ml) was added to each well carefully, then subsequently 10 µl of potassium acetate, 160 µl of distilled water and 10 µl of aluminum chloride were added. After 30 min of incubation at room temperature, a microplate reader was used to check the absorbance of the test extract at 415nm wavelength. Various doses of quercetin (2.5, 5, 10, 20 and 40 µg/ml) were employed as a positive control of the assay to generate a calibration curve, whereas methanol was used as a negative

control. The findings were reported as µg quercetin equivalent (QE)/mg of extract.

2.3.7 Evaluation of DPPH Free radical scavenging assay: The compounds' antioxidant capacity was tested using the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). A 10 µL of plant extract (4 mg/ml methanol) and 190 µL of DPPH solution (9.6 mg/100 methanol) were combined in order to achieve the reaction mixture's final concentrations of 200 µg/ml. The absorbance at 517 nm was measured using a microplate reader after 30 minutes of incubation at 37°C. To calculate the percentage of free radical scavenging activity, the study uses the formula below.

$$\text{Free Radical Scavenging Activity} = 1 - As/Ac * 100$$

Where, As and Ac, respectively, stand for the absorbance of the sample and the adverse control. Ascorbic acid was utilized as a positive control in the test, which was carried out in three iterations.

2.3.8 Evaluation of Total reducing power (TRP): The estimation of the reducing potential of extracts was done using a potassium ferricyanide colorimetric test. 250 µl of potassium ferricyanide (1% w/v in dH_2O) and 200 µL of phosphate buffer (0.2 M, pH 6.6) were added to 100 µL aliquot of every test extract 4 mg/ml methanol) before incubation at 50 °C for 20 minutes in a water bath. After adding 200 µL of trichloroacetic acid (10% w/v in distilled water) to every chemical test, the mixture was then centrifuged at 3000 rpm for 10 mins at room temperature. The supernatant of 150 ml was then added to 50 ml of 0.1% w/v $FeCl_3$ in distilled water in a 96-well plate. At 630 nm, the absorbance was then measured. Ascorbic acid (1 mg/mL) was used as the positive control while methanol was used as a negative control. Each sample's reducing power was given as the amount of ascorbic acid equivalent per mg of the test sample (µg AAE/mg).

2.3.9 Evaluation of Total antioxidant capacity (TAC): Using a phosphomolybdenum-based assay, the test sample's overall antioxidant capacity was calculated. 100 µL of test extract (4 mg/mL methanol) and 900 µL of TAC reagent (0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM ammonium molybdate) were mixed. methanol acted as a negative control. After that, the mixture was then incubated in a water bath at 95 °C for 90 min. After cooling, the absorbance of both test and standard solutions was then measured at 630 nm. The antioxidant potential was represented as µg AAE/mg of DW.

3. High-performance liquid chromatography analysis

Shimadzu HPLC system with controller (SCL-10AVP), degasser (DGU-14A), low-pressure mixer (FCV-10ALVP), pump (LC-10AT VP) in combination with 3D-PDA detector (SPD-M10A VP) and a software (LC solution) was employed to perform the RP-HPLC based analysis of polyphenols (total 13). Agilent Zorbax C8 (5 m; 4.6 250 mm) column was utilized. The binary gradient system was used to detect the polyphenols, employing mobile phase A (methanol: water: acetic acid: acetonitrile) in a ratio of (10:85:1:5) and mobile phase B (acetonitrile: methanol: acetic acid) in a ratio (40: 60: 1). At 1.2 ml/min, the flow rate was changed. 50 l was the injection volume. Furthermore, the gradient volume of the mobile phase B was 0-75 percent in the first 0-30 min, 75-100 percent in the next 0-31 min, 100 percent in the next 0-35 min and 0-36 min. Before injecting the new sample, the column was reconditioned for 10 minutes. Each standard had a concentration of 50 g/ml in methanol and the sample was made by dissolving 100 l in 1 ml of methanol. By comparing the sample's UV absorption spectrum and retention time to the standard, polyphenol concentrations were determined.

4. Statistical Analysis

All the above-mentioned experiments were performed in triplicates in a synchronized way and were repeated twice. To formalize statistical analysis, the mean value was determined in each experiment and the standard error was calculated (Microsoft Excel Program). All figures were created using the Origin program (8.5). The resultant data was reported as a mean standard deviation.

5. Results and Discussion

5.1. Synergistic Effects of UV-C and Melatonin on Biomass Production: Plants are capable of sensing and processing specific signals from their biotic & abiotic environments in order to achieve optimal growth and development[21]. Melatonin, chemically regarded as N-acetyl-5-ethoxytryptamine is a pleiotropic substance in living creatures that has a wide range of cellular and physiological functions[22]. Light, on the other hand, plays an important function in plant growth and development. The use of various light sources as an elicitor to enhance phytochemicals in pharmaceutical plants is receiving a plethora of attention. One of them is employing UV-C radiations, which have a function in governing the prompt expression of genes responsible for cell development and secondary metabolism[22].

The impacts of melatonin and UV-C radiation on the progression of Cichorium intybus callus culture were evaluated for optimal growth and biomass accumulation at numerous concentrations of elicitors **(Figure1 A and B)**. The findings illustrated enhancement in biomass production in Cichorium intybus callus culture under the impact of elicitor. Nevertheless, maximum biomass accumulation (fresh weight (FW): 269 g/L and dry weight (DW): (16.0g/L) was detected for UV-C (30 mins), while UV-C (60 mins) presented an insignificant response in callus culture, as obvious from the minimum biomass yield (FW: 140g/L and DW: 8.9 g/L). Notable reductions in biomass could be asserted due to irretrievable cellular damage, provoked by an extended period of radiation exposure, causing cell death. The advanced biomass accumulation could also be ensured by mutation induction in plant cells caused by UV-C exposure[17].

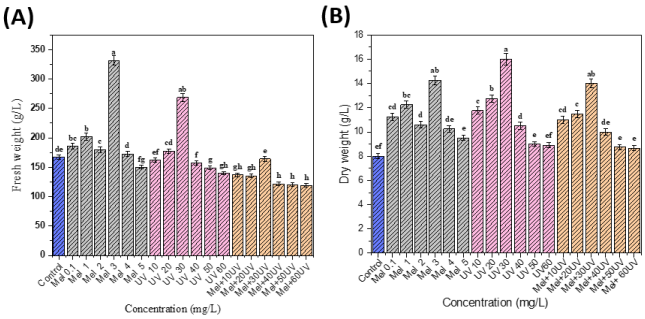

Figure 1: A) Biomass accumulation (fresh weight) under various concentrations of melatonin, UV-C, and Mel+UV-C, under optimized hormonal conditions and B) biomass accumulation (dry weight) under various concentrations of melatonin, UV-C, and Mel+UV-C, under optimized hormonal conditions. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically significant differences between the various experimental conditions ($p < 0.05$).

Under varied photoperiod regimes, UV-C treatments for 10-40 mins augmented proliferation in callus, however, UV-C doses for prolonged periods abridged cellular growth. The reduction in biomass accumulation with advanced UV-C treatments might be due to unalterable detrimental effects on cells, which could increase cellular death in plant-based cultures[23] and induction of oxidative stress[24] Photo-inhibition of the photosynthetic system in plants is also caused by prolonged exposure to bright light. Extreme light exposure causes an imbalance in photosynthetic O2 and CO2 fixation, chloroplast inhibition and lose fluorescence at PS II, as previously mentioned. Photo inhibition is usually caused by multifactorial light stress, which diverts the attention away from the QB proteins, which catalyzes the conversion of primary quencher (QA) to substantial PQ assembly through electron transfer[25].

Similarly, when Lepedium satvium cells were exposed to UV-C over long periods, cell growth and biomass proliferation were reduced[26]. When Linum usitatissimum cell cultures were exposed to UV-C radiations for 20 mins, an increase in biomass production was found[18] Moreover,[20] found that UV-C (20 min) accumulated the most biomass, but UV-C (60 min) had the least response in callus growth. Several tissue culture investigations of medicinal plants exposed to UV-C can be used to expand and verify our findings[27,22].

The application of melatonin as an elicitor at various concentrations yielded significant results. At 3 mg/L melatonin, supreme biomass (FW: 331.66 g/L and DW: 14.25 g/L) was identified, while minimum biomass (FW: 150.0 mg/L and DW: 9.4 g/L) was identified at melatonin 5 mg/L, signifying those minimum concentrations of melatonin have profoundly favorable effects on growth parameters of C. intybus callus culture **(Figure 2, Figure 3 and Figure 4)**. Whereas higher concentrations of melatonin inhibited the accumulation of biomass[28].

Figure 2: Callus morphology of Cichorium intybus (a) Control (b) Melatonin 0.1mg/L (c) Melatonin 1mg/L (d) Melatonin 2mg/L (e) Melatonin 3mg/L (f) Melatonin 4mg/L (g) Melatonin 5mg/L.

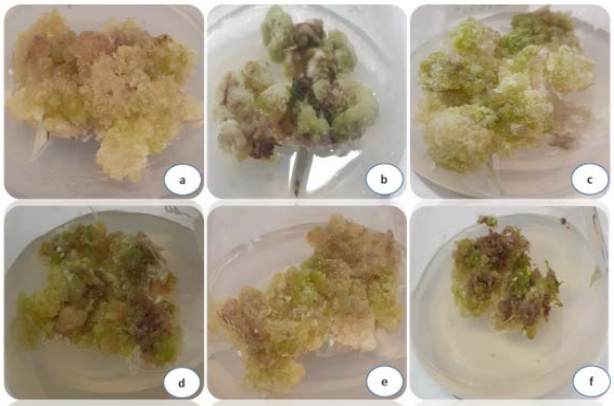

Figure 3: Callus morphology of Cichorium intybus (a) UV-C 10 (b) UV-C 20 (c) UV-C 30 (d) UV-40 (e) UV-C 50 (f) UV-C 60.

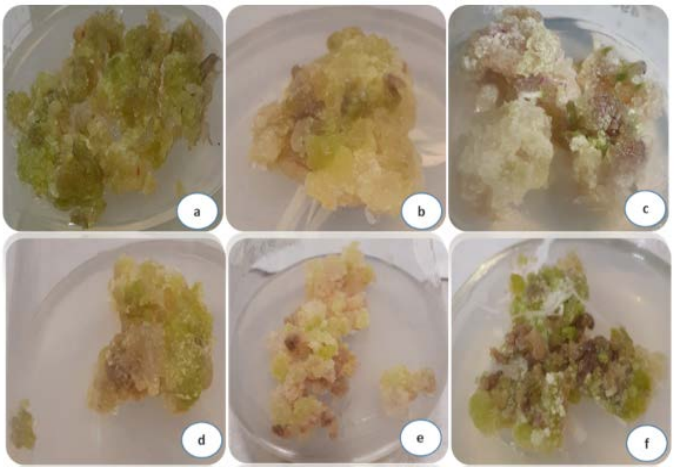

Figure 4: Callus morphology Cichorium intybus (a) Mel+10 UV-C (b) Mel+20 UV-C (c) Mel+30 UV-C (d) Mel+40 UV-C (e) Mel+50 UV-C (f) Mel+60 UV-C.

The greater concentration of melatonin may have resulted in the creation of reactive oxygen species (ROS), instigating cellular death and henceforth limiting cellular growth and proliferation[29]. The current findings are consistent with the previously established inhibitory effects of greater melatonin doses on biomass accumulation[20,28,30].

6. Influence of Melatonin, UV-C radiations and their combined effect on total phenolic and flavonoid production

To combat various biotic and abiotic challenges, plants have a variety of defensive chemical compounds that are released under stress. These encompass a wide range of secondary metabolites with low molecular weight, such as flavonoids and phenolics[31]. The present research was intended to scrutinize the impacts of elicitors on secondary metabolite production in *C. intybus* callus culture. Phenolic and flavonoid composition of *C. intybus* callus cultures have been reported to increase when treated with melatonin, UV-C and their combination. However, Mel + UV-C combined treatment showed higher phenolic and flavonoid production as compared to control and other applied treatments as illustrated in **(Figure 5, Figure 6, Figure 7 and Figure 8)**.

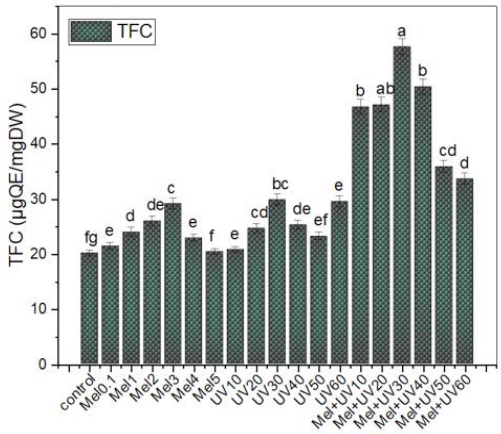

Figure 5: TFC of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically noteworthy differences amid the various experimental conditions ($p< 0.05$).

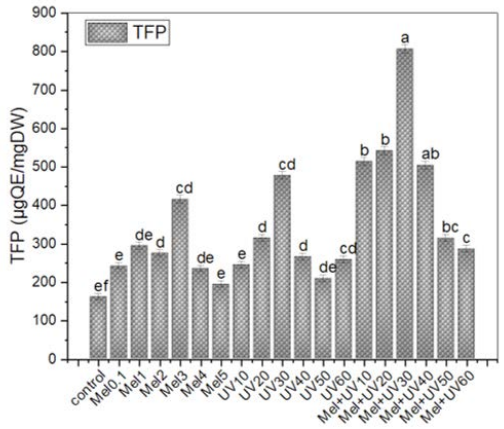

Figure 6: TFP of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically noteworthy differences amid the various experimental conditions ($p< 0.05$).

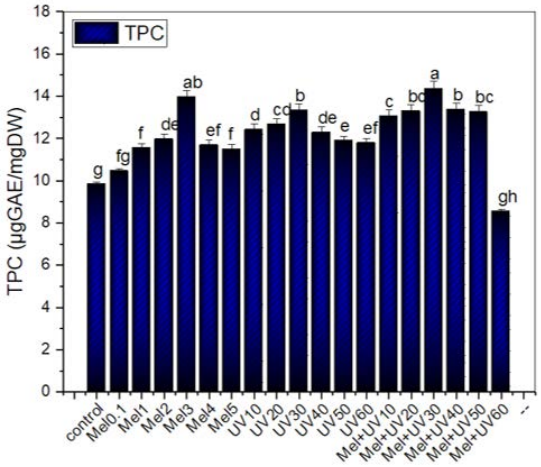

Figure 7: TPC of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically noteworthy differences amid the various experimental conditions ($p< 0.05$).

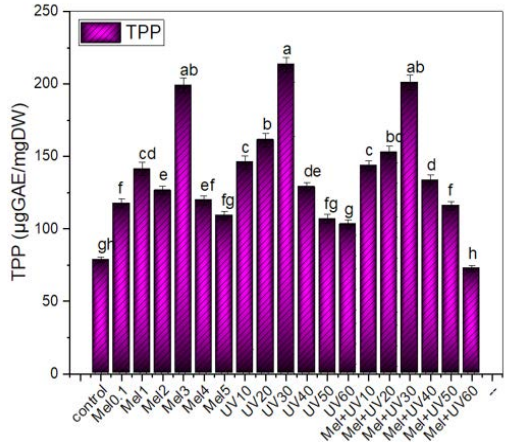

Figure 8: TPP of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically noteworthy differences amid the various experimental conditions ($p< 0.05$)

Maximum TPC (total phenolic content): 14.36 µgGAE/mg DW and TPP (total phenolic production): 201.4 µgGAE/mg DW) and TFC (total flavonoid content): 57.6 µgQE/mg and TFP (total flavonoid production): 806.4 µg/mg DW was identified in calli exposed to Mel (3mg/L) + UV-C (30 mins), whereas the minimum values of TPC (8.53 µgGAE/mg DW), TPP (72.50 µgGAE/mg DW), TFC (33.63 µgQE/mg DW) and TFP (285.85 µgQE/mg DW) were observed at a Mel (3mg/L) + UV- C (60 mins) exposure, as compared to the rest of all Mel+ UV-C treatments.

The application of several melatonin concentrations on the callus cultures of *C. intybus* was also inspected for polyphenols production. Mel (3 mg/L) significantly improved TPC (13.96 µgGAE/mg DW) and TPP (198.93 µgGAE/mg DW) and TFC (29.14 µgQE/mg DW) and TFP (415.24 µgQE/mg DW) accumulation compared to control (TPC: 9.83 µgGAE/mg DW, TPP: 78.64 µgGAE/mg DW, TFC: 20.17 µgQE/mg DW, TFP: 161.36 µgQE/mg DW). All melatonin treatments displayed a significant impact on the secondary metabolites accumulation in the callus culture of chicory. Melatonin plays a critical role in the expression of several genes that monitors the production of secondary metabolites and adds to the defense mechanisms used by plants to deal with stress[32]. Its interaction with catalases may increase H□O□ accumulation and ROS formation, leading to a rise in production that further activates secondary metabolic pathways.

Furthermore, maximum TPC: 13.33 µgGAE/mg and TPP: 213.28 µgGAE/mg and TFC: 29.85 µgQE/mg DW and TFP: 477.6 µgQE/mg DW accumulation was identified in calli which were treated with UV-C (30 mins), which is higher than control (TPC: 9.83 µgGAE/mg, TPP: 78.64 µgGAE/mg DW, TFC: 20.17 µgQE/mg DW, TFP: 161.36 µgQE/mg DW). There are some mechanistic explanations of how UV-C light affects the initiation and enrichment of plants' secondary metabolism that can be found in the literature. Some studies suggest that the galvanization of crucial enzymes like chalcone synthase and L-phenylalanine ammonia-lyase (PAL) can lead to a boost of the secondary metabolism when exposed to UV-C radiation. The primary steps in the biosynthesis pathways of several phenolics and flavonoids are catalyzed by such enzymes. The UV-C elicitation significantly increases the accumulation of flavonoids in response[33]. Due to their strong UV protection and antioxidant properties, flavonoids, amid all polyphenols, contribute towards an overriding role in plants' defense against photodamage. According to[33], among all phenolic compounds, UV-C treatments nearly endorsed and elevated the production of flavonoids. Additionally,[34] discovered that UV-C radiation upregulates FLS (flavonol synthase) and CHS (chalcone synthase), which are essential for the formation of flavonoids. At larger concentrations and varying light exposures, the accumulation of phenolics and flavonoids was inhibited. High levels of ROS production, disruption of cellular membranes, photo-inhibition, deficiencies in the metabolism of lipids, DNA damage and degradation of photosystem-II are some of the underlying molecular causes[30].

These current research findings are compatible with a plethora of literature reports on the possible impact of UV-C and melatonin treatments on innumerable medicinal plants[22,19,26,28,20].

7. Antioxidant assays (DPPH, TAC, TRP)

All plant species responded to stress by abruptly changing

their specific metabolic processes, which leads to the creation of ROS, which can harm the plant's essential components. In response to oxidative stress, plants also create several chemicals, including phenolic and flavonoids[35]. Herein, the antioxidant activities of the callus culture of Cichorium intybus subjected to various melatonin, UV-C radiations and combination of UV-C and melatonin regimes were examined one after the other using assays including DPPH, TAC and TRP. The DPPH test is an extensively employed method for evaluating the antioxidant potential of products made from plant cells because of its high sensitivity, mild clarity and practicability. DPPH (2,2-diphényl-1-picrylhydrazyl) is thought to be able to detect the existence of both ET (electron transfer) and HAT (hydrogen atom transfer) founded mechanisms for antioxidant potential[36]. DPPH basically measures the free radical scavenging activity that is represented as a percentage. Mel (3mg/L) + UV-C (30 mins) combinedly, UV-C (30 mins) and Mel (3mg/L) treatments, respectively, resulted in higher DPPH activities (92.1 %, 90.2 % and 89.7 %) when compared to the control 76.9 %; **(Figure 9)**.

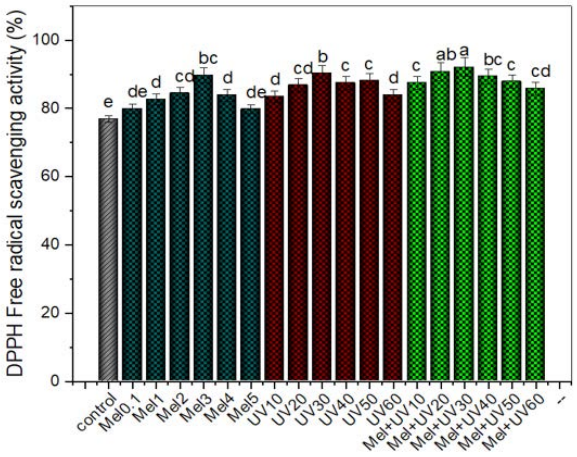

Figure 9: DPPH of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically significant differences between the numerous experimental conditions ($p < 0.05$).

These current results are consistent with those of[28], who found that Fagonia indica when treated with melatonin (10mg/L), revealed robust free radical scavenging activity. According to[18], cell cultures of Linum usitatissimum displayed the highest DPPH activity when it was exposed to UV-C (10 mins). Likewise,[20] reported the highest DPPH activity i.e. 93.8%, when callus cultures of Purple Basil were exposed to UV-C (50 mins).

The principal reason for melatonin's antioxidant effect is the activation of antioxidant enzymes. These enzymes serve a critical role in preventing oxidative damage to plants by improving the efficacy of the electron transport chain present in mitochondria. These *in vitro* antioxidant assay results have demonstrated an association amid the accumulation of phenolics and the antioxidants of callus extracts. The elevated antioxidant potential of callus extracts is likely due to an increase in the synthesis of secondary metabolites in reaction to elicitors, as previous investigations have noted similar events[37].

Moreover, highest TRP and TAC was detected in callus culture treated with Mel (3 mg/L) i.e. (212.8 µg AAE/mg; 146.7 µg AAE/mg) and UV-C (30 mins) i.e. (186.0 µg AAE/mg; 213.0 µg AAE/mg) respectively **(Figure 10 and Figure 11)**. And this could be aligned with higher secondary metabolite production.

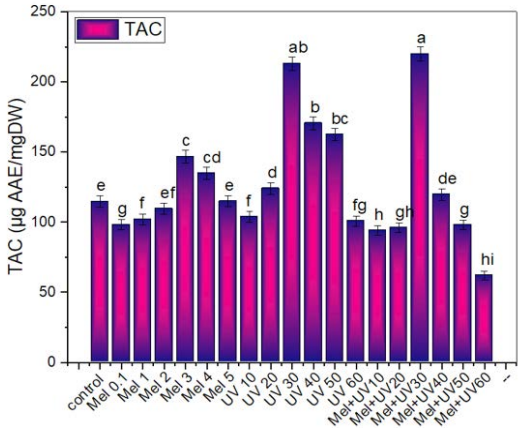

Figure 10: TAC of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically important differences between the numerous conditions ($p < 0.05$).

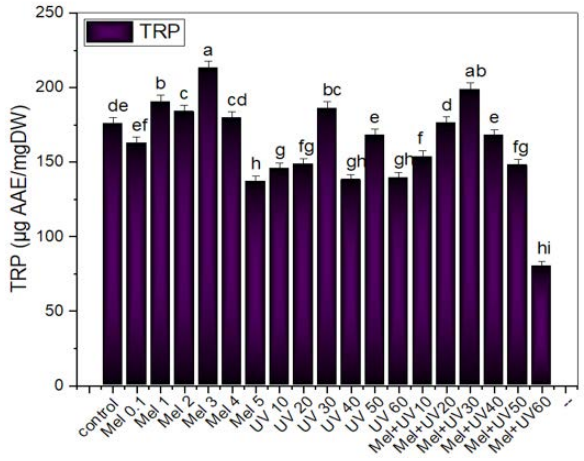

Figure 11: TAC of C. intybus callus cultures in response to different concentration of Melatonin, UV and their combined effect. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters show the statistically important differences between the numerous experimental conditions ($p < 0.05$).

8. Quantification of polyphenols by HPLC

Plants commonly produce phytochemicals, which serve as the plant's active components, when conditions are unfavorable. These phytochemicals are widespread across the entire plant kingdom and are indispensable for the existence, development and defense of plants[20]. When in vitro derived cells produce few secondary metabolites, several conditions call for higher output. This is probably due to the inhibition caused by enzymes or the transitory stationary phase[38].

Elicitors may serve as a stimulus for increased production of phytochemicals with significant economic value. Such metabolites can be accurately and precisely computed by employing HPLC. In the present work, a total of 13 phenolic compounds from Cichorium intybus extracts subjected to UV-C and melatonin were quantified. In comparison to melatonin and UV alone, melatonin and UV-C combined effect produced a

higher level of phytochemicals. Supreme secondary metabolites accumulation (10.993 µg/mg) was documented in cultures treated with a combination of Mel 3mg/L + UV-C 10 mins which are more than three folds higher than control (3.41 µg/mg D.W), followed by Mel 3mg/L + UV-C 20 mins (10.24 µg/mg) (Table 4.1). Likewise, UV-C (40mins) greatly heightened the accumulation of phytochemicals (6.93 µg/mg), followed by UV-C (30mins) i.e. (4.57 µg/mg) as compared to control.

Plants when exposed to UV radiation stress, trigger the production of vital plant phytochemicals through their defense mechanism. But it also causes the production of ROS, which quickly interacts with other macromolecules including proteins and lipids and damages the mitochondrial membrane[39].

Melatonin vigorously lessens the deleterious effect of UV-C radiations[40] by decreasing the leakage of electrons present in mitochondria and by invigorating the actions of the respiratory chain complexes damage[39]. Similarly, in the present research, the impact is considerable when treated with UV-C and melatonin synergistically; this is because of the success of melatonin to counter-act the negative impact caused by UV and their defense mechanism capable of producing other compounds. In C. intybus callus culture, the application of these elicitors may be a useful method for eliciting secondary metabolites.

Moreover, melatonin 3 mg/L also considerably enhanced phytochemical accumulation (6.35 µg/mg), which is almost two folds higher than control followed by melatonin 1 mg/L (5.15 µg/mg).

Cinnamic acid was found higher (3.878 µg/mg DW) in cultures treated with a combination of melatonin 3mg/L + UV-C 10 mins which is nearly six folds greater than control (0.643µg/mg DW), followed by 3mg/L + UV-C 10 mins (3.22 µg/mg DW). A similar trend was recorded for Luteolin biosynthesis.

Majority of secondary metabolites e.g. vanillic-acid (0.07 µg/mg DW), rutin (0.446 µg/mg DW), gallic-acid (0.725 µg/mg DW), catechin (0.915 µg/mg DW), entisic-acid (0.186 µg/mg DW), cinnamic-acid (1.95 µg/mg DW), luteolin (1.12 µg/mg DW) and myricetin (0.52 µg/mg DW) showed higher accumulation at melatonin 3 mg/L. Coumaric-acid (0.30 µg/mg DW) and caffeic-acid (0.15 µg/mg DW), accumulation was higher at melatonin 0.1 mg/L. All of these compounds have an extensive range of therapeutic qualities, including antibacterial, anticancer, anti-inflammatory, antioxidant and hepatoprotective actions according to recent research.

In the case of UV-C treatment majority of secondary metabolites displayed enhanced accumulation at UV-C (40 mins) followed by UV-C (30 mins) and UV-C (20 mins). *Catechins* **are well known to scavenge free radicals and chelate redox-active metals showed the highest accumulation (١،١٧ µg/mg D.W)** at UV-C (10 mins) as compared to all other treatments, which is about 5 folds higher than control (1.17 µg/mg; Table 4.1).

Plants are vulnerable to UV-C irradiation's harmful effects, which include the direct destruction of plastoquinone (PQ) in chloroplasts, disruption of mitochondrial function, DNA integrity, creation of ROS and the synthesis of peroxyl radicals. Due to their UV and antioxidative characteristics, a variety of phenolics and flavonoids contribute to minimizing the photodamage caused by UV-C radiations[41]. Additionally, it has been found that the UV triggers upregulation of the FLS & CHS enzymes, stimulation of many genes and phytoalexins, as well as the decoding of CHS, PAL, ANS and stilbene synthase, which are enzymes involved in the biosynthesis of phenolics[33]. Similar to other elicitors, UV-C radiations are generally thought to increase oxidative stress by producing ROS. ROS can have harmful effects on cell components. Based on their redox condition, ROS network physiological processes prior to damage by signaling. The ROS involved in the activation and control of metabolic processes is concomitant with the creation of precious secondary metabolites[42].

The current findings are consistent with the previously established reports[28,20,30]. Studies have shown that polyphenols can reduce the activity of oncogenes and the damaging effects of oxidative stress, **(Table 1)** which explains their function as anticancer, anti-inflammatory and neuroprotective agents[43].

Table 1: RP-HPLC based quantification of polyphenols in callus culture of *Cichorium intybus.*

Treatment	Conc.	Polyphenols quantified (µg/mg Dry weight)												
		Vanillic acid	Rutin	Gallic acid	Catechin	Syringic acid	Coumeric acid	Emodine	Gentisic acid	Caffeic acid	Ferulic acid	Cinnemic acid	Luteolin	Myricetin
Control		0.108 ± 0.004	0.265 ± 0.02	0.29 ± 0.016	0.25 ± 0.032	0.119 ± 0.006	0.024 ± 0.005	0.186 ± 0.015	0.159 ± 0.012	0.189 ± 0.019	0.027 ± 0.001	0.643 ± 0.11	0.937 ± 0.13	0.154 ± 0.02
Melatonin	0.1	0.025 ± 0.002	0.107 ± 0.01	Nd	0.231 ± 0.031	0.103 ± 0.006	0.303 ± 0.0019	0.319 ± 0.018	0.15 ± 0.011	0.158 ± 0.018	0.037 ± 0.002	1.072 ± 0.13	0.542 ± 0.1	0.064 ± 0.01
	1	0.069 ± 0.0034	0.325 ± 0.02	0.183 ± 0.01	0.464 ± 0.034	0.076 ± 0.005	0.066 ± 0.008	0.461 ± 0.019	0.226 ± 0.016	0.14 ± 0.016	0.065 ± 0.004	1.34 ± 0.14	1.21 ± 0.15	0.501 ± 0.04
	2	0.032 ± 0.0021	0.047 ± 0.01	0.65 ± 0.03	0.224 ± 0.031	0.1 ± 0.006	0.021 ± 0.005	0.136 ± 0.014	0.021 ± 0.007	0.063 ± 0.012	0.029 ± 0.001	0.634 ± 0.1	0.964 ± 0.13	0.161 ± 0.02
	3	0.071 ± 0.0035	0.446 ± 0.02	0.725 ± 0.04	0.915 ± 0.041	0.101 ± 0.006	0.088 ± 0.01	0.14 ± 0.014	0.186 ± 0.014	0.005 ± 0.002	0.06 ± 0.0037	1.95 ± 0.15	1.121 ± 0.14	0.525 ± 0.05
	4	0.018 ± 0.0016	0.258 ± 0.02	0.165 ± 0.01	0.748 ± 0.038	0.06 ± 0.004	0.022 ± 0.005	0.297 ± 0.017	0.101 ± 0.01	0.035 ± 0.008	0.032 ± 0.002	1.039 ± 0.13	0.436 ± 0.09	0.233 ± 0.03
	5	0.057 ± 0.003	0.102± 0.013	0.181± 0.012	0.214 ± 0.031	0.141 ± 0.007	0.258 ± 0.018	0.127 ± 0.013	0.102 ± 0.01	0.042 ± 0.009	0.121 ± 0.008	0.187 ± 0.05	1.08 ± 0.14	0.233 ± 0.03
UV-C	10	0.056 ± 0.0029	0.183 ±0.014	0.833 ± 0.05	1.178 ± 0.046	0.05 ± 0.003	0.087 ± 0.009	0.195 ± 0.015	0.159 ± 0.012	0.088 ± 0.013	0.033 ± 0.002	0.829 ± 0.11	0.676 ± 0.11	0.322 ± 0.04
	20	0.063 ± 0.0032	0.339 ± 0.02	0.227 ±0.013	1.052 ± 0.044	0.048 ± 0.003	0.102 ± 0.01	0.361 ± 0.018	0.172 ± 0.013	0.086 ± 0.013	0.032 ± 0.002	0.947 ± 0.12	0.743 ± 0.12	0.216 ± 0.03
	30	0.015 ± 0.0015	0.101 ±0.013	0.335 ±0.017	0.71 ± 0.038	0.072 ± 0.004	0.001 ± 0.001	0.258 ± 0.016	0.213 ± 0.015	0.054 ± 0.011	0.058 ±0.0037	1.986 ± 0.15	0.718 ± 0.12	0.007 ± 0.009

	40	0.035 ± 0.0022	0.064 ± 0.01	0.244 ±0.014	0.825 ± 0.039	0.068 ± 0.004	0.03 ± 0.006	0.674 ± 0.02	0.202 ± 0.014	0.236 ± 0.02	0.042 ± 0.003	1.009 ± 0.13	3.334 ± 0.2	0.112 ± 0.01
	50	0.02 ± 0.0018	0.187 ±0.015	0.136 ±0.011	0.336 ± 0.033	0.016 ± 0.002	0.016 ± 0.005	0.147 ± 0.014	0.094 ± 0.009	0.044 ± 0.009	0.109 ± 0.006	0.574 ± 0.1	0.345 ± 0.08	0.118 ± 0.01
	60	0.033 ± 0.0021	0.294 ± 0.02	0.344 ±0.017	0.001 ± 0.01	0.054 ± 0.003	0.027 ± 0.006	0.081 ± 0.012	0.153 ± 0.011	0.05 ± 0.01	0.034 ± 0.002	0.632 ± 0.11	0.082 ± 0.04	0.153 ± 0.02
Mel 3 + UV-C	10	0.071 ± 0.0035	0.844 ±0.035	0.373 ± 0.018	0.782 ±0.039	0.231 ± 0.001	0.095 ± 0.01	0.194 ± 0.015	0.395 ± 0.02	0.179 ± 0.018	0.133 ± 0.01	3.878 ± 0.2	2.526 ± 0.17	1.282 ± 0.08
	20	0.063 ± 0.0032	0.693 ± 0.03	0.435 ± 0.02	0.547 ± 0.036	0.22 ± 0.009	0.081 ± 0.009	0.108 ± 0.013	0.318 ± 0.019	0.136 ± 0.016	0.127 ± 0.009	3.221 ± 0.18	3.244 ± 0.19	1.046 ± 0.07
	30	0.056 ± 0.0029	0.694 ± 0.03	0.347 ±0.017	0.483 ± 0.035	0.178 ± 0.008	0.199 ± 0.017	0.02 ± 0.003	0.297 ± 0.018	0.146 ± 0.017	0.121 ± 0.008	3.27 ± 0.19	2.701 ± 0.18	0.734 ± 0.06
	40	0.075 ± 0.0038	0.51 ± 0.026	0.272 ± 0.015	0.415 ± 0.034	0.042 ± 0.0025	0.072 ± 0.008	0.125 ± 0.014	0.131 ± 0.01	0.126 ± 0.015	0.079 ± 0.005	2.168 ± 0.16	1.589 ± 0.16	0.403 ± 0.04
	50	0.045 ± 0.0025	0.484 ±0.025	0.29 ± 0.016	0.301 ± 0.033	0.182 ± 0.009	0.051 ± 0.007	0.117 ± 0.013	0.253 ± 0.017	0.098 ± 0.014	0.077 ± 0.005	2.216 ± 0.17	0.909 ± 0.13	0.405 ± 0.04
	60	0.064 ± 0.0033	0.337 ± 0.02	0.417 ± 0.02	0.11 ± 0.024	0.031 ± 0.002	0.025 ± 0.006	0.117 ± 0.013	0.123 ± 0.01	0.07 ± 0.012	0.031 ± 0.002	0.983 ± 0.13	1.064 ± 0.14	0.211 ± 0.03

9. Conclusion

In the present investigation, callus cultures of C. intybus under the combined effects of UV-C and melatonin revealed an evident pattern. Melatonin and UV-C considerably impacted biomass production and enhanced the accumulation of polyphenols. Overall, when compared to their combined effect, melatonin and UV-C separately generated the highest biomass output. However, the highest levels of TPC, TFC and antioxidant activity were observed in cultures that had been exposed for 30 minutes to UV-C together with 3 mg/L of melatonin. Melatonin (5mg and UV-C (60 mins) were reported with minimum sound effects on the enhancement of biomass and phytochemicals. The recent research be responsible for a comprehensive understanding of the methodology for the production of biomass, phytochemicals and antioxidant enzymes in C. intybus in vitro cultures under melatonin and UV-C treatment. To improve the synthesis of secondary metabolites in C. intybus at commercial scales and to better understand the impact of UV-C and melatonin, several high throughput techniques must be used.

10. Author Contributions

M.J.H performed plant tissue culture experiments, TPC, TFC, DPPH, TAC and TRP activities. Y.A assisted M.J.H with major experiments and other biochemical assays. S.B and A.R assisted M.J.H in sample preparations. G.Z assisted MJH with TPC, TFC and DPPH. H.A, A.Z and I.H performed extraction for HPLC and conducted HPLC. B.H.A conceived the idea, supervised the research and reviewed the paper critically.

11. Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

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