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Towards the Biopharmaceutical Potential of Melatonin-treated Callus Cultures of Isodon rugosus

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Abstract

Medicinal plants are a source of valuable natural bioactive compounds. To enhance the production of these metabolites, elicitation provides an excellent approach. Herein, the influence of melatonin was investigated on callus cultures derived from pharmacologically important plant Isodon rogusus. In the current data at the 5 µM concentration of stem-derived callus cultures, we observed a significant linear correlation between two parameters (for example, TPC 230.58 ± 46.45 mg/g DW and TFC 44.68 ± 15. 24 mg/g DW). Moreover, we also observed some physiological and morphological changes in callus cultures upon treatment with melatonin. Being a natural antioxidant property of melatonin, it can prevent the degradation of red blood cells, which in turn protects red blood cells from oxidative damage at a concentration of 5 µM and is considered a safe dose. HPLC screening analysis revealed the presence of several important metabolites, such as rosmarinic acid, caffeine acid, plectranthoic acid, betulinic acid and linoleic acid, which showed inhibitory activity against alpha-amylase (42.50 %) and cholinesterase enzyme (AChE 51.364 ± 1.11 % and BChE 40.188 ± 0.47 %. The enzymatic activities (SOD and POD) increase the antioxidant defense system of I. rugosus by scavenging the content of H_2O_2 and OH^- radicals. From a practical point of view, this study was conducted to provide sufficient experimental evidence to demonstrate that this decrease in antioxidant activities and cytotoxicity effect may be due to a reduced content of antioxidant active ingredients i.e., TPC and TFC resulting from externally applied melatonin.

Keywords: I. Rugosus; Melatonin; Hemolytic Activity; Hepg2 Cell Line; A-Amylase; Cholinesterase; Antioxidant Enzymes

Introduction

Plants globally have been adopted as pharmaceutics beside food source since ancient times mainly because of their analeptic health attributes (Khan et al 2019 a). Different cultural and traditional practices have squandered more than 70 thousand plant species for medicinal and feeding purposes (Abbasi et al, 2019). In Pakistan, around 34 % of all plants are reported as medicinal species where 400 to 600 among 6000 flowering plants possess enormous phyto-medicinal significance (Khan et al, 2007). The plants belong to labiateae or lamiaceae family, are utilized for several therapeutic objectives including anthelmintic, antibacterial and analgesic (Naghibi et al, 2005). I. rugosus (Wall. ex Benth), is a renown specie of this family that is locally famous as sperkai, boi or phaypush and exist in Northern areas of Pakistan. Morphologically, the plant is a branched shrub, aromatic and 1 to 5 feet in height with ovate leaves and dense hairs spanned over ventral side. The flowering followed by ripening of seeds occurs during July-October (Zeb et al, 20014). Traditionally, it has been exploited for multitude of therapeutic purposes such as antiseptic, anti-diarrheal, anti-cancer, hypoglycaemic, anti-microbial, anti-inflammatory and anti-diabetic (Ajmal et al., 2012, Janbaz et al., 2014, Sher et al., 2011). Traditionally, it has been exploited for multitude of therapeutic purposes such as antiseptic, anti-diarrheal, anti-cancer, hypoglycaemic, anti-microbial, anti-inflammatory and anti-diabetic (Ams et al, 1990; Zeb et al, 2014, Abbasi et al 2019; Azmi et al, 2006). The drops of its leaf derived extract are used as ear pain reliever while the same extract is known effective in treatment of scabies (H, Sher et al, 2011). For instance, the extract is also used in hypertension, rheumatism, fevers and toothache. It has also been recently investigated for its anticholinestrase and antioxidant potential by Zeb et al. (2014). Medicinal plants are thought to be constantly confronted with biotic and abiotic environmental stresses, in order to develop a defense system against pathogenic attacks, signaling, stimulatory and inhibitory effects on enzymes, plant metabolism and growth in higher plants (Bennett and Wallsgrove 1994). Structural-based classification of polyphenolic compounds has been characterized into several groups such as phenolic, flavonoids, terpenoids, steroids, and alkaloids (Harborne 1999).

Alzheimer's disease (AD) is a chronic neurodegenerative disease that has affected about 20 million people over the world. It is distinguished via mainly with neural degradation resulting in malfunction among neurotransmitters that leads to slant the acetylcholine (AChE) levels and failure of cognitive functions (Kamal et al, 2006; Zeb et al, 2014 b). Cholinesterase hydrolyzes acetylcholine into choline and acetic acid (Topal et al. 2016). Gülçin et al. (2016). The application of inhibitors for two key enzymes; acetylcholinesterase (AChE) and butyrylcholinestrase (BChE), responsible for neurotransmitters degradation is a conceivable treatment to restore ACh levels and reinstate neurotransmission (Giacobini, E. (1998; Zeb et al, 2014 b). Various types of synthetic drugs employed for its treatment express severe side effects like hepato-toxicity, gastrointestinal issues that emphasize further on to discover best alternative natural inhibitors (Oh et al, 2004; Schulz, et al, 2003). In addition to the damages to key macromolecules like DNA, RNA, proteins and enzymes induced by excessive reactive oxygen species (ROS), the oxidative stress has been disclosed also in occurrence of neurodegenerative syndromes including AD (Zhu et al, 2004; Lobo et al, 2010). Due to the toxic side effects of synthetic drugs/antioxidants, herbal remedies are usually adopted as anti-oxidative phytonutrients that express protective health attributes (Barlow et al , 1990; Zeb et al, 2014 b).

The tremendous and historical adaptation of plants as medicinal is due to the range of significant phytochemicals they comprise; mainly the secondary metabolites. Secondary metabolites in plants arise from primary compounds and are widely employed in pharmaceutics, cosmetics, nutraceuticals and other industrial purposes (Seigler 1998; Yang et al. 2018). Structure based classification of polyphenolic compounds has been characterized into several groups such as phenolic acids, flavonoids, terpenoids, steroids, and alkaloids (Harborne 1999). However, Plants are thought to have been continuously confronting biotic and abiotic stress conditions to develop defense system by several mechanisms including signaling and stimulatory pathways, enzymatic reactions to maintain plant growth and metabolism (Bennett and Wallsgrove 1994). Being the frontline products of defense response, phenolics and flavonoids protect plants under stress because of their strong antioxidant capability, while plants also have evolved

enzymatic as well as non-enzymatic free radicals scavenging systems (Khan T et al 2019 a).

However, the main issues for developing pharmacological products are the lesser content availability, downstream procedures and esteemed fluctuations (Khan T 20019 b). Recently, advances in plant tissue culture techniques have overcome several inconveniences at industrial level and enabled efficient production of these bioactive compounds irrespective of climate conditions (Zhao 2005; Ruby Tiwari and Rana 2015). Propagation of secondary metabolites by biotic and abiotic elicitation is one of the few approaches recently being adopted to intensify the potential yield and to ease the net cost (Angelova et al. 2006; Yin et al. 2013; lee et al. 2014). Such approach involves application of trace amounts of elicitors to generate or further enhance the biosynthesis of these metabolites. Ramakrishna and Ravishankar (2011) reported the role of several biotic and abiotic agents on the production of secondary metabolites including melatonin, methyl jasmonate, salicylic acid, light, temperature, serotonin, plant growth regulators, cold, salt and chemical stress etc.

Melatonin (N-acetyl-5-methoxytryptamine), is a biogenic amine that can be found in plant and animal kingdom. A number of studies have been reported on melatonin role in plant growth as well as defensive responses under stress conditions (Khan etal 2019 b). It possess distinct analogy with indole-3-acetic acid and used as an alternative (Trejo-Espino et al, 2011). Tan et al. (1993) reported that, in nature, melatonin is a potent and high-capacity free radical scavenger, as compared to vitamin E, while other studies have proved its defensive role in salt stress, withstand cold caused injury and drought, and metals contaminated soils (Ullah et al, 2020; Khan et al 2019b, Wei 2015). Sarropoulou et al. (2012) decoded other traits of melatonin and found that exogenously applied melatonin at low concentration enhances proline and photosynthetic pigments in plants. Additionally, its growth promoting and protective role reported previously include hormonal networking under darkness (Kolár and Macháckova 2005), cell division and spindle fiber formation during mitotic division (Murch and Saxena, 2002). For instance, Galano et al. (2013) observed that melatonin not only scavenge ROS but also reactive nitrogen species (RNS) which discriminates it from other antioxidants. Under stress conditions, ROS are produced which causes DNA damage, enzyme inactivation and oxidative degradation of lipids (Fischer et al. 2013; Zheng et al. 2015). Although, Melatonin protects the plant from the undesired functions of ROS by regulating the enzyme serotonin N-acetyltransferase (SNAT) which expresses during the stress conditions (Byeon and Back 2014). While, previous studies only focused on controlling the negative effects of ROS instead of eliminating them from the biological systems (Considine et al. 2015).

Hence, the current study planned to investigate the melatonin effects on I. rugosus in-vitro cultures; its growth promoting potential and phytochemicals enhancement by biomass, TPC and TFC analysis. For instance, to elaborate its effects on free radical scavenging activity, antioxidants enzymes, antioxidants capacity and reducing potential. Moreover, to explore the inhibitory potential of melatonin treated extracts against cholinesterases [acetylecholinestrase (AChE) and butyrylcholinestrase (BChE)], RBC hemolysis, cytotoxicity in HepG2 cell lines and alpha amylase activities. To the best of our knowledge, this is the first report exploring the direct implication of melatonin elicited extracts of I. rugosus in vitro cultures in prevention strategy for Alzheimer's, Diabetes and hepatocellular carcinoma. To depict the hepato-protective activity of melatonin treated cultures of I. rugosus against HepG2 cell line and inhibitory activity for cholinergic activity to justify the treatment of Alzheimer's symptoms.

Material and Methods

Plant material

Seeds of I. rugosus were collected from the wild plant species located at dry-hill areas of Khyber Pakhtunkhwa province in Pakistan.

Seed germination and Callus culture

Seeds were germinated in a sterile way on MS (Murashige and Skoog 1962) medium containing 30 g/L (w/v) of sucrose and 8 g/L (w/v) of agar. pH of the medium was adjusted to 5.6-5.8. After 5 weeks, explants were inoculated on MS medium supplemented with different concentration of plant growth regulators (PGRs) in the presence of

different concentration (1, 2, 3, 4, 5, 10, 50 and 100 µM) of melatonin. Taking inspiration from our previous study (Abbasi et al. 2019), we continued to use a mixture of Thidiazuron (TDZ) and α-naphthalene acetic acid (NAA) in a ratio of 1:3 mg/L. Subsequently, after 5 weeks, data were collected taking fresh and dry weight of callus cultures derived from explants for supplementary examination. The calli obtained at all the tested concentrations of melatonin were green and compact.

Phytochemical screening

At the end of 5 weeks, fresh callus cultures grown at different melatonin treatments were harvested for the determination of fresh weight (FW). Then, the callus cultures were dried at 35-37 °C for 24-48 hrs. Finally, the dry weight (DW) was determined. The phytochemical screening of DW samples was done for triterpenes/steroids, alkaloids, flavonoids, saponins, tannins, and phenolic acids. For the quantitative analysis, color intensity was used as analytical response to these tests.

Total Phenolic Content (TPC)

TPC was determined by employing a slightly modified protocol of Singleton and Rossi (1965). Folin-Ciocalteu (FC) reagent was used to estimate the TPC and was expressed as GAE (Gallic acid equivalent mg/mL). Briefly, 20 µL of sample was mixed with 90 µL of FC reagent and incubated at room temperature. After 5 min of incubation, 90 µL of sodium carbonate (6% w/v) was added to the above mixture. The spectral analyzer (Halo DR-20, UV-Vis spectrophotometer, Dynamica Ltd., Victoria, Australia) was used to measure the absorbance at 630 nm. The results were expressed in mg of Gallic acid as standard (0-50 µg-mL, R2 = 0.968), equivalent to GAE per 100 g of dry sample. Total Phenolic Production (TPP) was then estimated using the following formula:

$$TPP\ (mg/L) = DW\ (g/L) \times TPC\ (mg/g)$$

Total Flavonoid Content (TFC)

TFC was determined using the previous aluminium chloride colorimetric method with slight modification (Chang et al. 2002). Briefly, 20 µL of extracted sample was mixed with 10 µL of 10% $AlCl_3$ (w/v) and 10 mL of CH_3CO_2K (1M potassium acetate), monitored by the addition of 160 µL of dH_2O. Before taking absorbances, samples were incubated for 30 min at room temperature. The optical density was determined by means of a spectrophotometer at λmax = 415 nm. The level of flavonoid concentration was calculated from the calibration plot symbolized as mg quercetin equivalent/g of sample. Total Flavonoid Production (TFP) was then estimated using the following formula:

$$TFP\ (mg/L) = DW\ (g/L) \times TFC\ (mg/g)$$

Evaluation of antioxidant activity

Free Radical Scavenging Assay

For this assay, previously documented protocol of Lee et al. (1998) was used with slight modification. 20 µL of sample and 180 µL of DPPH reagent were mixed and the absorbance was measured at 517 nm, after incubation of one hour. Ascorbic acid was used as control. The following formula was then used to determine the free radical scavenging activity:

$$\%\ scavenging\ DPPH\ free\ radical = 100 \times (Abc\text{-}Abs/Abc)$$

Where, Abc denotes absorption of the control, while Abs denotes the absorption of the sample.

Total Antioxidant Capacity (TAC) (Phosphomolybdenum method)

TAC (total antioxidant capacity) of tested samples was calculated by standard method after slight modification (Umamaheswari and Chatterjee 2008). For examination, the mixture contained 100 µL aliquot of each sample, 180 µL of phosphomolybdenum reagent (4 mM), and 0.6 M sulfuric acid. After incubation for 90 min at 95 °C, the samples were cooled to room temperature and absorption was measured at 695 nm. For the calibration curve, methanol was taken as a negative control and ascorbic acid as a positive control. The results of the total antioxidant capacity were then expressed as equivalent to alkaline ascorbic acid (AEAC: Ascorbic acid Equivalent Antioxidant Capacity)

Total Reducing Power analysis (TRP)

To measure the reducing potential of the tested

samples, protocol of Ahmed et al. (2017) and Abbasi et al. (2019) was followed. Briefly, 40 µL of each sample from the stock solution (4 mg/mL samples in DMSO) were taken in Eppendorf and phosphate buffer (0.2 M, pH 6.6) plus 1% (w/v) potassium ferricyanide were then added. Finally, 10% trichloroacetic acid (w/v) was added and centrifuged for 10 min at 5760 x g (Spectrafuge 24D microcentrifuge).

Before collecting the supernatant material, the mixture was incubated for 20 min at 50 °C. The floating material (166.66 µL) was mixed with 50 µL of 0.1% (w/v) ferric chloride solution. 33.3 µL ferric chloride (0.1%) was then added into each well of microplate and reading was taken at 630 nm using a microplate reader. Ascorbic acid and methanol were taken as positive and negative controls, respectively. The results were expressed as alkaline ascorbic acid equivalent (AEAC)

ABTS Scavenging Analysis

For producing ABTS radical cationic suspension, equal amount of ABTS (7.4 mM) and $K_2S_2O_8$ (2.6 mM) were taken and allowed to react for 16 hrs in the dark. After that, 1.0 mL dH_2O was added; meanwhile, the reading was taken at 734 nm to attain the reading of 0.73. At the end, 150 mL of sample extracts were added in 2850 mL of ABTS. After incubation for 6 min, reading was taken again at 734 nm. ABTS was expressed as TEAC (Trolox-C equivalent antioxidant capacity), the entire procedure was followed from Abbasi et al. (2019).

Ferric ion Reducing Antioxidant Power (FRAP) Assay

Reduction ability of the callus extract of I. rugosus was expressed as mg/ascorbic acid equivalents (AAE)/mL. Protocol of Benzie et al. (1996) and Abbasi et al. (2019) with brief modification was followed. For the stock solution, FRAP was formulated by mixing 25 mL of acetate solution (300 mg, pH 3.6), 2.5 mL of TPTZ (10 mM 2,4,6-tri(2-pyridyl) -1,3,5-triazine), 2.5 ml of $FeCl^{3+}$ HCl. At the end, 150 mL of each sample was added in 2850 ml of reducing solution and the solution was allowed to react for 2 h in the dark. Absorbance was then taken at 593 nm.

Anti-Diabetic Assay (α-Amylase Inhibition Assay)

Modified method of Sangeetha and Vedasree (2012) was used to determine the α-amylase inhibition assay. In brief, the reaction mixture was consisting of 10 µL of test sample and 15 µL phosphate buffer and this mixture was then allowed to react with 40 µL starch with 25 µL α-amylase enzyme. After 30 min of incubation at 50 °C, 20 µL of 1 M hydrochloric acid and 90 µL of iodine solution were added gradually to each 96 well of the microplate readers. Acarbose was used as a reference, while the extract solvent was used as a reference. At 540 nm, absorbance examination was done by means of spectrophotometer and α-amylase enzyme inhibition was measured in percentage.

Enzymatic Antioxidant Activities (SOD & POD)

About 100 mg of fresh weight samples were homogenized with cold 50 mM/L phosphate buffer saline solution (pH 7.8). Subsequently, the blended mixture of each samples was centrifuged at 4 °C at 10,000 rpm for 15 min (approximately equal to 8944 x g). At the end, 1 mL of extracted supernatant was used as crude enzyme extract for further testing. To estimate the activity of peroxidase, we followed the protocol of Lagrimini (1991) with slight modification. The activity of guaiacol peroxidase (POD, EC 1.11.1.7) at 470 nm was determined. In the presence of H_2O_2, the POD enzyme participates in the conversion of guaiacol to guaiacol tetra. 200 µL of the reaction mixture was prepared by adding 40 µL of phosphate buffer (50 mM of pH 7.0), 20 µL of guaiacol (100 mM), 100 µL of distilled water and 20 µL of H_2O_2 (27.5 mM). The blank was prepared with the same concentration of ingredients but without extracting the enzyme. Similarly, superoxide dismutase (SOD; EC1.15.1.1) activity was performed by using previous method (Giannopolitis and Ries 1977; Panda and Khan 2004). Catalyzed reaction of SOD estimation is based on the reduction of NBT (nitroblue tetrazolium). The reaction mixture contained 20 µL of EDTA (1 mM), 30 µL of methionine (130 mM), 20 µL of NBT (0.75 mM), 78 µL of Phosphorus buffer (pH 7), 2 µL riboflavin (0.02 mM) and 60 µL of enzyme extract solution. After exposure to fluorescent light for 7 min, the optical density was measured at 560 nm. Blank contained all the chemicals expect enzyme extract.

In-vitro Assay of Cellular Toxicity Against HepG2 Cell Line

Cell Lines and Cell Culture

For the human liver cell line, ATCC HB-8065 (American Type Culture Collection; Manassas, VA, USA) was used to measure the cytotoxicity of melatonin treated samples. The cell line was maintained at 37 °C and 5% CO_2 in DMEM (Dulbecco's Modified Eagle Media) enriched with 2 mL L-glutamine, 2 mM $C_3H_3NaO_3$ (Na-pyruvate), $C_9H_{11}N_2O_4S$ 100 U/mL (penicillin), 100 µg/mL streptomycin and 10% of FCS (fetal calf serum) under humidity of 5% CO2. HepG2 cells were harvested at room temperature for 1 min with 0.5 mM trypsin-EDTA solution. The cells were sub-cultured at regular intervals to achieve confluency of 80–90%. The research protocol was previously discussed in Siddiquah et al. (2018) with a slight modification being done.

Cell Viability Analysis

For the in vitro viability and cytotoxicity of HepG2 cells for I. rugosus, SRS (sulforhodamine-B) protocol was followed as described by Buranrat et al. (2017). Callus extracts were dissolved in DMSO prior to experiment. Cells were grown in a microtiter plate of 96-well at a density of 15000 cells per well and allowed to stick for 24 h at 37 °C. The cells were then treated with 200 µg/mL of each sample for 24 h. Cells were fixed with ice-cold 10% $C_2HCl_3O_2$ (trichloroacetic acid-TCA) at 4 °C. After incubation for 1 h, cells were washed with dH_2O thrice and then air dried. 0.01% sulforhodamine-B dye was used at room temperature for staining for 30 min and washed with 1% CH_3COOH (acetic acid) thrice to remove unbounded dye, followed by drying of the plates. Afterwards, unbound dye was dissolved in 10 mM Tris (100 µL/well) base solution (pH 8) at room temperature for 5 min. In the current experiment, untreated cells and Dimethyl sulfoxide (DMSO 1%) were used as controls (at 1% or more, toxic effects have been reported depending on the type of cell line used. That's why there is a slight difference between untreated cells and DMSO dissolved untreated HepG2 cells).

Cell viability was monitored prior to Tris treatment and photographs were taken with an Olympus CK2 light microscope armed with digital camera. Absorbance was measured at wavelength of 565 nm (Platos R 496, AMP). The experiment was repeated twice with triplicates for each sample in independent experiments and their standard deviations were calculated. To calculate the percent viability relative to untreated sample, the succeeding formula was used:

Cell viability (%) = (Absorbance of Sample-Absorbance of sample control)/ (Absorbance of untreated Cells-Absorbance of media only) ×100

Percent inhibition was calculated by formula:

Cell inhibition (%) = 100 – Cell viability (%)

In Vitro Cholinesterase Inhibitory Analysis

To evaluate the inhibitory activity of cholinesterase of I. rugosus callus extract, previously modified Ellman's protocol was followed. Concisely, 140 µL sodium phosphate buffer (0.1 M, pH 8.0), test samples 20 µL and 20 mL AChE enzyme (0.09 U/mL) were mixed and then kept for 15 min. After that, in 96-well microtiter plates, 10 µL DTNB (10 mM) and 10 µL ATCI (14 mM) was added. Absorbance was measured at 412 nm for 30 min after initiation of the enzymatic reaction. The same procedure was followed in case of BChE testing with butylrylcholinesterase enzyme, S-butyrylthiocholine chloride as a substrate, and for reference, physostigmine was used. All the solutions were prepared in 1% DMSO (1 mg/mL). Cholinesterase inhibitory activities were evaluated at 50% inhibition (50 µg/mL). For further calculation, more than IC_{50} concentration was considered as positive inhibition %.

HPLC Analysis

Quantification investigation of I. rugosus callus extracts was done by HPLC analysis. The whole system was composed of Photodiode Array Detector (PAD), Metachem Degasit degasser, auto-sampler of Varian Prostar 410, Varian Prostar 230 pump and controlled by Galaxie version 1.9.3.2 software. For HPLC, the sample was prepared by using the following protocol: in 500 µL of high-quality HPLC methanol, 100 mg of dry homogenous powder was added using Ultra-Turrax T25 basic mixer and T25 basic blender. The USC1200TH ultrasonic bath was used for one hour at 25 ± 2 °C, frequency of 30 kHz with 400 watts of electrical power, equipped with a digital timer, frequency and temperature control. The suspension was collected after centrifuga-

tion for 5 min at 10,000 rpm and then evaporated and resuspended in pH 4.8 citrate-phosphate buffer of 1 mL (0.1M). β-glucosidase 5 units/mL was incubated for 4 h at 37 °C to release aglycone for chromatograms. Each sample of HPLC was centrifuged for 5 min at 10,000 rpm (approximately equal to 8944 x g) (Spectrafuge 24D microcentrifuge). The floating material was filtered using a 0.45 µm syringe filter. Chromatographic separation was achieved with a Purospher STAR (Merck) RP-18 column (5 µm; 250 x 4.0 mm) at 35 ° C. The mobile phase was programmed using a mixture of solvent A (methanolic solution) and solvent B (gradient HPLC water) under acidic conditions (0.05% formic acid). Linear gradient ranging from 5:95 v/v (solvent A) to 100:0 v/v (solvent B) with a flow rate of 1.30 mL/min was applied. Fluorescence detector was programmed for excitation at 288 nm. Pentacyclic triterpenoids and phenolic compounds from I. rugosus were identified by an assessment with commercially prepared parameters and concentrations were calculated using 5-point calibration curves (R > 0.999)

Hemolytic Activity

Modified protocol of Yang et al. (2005) was followed for the in vitro hemolytic activity. For the preparation of erythrocytes suspension, EDTA-treated fresh blood was used. The blood was centrifuged at 1500 rpm for 3 min. 4% erythrocyte suspension was prepared in sterile phosphate buffer saline for hemolytic study. For hemolytic activity, the crude sample extracts of 20 µL, 15 µL, 10 µL, and 5 µL were mixed with 180 µL, 185 µL, 190 µL and 195 µL of erythrocytes cells, respectively, to obtain volume of 200 µL in 1.5 mL Eppendorf tube. 100 mL of the supernatant was collected after centrifugation at 10,000 rpm at room temperature and then transferred into microtiter plate for measuring absorbance at 540 nm (Bio-Tek ELX800). The hemolysis percentage is calculated using the formula with the determined IC_{50} value:

% Hemolysis = [Sample Abs - Negative control Abs /Positive control Abs - Negative Control Abs] × 100

Statistical Analysis

The Phytochemical analysis, enzymatic assays, anticancer, anti-Alzheimer and antioxidant radical scavenging data were analyzed using ANOVA (Contrast Analysis), followed by the DMTR test (Duncan Multimeter Range) to evaluate the media difference at a 5% significance level. All the presented results were expressed in means ± SD (Fowler and Cohen 1990).

Results and Discussion

Effect of Melatonin on Callus Induction Frequency

In pre-optimized medium (TDZ and NAA at the ratio of 1.0:3.0 mg/L), different concentration of melatonin (1.0, 2.0, 3.0, 4.0, 5.0, 10, 50, and 100 µM) were used to verify the effectiveness of callus induction and subsequent production of secondary metabolites. Stem explants were used for the establishment of calli. After 10-12 days, frequency of callus induction was examined at the wound sites of explants fortified with melatonin and those without melatonin treatment as controls. Induction frequency was high at low concentration of melatonin: 1.0 µM up to 5.0 µM, whereas a progressive decline in callus induction was observed at higher concentrations (10, 50 and 100 µM). The data documented in Table 1 represents the physical properties of callus at the end of 4th week, encompassing the size, color and texture of calli. Significant amount of induction frequency was observed in all treatments with slight morphological differences. Typically, calli were greenish white and green at low concentrations (1-5 µM) while light brownish in color at higher concentration (10-100 µM) of melatonin, as reflected in Figure 1. According to previous literature, stem-derived callus is as friable as illustrated by Pereira et al., (2013) and Abbasi et al., (2019), but the callus cultures grown in medium supplemented with melatonin in our study had a compact texture.

Table 1: Effect of Melatonin at different concentrations on I. rugosus stem-derived callus

15 mg/ml	10mg/ml	5mg/ml	IC 50 mg/ml
-2.22	-3.13	-5.04	>500
-0.04	-10.68	-14.68	>500

-7.22	-9.59	-12.59	>500

Note: Sufficient callus (++++), Moderate callus (++), MS; Murashige and Skoog Medium, LG; light green, C; Compact, Mela; Melatonin

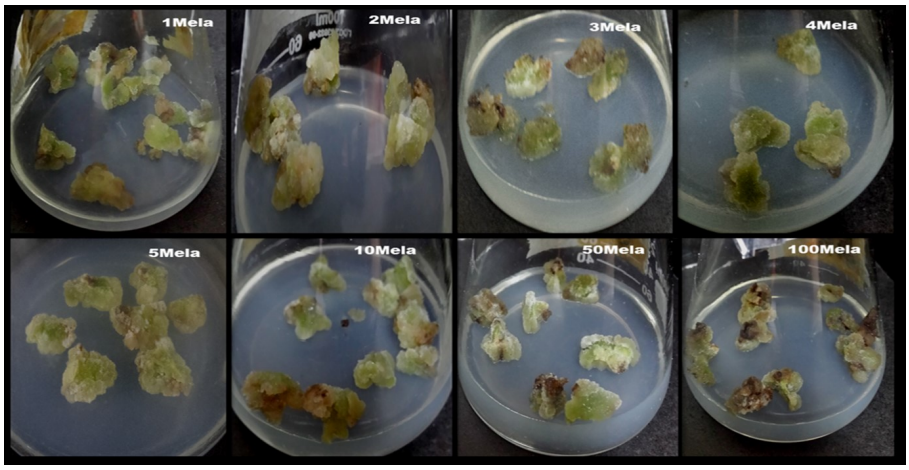

Figure 1: Induction of friable stem callus after 4 weeks of culturing on MS medium supplemented with different concentration of melatonin at optimized combination of TDZ+NAA (1:3 mg/L)

Melatonin and TDZ have been reported as effective bio-regulators in in vitro cell and tissue cultures. These bio-regulators, when exogenously applied to various plants, perform vibrant roles in organogenesis and callus induction (Guo et al. 2011; Murch et al. 2001). Pelagio-Flores et al. (2011) briefly explained the role of melatonin as auxin type plant growth regulator. So far, there is no research data available which specifies the effect of melatonin alone or in combination with other growth regulators such as NAA and TDZ on growth aspects, especially in case of I. rugosus.

Effect of Melatonin on Biomass Accumulation

Melatonin biologically regulates diverse physiological processes in plants. It has been identified in various plant tissues, including root, stem, leaves, flowers, fruits and seeds (Reiter et al. 2015). Arnaud and Hernández Ruiz (2017) recently suggested another characteristic of melatonin that exhibits an auxin-like activity that promotes or inhibits root growth. Note that growth inhibition occurs only at high concentrations of melatonin (> 10 μM) in the roots. Similar to our findings, there was synergistic effect between melatonin concentration and biomass accumulation of secondary metabolites. There was a gradual increase in fresh and dry weight of callus-derived culture but at higher concentration of melatonin, biomass accumulation declined gradually, which is evident in Figure 2.

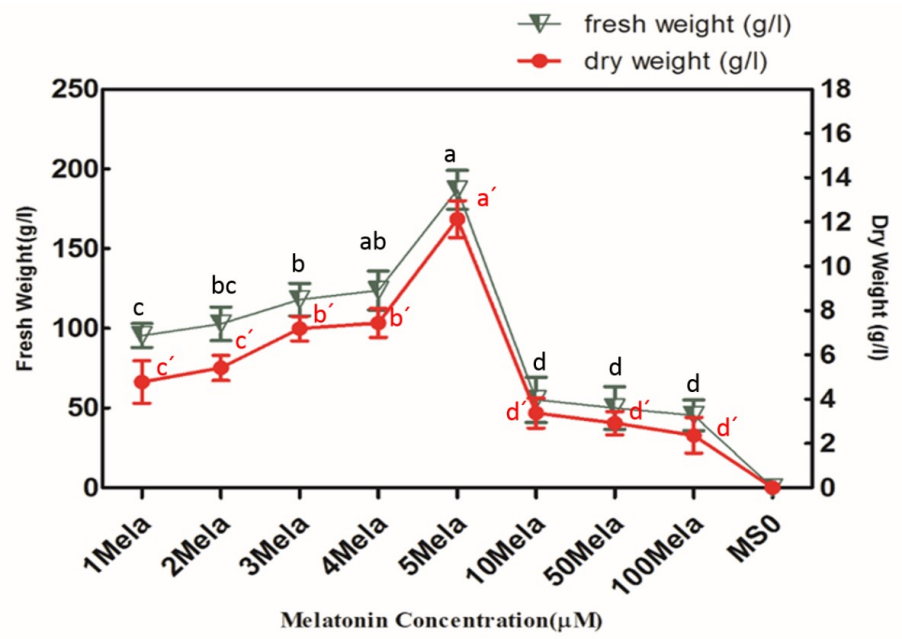

Figure 2: Growth curves analysis of fresh and dry weight of I. rugosus calli after 4 weeks of culturing on MS medium supplemented with different concentration of melatonin elicitor. The graph points and error bars represent, respectively, mean and SD from three experiments. Different letters indicate probability differences of 0.05 between the parameters

Maximum biomass accumulation was observed at 5 µM melatonin concentration with FW 195.56 mg/L and DW 12 mg/L, while at high concentration (100 µM) melatonin, biomass accumulation was 52.24 mg/ FW and 2.9 mg/L DW. At this concentration, browning of callus was observed due to oxidation of phenolic compounds with poor growth, ultimately leading to cell death. The efficiency of melatonin is not clearly understood in higher plants. However, the growth and evolution of these medicinal plants has been transformed by the antioxidant activity, stability of the membrane and the up- and down-regulated genes (Zhang et al. 2014).

Estimation of Total Phenolic and Flavonoid Contents

In this report, we observed a trend in the accumulation of phenolic compounds that was directly proportional to the accumulation of DW biomass and the concentration of melatonin used. Concentration of Melatonin at high rate decreased the number of phenolic compounds such as 10 µM (200 mg/g DW), 50 µM (187.61 mg/g DW), and 100 µM (100.4 mg/g DW). Whereas, concentration below 10 µM enhanced the accumulation of phenolic compounds like 1 µM (175.52 mg/g DW), 2 µM (179.9 mg/g DW) 3 µM (185 mg/g DW), 4 µM (209.85 mg/g DW), 5 µM (230.58 mg/g DW), as illustrated in Figure 3a. It mainly played an important role in adapting the plants to the changing environment, as it generally did not participate in the natural growth of the plant. But in current data, TDZ and melatonin might elicit stress on callus culture, and as a result, increased the production of phenolic content, flavonoids and various antioxidants to overcome the stress state. Khan et al. (2016) confirmed that the accumulation of these secondary metabolites was due to the activation of phenylpropanoid pathway. On the other hand, overall similar trend was observed in case of flavonoid accumulation, as in case of phenolic compounds. In our study, we examined that, at the same concentration of melatonin highest accumulation of flavonoid was observed at 5 µM (44.68 mg/g DW). Furthermore, low quantity of flavonoids was produced at 10 µM (29.98 mg/g DW), 50 µM (23.95 mg/g DW), and 100 µM (20.21 mg/g DW) of melatonin as seen in Figure 3. It was assumed that at higher concentration of melatonin, in the presence of TDZ, excessive amount of ethylene was released which alternatively decreased the production quantity of these precious secondary metabolites (Shibli et al. 1997). Current data also showed that the production of these secondary compounds is highly dependent on the accumulation of dry callus mass with increasing melatonin concentration. Application of elicitor i.e., melatonin, can be a

strategy in scaling-up the polyphenol compounds production (Wang and Frei 2011). It was hypothesized that melatonin stress stimulated the phenylpropanoid metabolism of I. rugosus (Cvikrová et al. 2003). More research is required to confirm this statement.

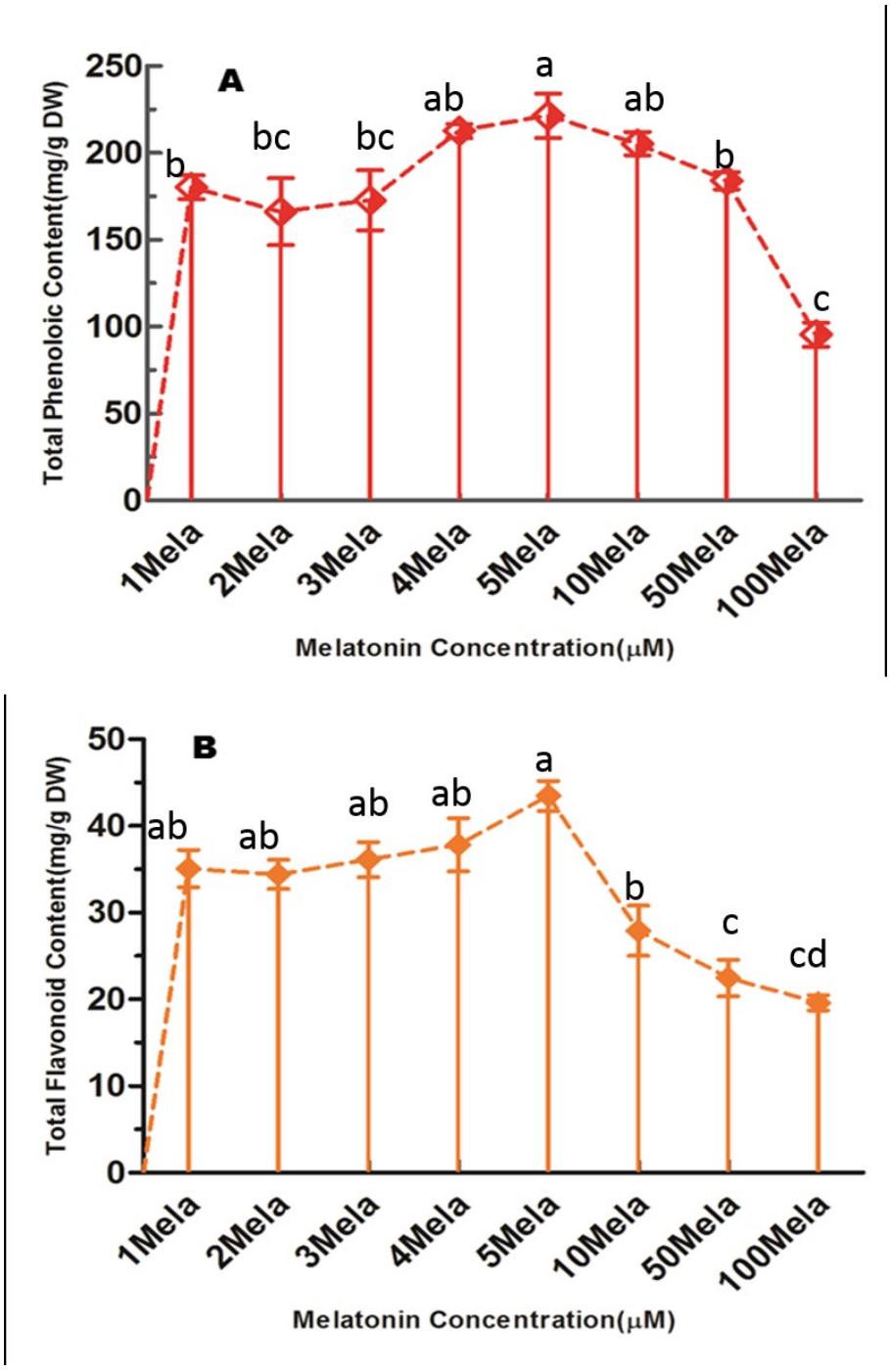

Figure 3: Effect of different concentrations of melatonin on \ total phenolic content (a) and (b) total flavonoid content in I. rugosus calli. Data represent means ± SD from three independent experiments. Different letters indicate probability differences of 0.05 between the parameters

Also, a similar trend was observed (TPC and TFC) in the overall production of these melatonin metabolites. In all samples treated with melatonin, we observed a significant increase in the metabolism of secondary metabolites (phenolic and flavonoids), which resulted in an increase in the overall production of these metabolites as seen in Figure 4. From the literature, we know that the accumulation of phenol in plants is methodically related to environmental

pressure. Being an important natural antioxidant, melatonin can protect plants from environmental stress to provide a radical-free climate. Posmyk & Janas (2009) briefly studied the protective role of melatonin in medicinally impotent plants.

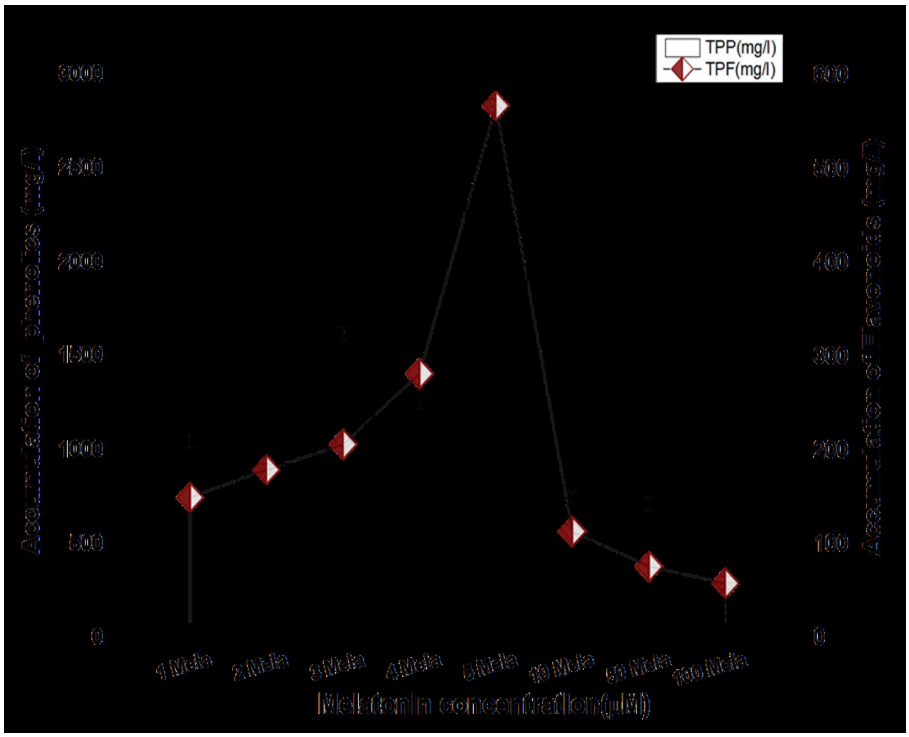

Figure 4: Production of phenolic and flavonoids in callus cultures of I. rugosus in response to different melatonin concentrations. Data represent means ± SD from three replicates. Different letters indicate probability differences of 0.05 between the parameters

Effect of Melatonin on DPPH Antioxidant Activity and Dry Mass

During oxidative stress, excessive quantity of ROS and other free radicals are accumulated in-vivo which might cause impairment directly or indirectly to the body (Chen et al. 2009). The production of ROS at moderate or low levels produces beneficial effects that include various physiological functions, such as immune function, various signaling and redox regulation pathways. But the formation of these reactive free radical species in higher amount causes destructive effect e.g. hydroxyl (OH), peroxynitrite (NO_3), oxygen (O_2) and nitric oxide (NO). Plant secondary metabolites of pharmacological importance have been used as a primary antioxidant to suppress these toxic radicals from the body that cause various metabolic syndrome (Tan et al. 2007). A positive correlation was observed between antioxidant DPPH and DW of melatonin-treated callus (g/L). The scavenger activity of melatonin was increased with the increase in dry mass at low concentration.

This increase in DW confirms the accumulation of secondary metabolites which in return increases the efficacy of melatonin at 1 µM (91.3 µM TEAC), 2 µM (92.2 µM TEAC), 3 µM (92.7 µM TEAC), 4 µM (92.5 µM TEAC) and 5 µM (93.4 µM TEAC). Whereas increased concentration inhibited the DPPH activity as the dry mass was also decreased: 10 µM (92.4 µM TEAC), 50 µM (92.1 µM TEAC), 100 µM (91.9 µM TEAC), as shown in Figure 5. It was estimated that, due to high potent antioxidant activity, melatonin effortlessly crosses the cell membrane, blood and brain barriers. It results in stable end-products originated from reacting with free radicals, as it cannot reduce to its initial state, as reported by Pandi-Perumal et al. (2006). Adil et al. (2015) also reported that combination of melatonin with auxin increases shooting and rooting response by 3-fold. Xu et al. (2017) examined similar findings that high concentration of melatonin enhanced the antioxidant capacity and polyphenolic compounds of Grape Berries. So far, there is no data reported which directly associate the level of melatonin, DW and DPPH free-radical scavenging activity, al-

though a possible association between them has been suggested. This study provides the evidence that melatonin enhances antioxidant capacity, at least partially, via promoting polyphenol accumulation.

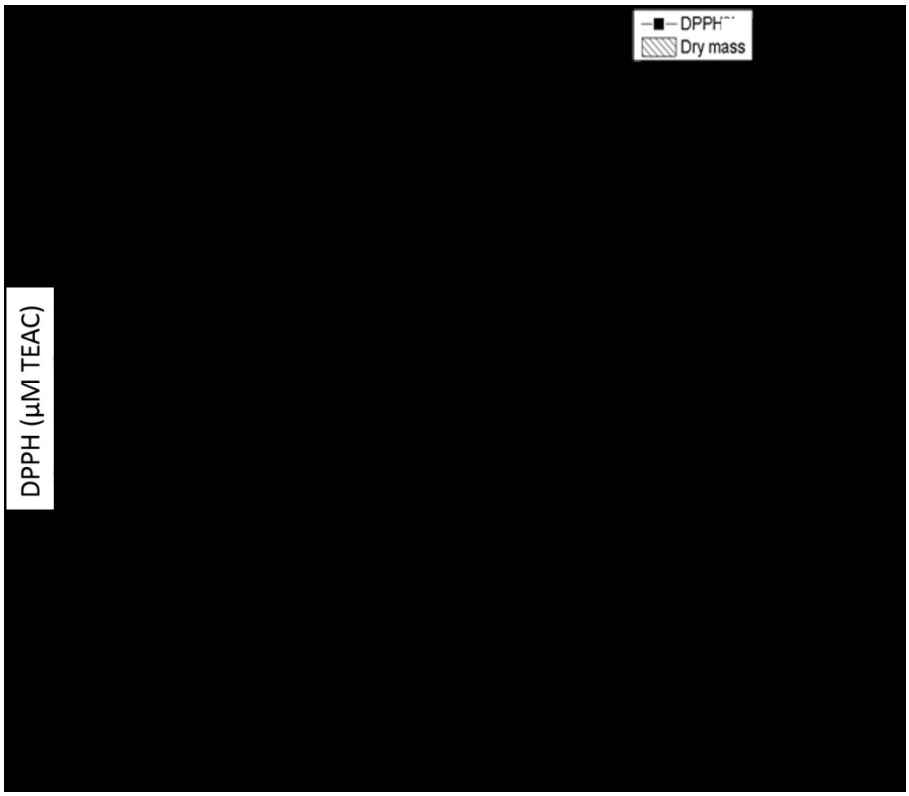

Figure 5: Effect of melatonin treatment on dry mass and free radical scavenging potential of callus extract. Data represent means ± SD from three independent experiments. DPPH is expressed in Trolox C µM Equivalent to Antioxidant Capacity (TEAC). Different letters indicate probability differences of 0.05 between the parameters

Effect of Melatonin on Enzymatic Antioxidant Assays

The production of antioxidant enzymes is secondary to the ecological pressure of plants. These enzymes absorb ROS's effect to benign derivatives, the first step toward the defense system against the toxic state (Posmyk 2009). Superoxide Dismutase (SOD) and Peroxidase (POD) activities significantly varied with low and high concentration of melatonin (Figure 6). SOD enzyme was gradually decreased with the increase in concentration of melatonin. At low concentration (1 µM) of melatonin, the SOD activity was 0.094 (nM/min/mg FW), whereas, at 100 µM Melatonin concentration, SOD was 0.04 (nM/min/mg FW). However, POD showed positive relation i.e. with the increase in concentration of Melatonin, the POD activity was increased gradually, such as, at low concentration (1 µM) of melatonin, it was 0.15 (nM/min/mg FW) and at 50 µM melatonin, the POD was estimated as 0.0659 (nM/min/mg FW). Parallel to current observation, Sun et al. (2018) investigated that POD activity progressively increased with increasing Al^{3+} amount higher than 40 mg/L in both old and young Tieguanyin leaves.

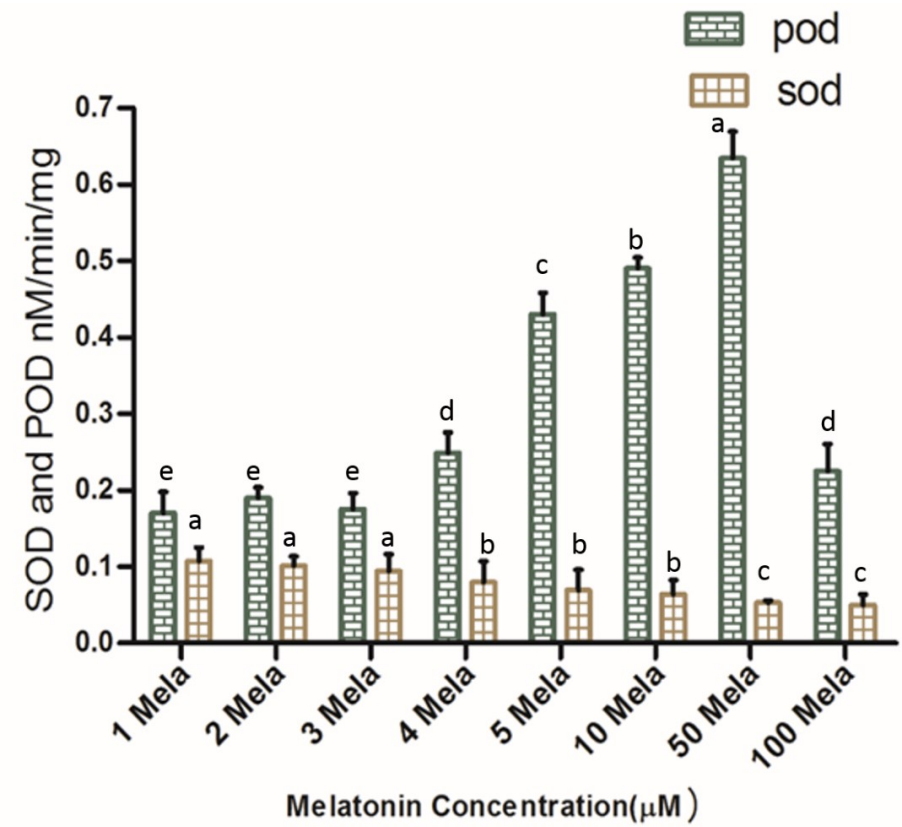

Figure 6: Effect of melatonin treatments on SOD and POD activities in fresh I. rugosus calli extracts. Data represent means ± SD from three independent experiments. Different letters indicate probability differences of 0.05 between the parameters

POD is naturally existing iron-bearing metallic protein, oxidoreductase enzyme in plants. Besides involved in removing ROS, it catalyzes decomposition of H_2O_2 and other phenols. That's why, we hypothesized that the increase in concentration of melatonin increases the phenolic compounds which in turn stimulates the POD activity. Meanwhile, POD actively interrelates with SOD to competently eradicate ROS involved in metabolism. SOD suppresses the anion O^{2-} radicals to defend cells from oxidative impairment. SOD efficiently works at low concentration but at high concentration of melatonin, SOD efficacy slowdown, which may be due to stress caused by over-accumulation of melatonin. Sun et al. (2018) stated the same observation; SOD activity was progressively reduced at higher quantity of Al^{3+} (200 mg/L). Hence, in current data, we observed and postulated that inverse relation of SOD and POD means that at low or high concentration of melatonin, the activity of both enzymes effectively combat and strive to reduce the adverse condition of ROS. Therefore, in the present data, we observe and hypothesize that the inverse relationship between SOD and POD means that at low or high concentrations of melatonin, the activity of both enzymes actively combats and seek to reduce the adverse condition of ROS.

Trend in Antioxidant Capacity and Total Reducing Power Assays

In addition to natural antioxidants, it has been revealed that many phenol derivatives contain high levels of antioxidant activity. The reducing ability of sample extracts to reduce ferric ions into ferrous ions (Fe^{+3} to Fe^{+2}) will possibly assist as an important sign of its potential antioxidant activity (Choi et al. 2010). I. rugosus displayed strong reducing capacity at low level of melatonin, such as at 1 μM (250.44 μM AEAC), 2 μM (249.73 μM AEAC), 3 μM (248.61 μM AEAC), 4 μM (248 μM AEAC) and 5 μM (252 μM AEAC) concentrations, as shown in Figure 7. High concentration decreased the reducing capacity due to low accumulation of phenolic compounds. This reduction capacity of extracts indicates the ability of electron donating ability of phenolic compounds. At the same time, during the lipid peroxide pathway, they also reduced the oxidative intermediates. As a result, they act as primary and secondary antioxidants. Benslama and Harrar (2016) reported the same

observation in two medicinal plants. They studied that methanolic extract of Z. album (Zygophyllaceae) has strong reducing capacity (2399.65 ± 12.31 µg AAequ/mg E) and free radical scavenging activity (EC50 = 0.096 ± 0.001) due to its rich content of polyphenols and flavonoids, as compared to A. scoparium capacity (Chenpodiacea). A positive relationship was observed between the total reduction of phenolic compounds and the concentration of melatonin. In redox reaction, mechanism behind reducing ability of extracts was electron transfer capacity of secondary metabolites to split free radicals into less reactive oxygen species (inert products) and water molecules. Maksimović et al. (2005) proved that phenolic compounds act as natural antioxidants, owing to their ability to inhibit, reduce and scavenge free radicals. Total antioxidant capacity also depends upon polyphenolic elements of medicinal plants. Positive relation was seen at low concentration (1 µM (100.71 µM AEAC) and higher concentration (5 µM (60.33 µM AEAC), 100 µM (30.44 µM AEAC) of melatonin.

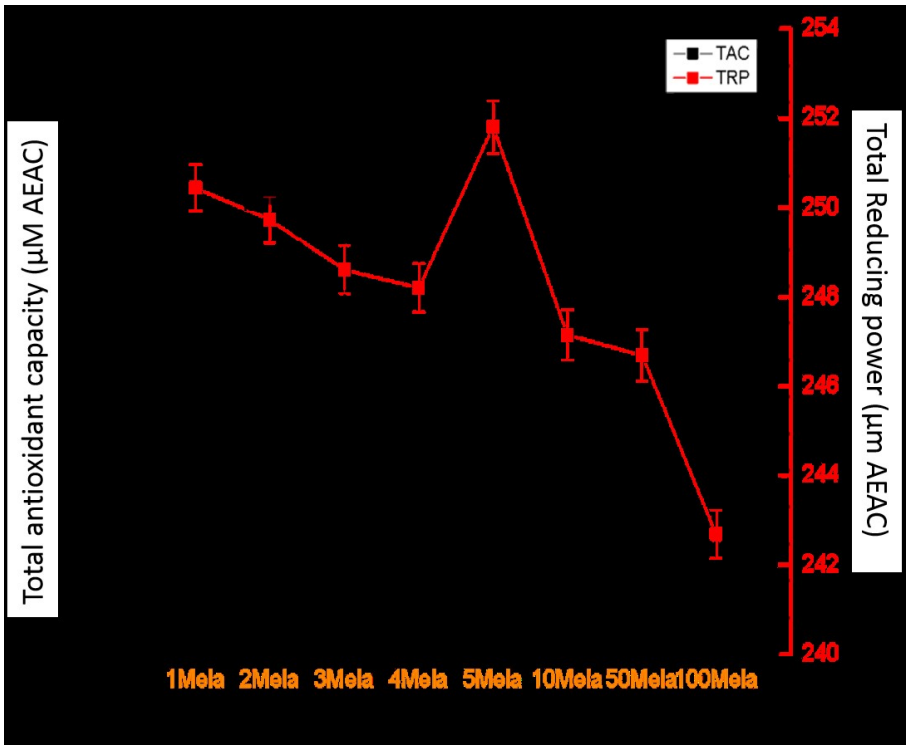

Figure 7: Effect of melatonin treatment on TAC (A) and TRP (B) activities in the calli extract obtained from stem-derived callus of I. rugosus. Data represent means ± SD from three independent experiments. TAC and TRP is expressed in Ascorbic acid µM Equivalent to Antioxidant Capacity (AEAC). Different letters indicate probability differences of 0.05 between the parameters

Another stimulating and encouraging aspect to study the total antioxidant and reducing capacity was the structural peculiarities and configurations of melatonin and NAA. In this study, we observed that due to its effective melatonin reducing ability, it significantly changes free radicals into inactive radicals. Koepfli et al. (1938) described the structural requirements of chemical compound exhibiting auxin containing properties. They reported that these compounds must contain aromatic unsaturated ring with an aliphatic side chain and -COOH group in the side chain like NAA and IAA. NAA carries a carbonyl group with an electronically charged region in -COOH separated by (approximately 0.5 nm) a positively charged ring structure. This distance plays a very important role in the activity of auxin. Auxins contain essentially 3 important requirements, such as the planer polycyclic aromatic ring, the binding site of negative charge carboxylic acid and the hydrophobic site that separates the two binding sites. Like NAA, the configurational structure of melatonin contains a carbonyl group with an electric charge region and a hydrophobic group, which were hypothesized by Arnao and Hernández-Ruiz (2006). Therefore, it was suggested that the reduction in the antioxidant capacity of NAA and melatonin depends on the redox properties, hydrogen donors and aliphatic side

chains, the COOH group plays a vital role in reducing the risk of various human diseases.

Trends in ABTS and FRAP Assays

Reproducibility trend of the antioxidant activities such as ABTS and FRAP assays of I. rugosus were measured by using methanolic callus extracts (Figure 8). The ABTS scavenging capacity depends directly or indirectly on the concentration, chemical composition and the polymerization levels of antioxidant agents e.g., polyphenols and confirmed the presence of pentacyclic terpenoids and phenolic compounds as identified by HPLC analysis of callus extracts. ABTS scavenging value at 4 µM melatonin was 550.96 ± 13.22 (µM TEAC), FRAP (µM TEAC) 772.98 ± 12.34, and at 5 µM melatonin, ABTS 525.75 ± 13.43 (µM TEAC), FRAP (µM TEAC) 750.37 ± 12.98. Mechanism behind the antioxidant activity was to accept or denote the electrons directly. In this way, elimination of unpaired electron from the highly interacting radicals to eliminate the formation of frees radicals, leads to the complete detoxification. Yen and Chen (1995) briefly described the strength of reduction of the sample extracts based on the direct transmission of electrons in the reduction reaction of $Fe^{3+}(CN-)^6$ $Fe^{2+}(CN-)^6$. The index of ABTS radicals were considered to be more reactive as compared to DPPH by single transfer of an electron at specific absorbance of 734 nm (Liü et al. 2009).

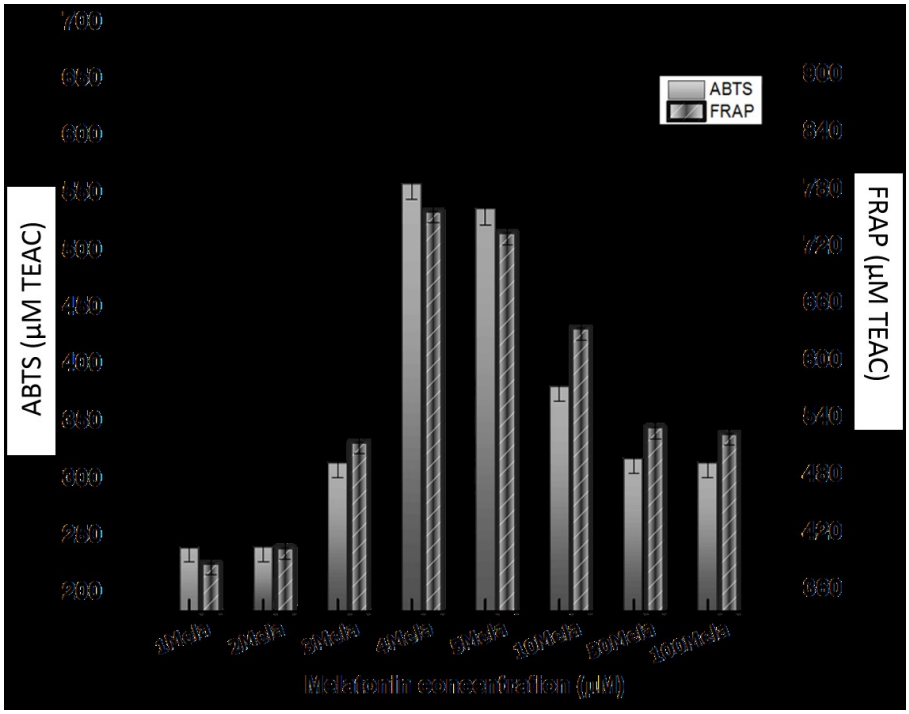

Figure 8: Effect of melatonin treatment on ABTS and FRAP activities in the calli extract obtained from stem-derived callus of I. rugosus. Data represent means ± SD from three independent experiments. ABTS and FRAP is expressed in Trolox C µM Equivalent to Antioxidant Capacity (TEAC). Different letters indicate probability differences of 0.05 between the parameters

Trend in α-Amylase Inhibition Assay

Diabetes is a collection of carbohydrate metabolic disorder which occurs as a result of carbohydrate, fat, and protein metabolic abnormalities (Craig et al. 2009). According to literature, hyper-glycaemia is the chief reason of diabetes mellitus which in return generates ROS (Patel et al. 2011). In present study, we observed that low to high concentration of melatonin gradually showed elevated level of inhibition which was respectively decreased at higher concentration i.e. 1 µM (34.17 %), 2 µM (36.19 %), 3 µM (35.29 %), 4 µM (38.50 %), 5 µM (42.50 %), 10 µM (39.21 %), 50 µM (29.16 %), and 100 µM (21 %), as shown in Figure 9. Previous data have demonstrated the vital role of medicinal plants in the treatment of diabetes. Mainly, in developing countries like Pakistan, people have limited access to modern and expanded resources for treatment. Therefore, the present study provides valuable information about the pres-

ence of numerous possible potential bioactive phytoconstituent inhibitors of this enzyme such as phenols, terpenes, and flavones etc. in I. rugosus. These inhibitors help to identify enzymes which hydrolyze carbohydrates e.g. α-amylase from extract.

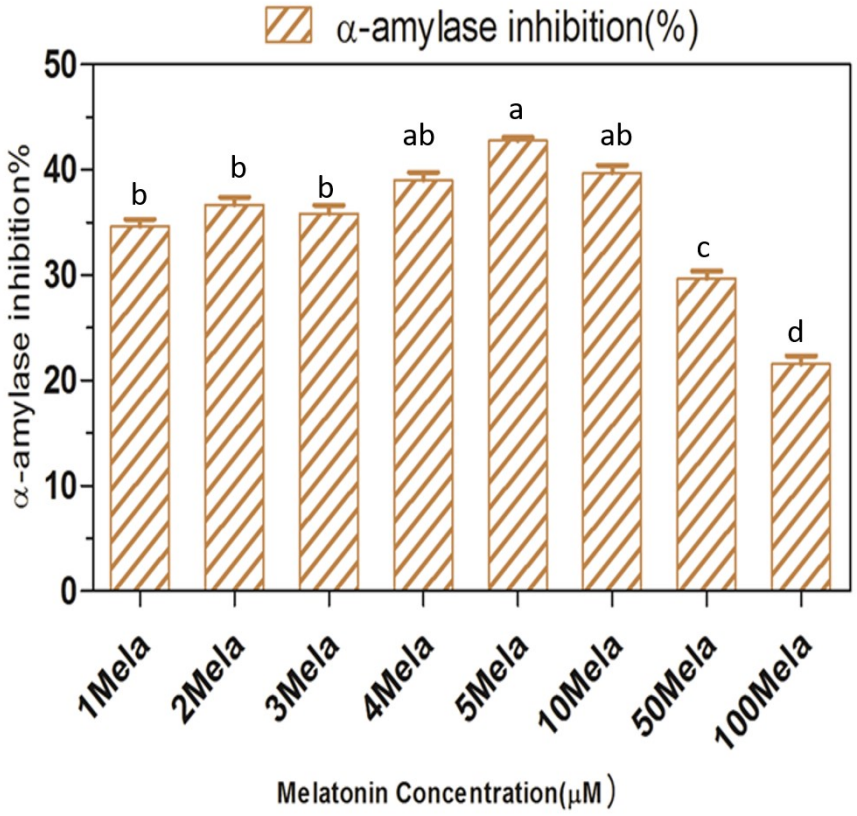

Figure 9: Effect of melatonin treatment on α-amylase inhibition potential of callus extract from I. rugosus. Data represents means ± SD from three independent experiments. Different letters indicate probability differences of 0.05 between the parameters

In diabetes, ROS induced membrane damage, lipid peroxidation and β-cells, thus the presence of antioxidant secondary metabolites in plants might prevent against diabetes. In concordance with the findings of other observation to some extent, Zhang et al. (2017) studied that α- and β-amylase were considerably amplified (14.5% and 23.5%, respectively) on seed germination, after exposure of exogenous melatonin application in comparison to NaCl treatment. It is concluded that, at the present-day, there is limited systematic observation available to figure out the efficacy of melatonin related to α-amylase analysis. As far as we know, this is the first action to show the relationship of melatonin and α-amylase, so the current results may prove beneficial in the future.

Cytotoxicity

Effect of Melatonin-Treated and Untreated Callus Extracts on Cell Viability

The viability test determines the number of viable cells in a population group. Combination of total number of cells and the number of viable cells an accurate signal to the physical strength (health) of hepatic cell culture. Cytotoxicity of melatonin-treated callus culture was slightly higher (% viability of 60.46 %) as compared to callus cultures grown without melatonin treatment (% viability of 87.20 %). Morphological changes such as long fibroblasts type cells were observed in both cases, as can be seen in Figure 10a. The rate of cellular inhibition was high in the presence of melatonin-treated callus culture due to its ability to eliminate free oxygen radicals. Girish et al. (2013) also interpreted that melatonin was involved in triggering platelet apoptosis.

They concluded that 100 µM melatonin significantly decreased MTT activity, proposing its cytotoxic nature. 50 % reduction in the viability of platelets confirmed its cytotoxic effect (Girish et al. 2013). Also, appreciable rate of total antioxidant capacity (TAC), α-amylase inhibition and satisfactory reducing power (TRP) magnify the apoptotic rate to protect the cells and tissues from radical damages. At different physiological levels, anti-proliferative and cytotoxicity activities of melatonin firmly depend on intracellular redox state. It performs complex machinery of inhibition of proliferation and stimulation of apoptosis (Proietti et al. 2012). The presence of a low-molecular weight melatonin receptor with a specific configuration allows melatonin to diffuse effortlessly in both extracellular fluid and cells (Mangelsdorf and Evans 1995). It was also reported that the toxic effect of melatonin on cells was associated with phosphorus binding with AMP, ADP and ATP (Mills et al. 2005; Cardinali et al. 1993).

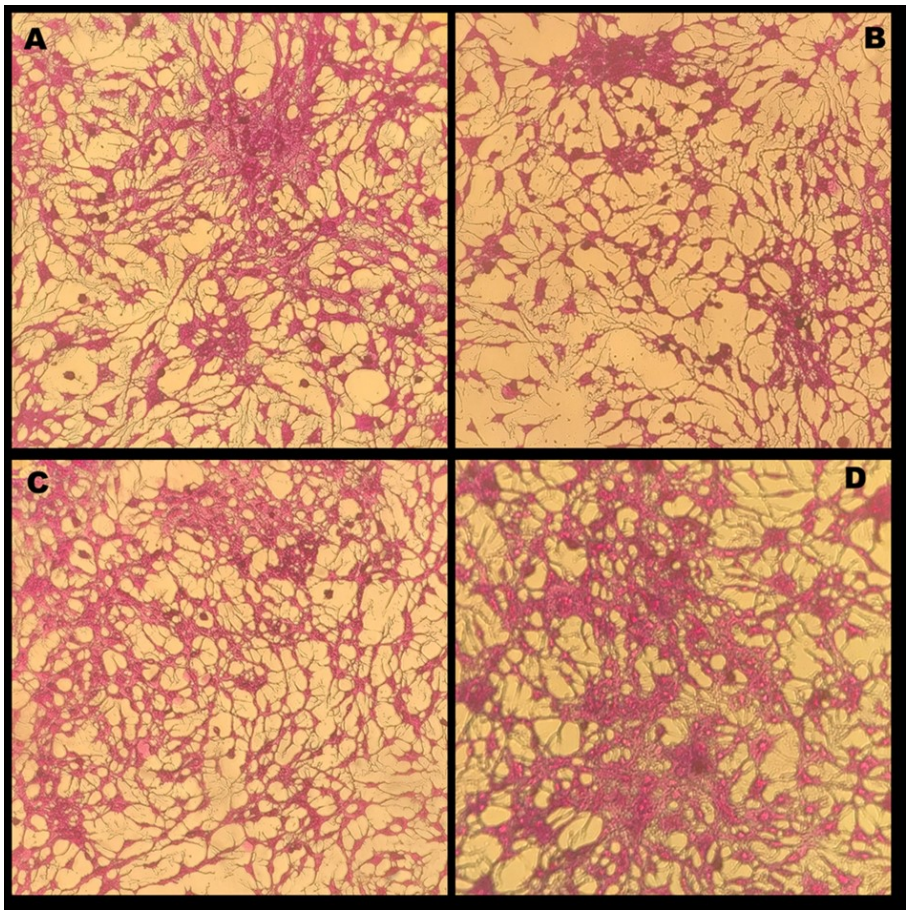

Figure 10a: Cytotoxic effects of melatonin untreated (a) and treated (b) callus extracts on HepG2 cells upon 24 h treatment with 200 µg/mL concentration. (c) DMSO (solvent) and (d) untreated HepG2 cells were included as controls

Statistically, in the current data, melatonin-treated callus culture showed a significant inhibitory rate of 39.54%, while the inhibitory rate without melatonin was 12.79 % (TDZ + NAA 1:3 mg), as seen in Figure 10b. Results of Fu et al. (2011) supported our observation that 100 µM melatonin prevents the proliferation of neural stem cells and promotes their differentiation. They examined that this inhibition at high concentration of melatonin is strongly linked with the antioxidant properties of the indole. Ozdemir et al. (2009) and Martín-Renedo et al. (2008) reported the similar conclusion that dose of 1 µM indole intensely showed inhibition of cell proliferation in HepG2 cells. We believe that this is the first work that shows the in vitro cytotoxicity comparison of melatonin-treated and untreated callus extract.

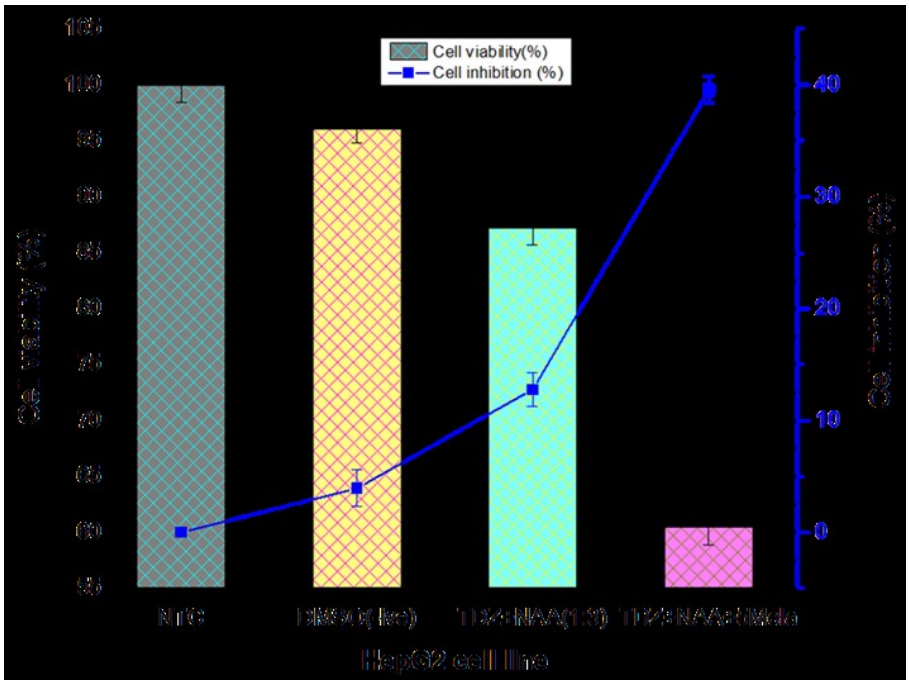

Figure 10b: Effect of melatonin treatment on the viability and inhibition of HepG2 cells compared to untreated controls (Means ± SD). Each sample was studied in triplicates and experiment was performed thrice

Quantitative HPLC Analysis

I. rugosus is an important reservoir of several important bioactive molecules. It may include several types of polyphenols such as mono & sesquiterpenes, triterpenes, phenolics, alkaloids, flavonoids, saponins, glycosides and essential oil. At commercial level, for consumption of these valuable compounds, it is essential to obtain in-vitro culture and HPLC extraction protocols to determine these compounds in efficient and reproducible ways (Weyerstahl et al. 1983; Tiwari et al. 2008).

Hereby, we aim to estimate the production of phenols e.g. Rosmarinic aicd (RA) and caffeic acid (CA) and pentacyclic triterpenoids e.g. Plectranthoic acid (PA), betulinic acid (BA) and oleanolic aicd (OA) in I. rugosus callus cultures by HPLC. Figure 11 displays the proposed biosynthetic pathway of triterpenoids and phenolic compounds. Davies (1995) reported that, on the physiological level, abiotic and biotic stresses generate "Oxygen Paradox", an atmosphere rich in O_2. At the same time, this aerobic pressure fully depends on molecular reduction of O_2. Alternatively, high concentration of oxygen causes lethal effect on our life. Therefore, accidental partial reduction of oxygen by electron transfer measured as an important factor which makes supply of oxygen dangerous. As a result, it releases various harmful highly reactive species instead of forming H_2O e.g. O^{-2}, H_2O_2 and $^{\cdot}OH$ (Demidchik 2015). We monitored that melatonin added stress condition which alternatively increased the production of PAL (phenylalanine ammonia-lyase) activity, leading cause of important phenolic and pentacyclic triterpenoids. Literature revealed that chemical signals escalate the expression of PAL enzyme, as a result improving the production of carbon-based (terpenoid and phenolic)/nitrogen-based (alkaloids) metabolites (Jeyaramraja et al. 2003; Strissel et al. 2005; Scheible et al. 2004; Dixon and Paiva 1995). In the current study, we observed positive relation at low concentration of elicitor on pentacyclic triterpenoids, for example, 1 µM melatonin [BA 96.44 ± 6.11 (µg/g of DW), OA 460.14 ± 22.23 (µg/g of DW), PA 342.88 ± 22.24 (µg/g of DW)]; 3 µM melatonin [BA 126.14 ± 14.25 (µg/g of DW), OA 518.86 ± 24.51 (µg/g of DW), PA 379.4 ± 21.31 (µg/g of DW)]; 5 µM melatonin[BA 267.33 ± 11.14 (µg/g of DW), OA 900.42 ± 21.17 (µg/g of DW), PA 1068.19 ± 22.34 (µg/g of DW)]. Conversely, at high concentration of melatonin, all the compounds decreased such as 10 µM melatonin [BA 369.73 ± 13.21 (µg/g of DW), OA 747.29 ± 20.19 (µg/g of DW), PA 661.66 ± 22.31 (µg/g of DW); 100 µM melatonin [BA 212.61 ± 11.32 (µg/g of DW), OA 475.38 ± 22.21 (µg/g of DW), PA 400.14 ± 24.23 (µg/g of DW), which is evident in Figure 12.

In the matter of phenolic, no positive relation was observed. At low concentration, CA was very high but gradually decreased with the increase in melatonin conc. e.g. 1 mela (µM) 70.81 ± 1.51 (µg/g of DW), 3 mela (µM) 64.42 ± 1.22 (µg/g of DW), 5 mela (µM) 48.37 ± 1.23 (µg/g of DW) and 100 mela (µM) 41.24 ± 1.23 (µg/g of DW) (Figure 3.13). While, in RA, maximum concentration was seen at 4 mela (µM) 5312.64 ± 95.66 (µg/g of DW). Thus, HPLC analysis was used to detect the amount of some important compounds of I. rugosus showing good antioxidant potency, as revealed by POD, SOD and DPPH assays.

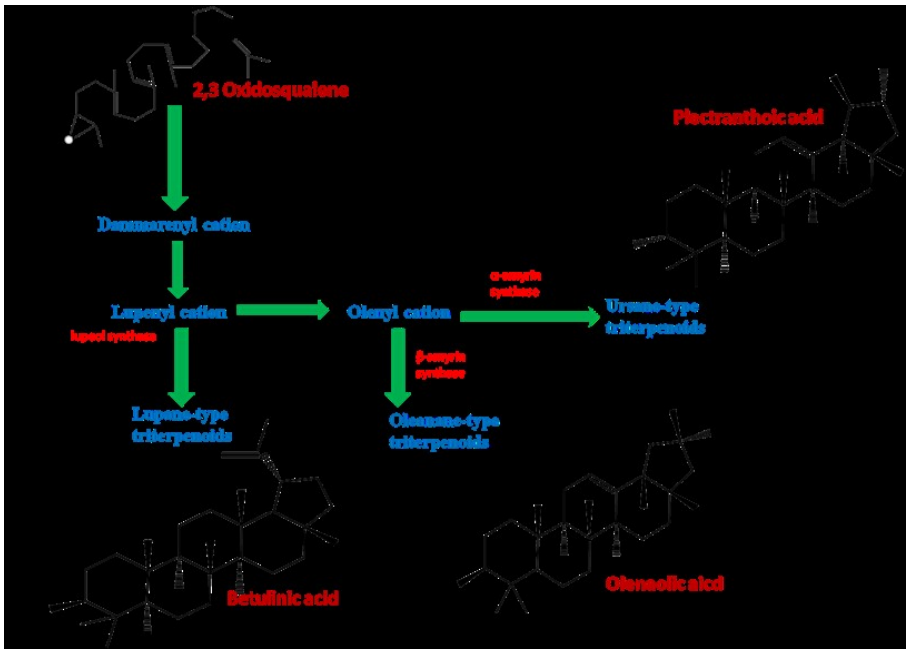

Figure 11: A proposed biosynthetic approach leading to the formation of (a) pentacyclic triterpenoids from precursor 2, 3 Oxidosqualene (b) Rosmarinic acid and Caffeic acid from Shikimic path

Anti-Alzheimer Potential of Metabolites Isolated from I. rugosus Callus Cultures

As shown in Figure 13, the inhibitory activity of 2 types of cholinesterase were used to measure the Anti-Alzheimer potential from the methanolic extracts of melatonin-treated callus of I. rugosus such as AChE (acetylcholinesterase) and BChE (butyrylcholinesterase). We examined the cholinesterase inhibitory effect of all HPLC-isolated compounds. However, only rosmarinic acid showed significant IC_{50} inhibition (50 µg/mL) AChE 81.29 ± 2.34 and BChE 72.31 ± 3.85. While all the other compounds were considered as inactive inhibitors against cholinesterase enzymes which showed 30 % less inhibition. Among the various neurodegenerative disorders, Alzheimer's disease is considered as one of the most widely spread pathological condition with clinical characteristics i.e. defect of brain neurons and loss of memory (Alzheimer's Association 2014). One of the most effective methods used to study the Alzheimer disease is to conserve the synthesis of acetylcholine by means of cholinesterase enzyme inhibition (Lahiri et al. 2002). Sizable inhibitory activity of melatonin was observed with cholinesterase enzymes (AChE and BChE) against Alzheimer's with inhibition % (AChE) 56.986 ± 1.12, (BChE) 35.307 ± 0.46 at 4 µM melatonin and (AChE) 51.364 ± 1.11, (BChE) 40.188 ± 0.47 at 5 µM melatonin concentrations. Literature supported few reports which described the inter-crossed relation of pentacyclic triterpenoids and cholinesterase inhibitory activity against Alzheimer (Dumont et al. 2009; Patil et al. 2011; Wilkinson et al. 2011; Zhang et al. 2012;. Hasnat et al. 2013) unified inhibitory activity of acetylcholinesterase with polyphenolic compounds (r2 = TPC 0.7736 and TFC 0.7755) of Ganoderma lucidum grown on germinated brown rice (GLBR) extract. In brain, there are a number of cholinergic areas used to control several functions. Scientifically through synapses, nerve cells stay connected to each other throughout the nervous system in all vertebrates including humans and also in-

sects, by the release of signaling chemicals termed as neurotransmitters. People with dementia, also known as Alzheimer's disease, in their brain possess low levels of acetylcholine (Chung et al., 2001; Piazzi et al., 2008). Cholinesterase's enzymes: AChE and BChE dissect acetylcholine and enhance the memory. Therefore, low concentrations of I. rugosus callus extracts were used to inhibit the cholinesterase's enzymes and to increase the availability of acetylcholine transmitter used for communications between brain cells.

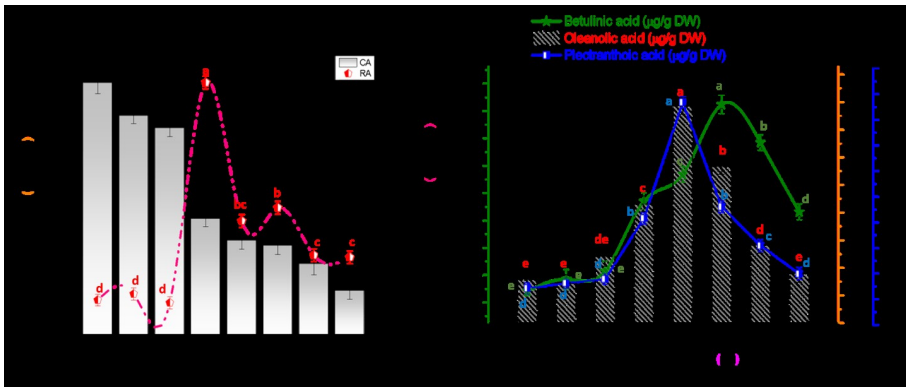

Figure 12: Quantitative HPLC analysis showing accumulation of (a) phenolic and (b) pentacyclic triterpenoids in melatonin-treated callus extracts of I. rugosus. Each sample was studied in triplicates and experiment was performed twice. Different letters indicate probability differences of 0.05 between the parameters

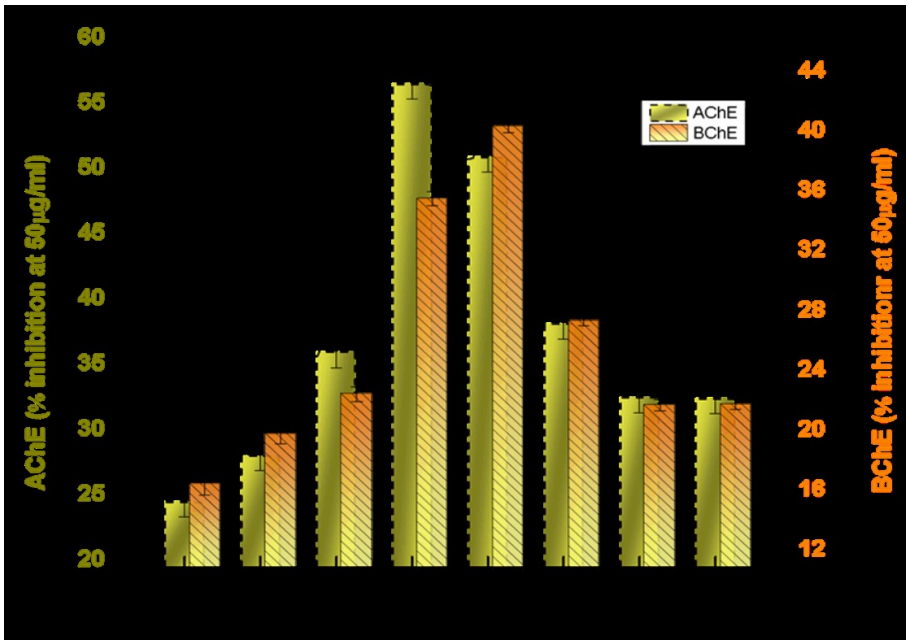

Figure 13: Rosmarinic acid AChE and BuChE inhibition efficiency of melatonin-treated I. rugosus callus cultures. Each sample was studied in triplicates and experiment was performed twice. Different letters indicate probability differences of 0.05 between the parameters

Hemolysis of Red Blood Cells

Hemoglobin in red blood cells are sensitive to the damage caused by ROS. Through auto-oxidation, it becomes methemoglobin (MetHb), resulting in the production of O_2. These oxidizing agents are responsible for many of the etiological factors (Harman 1992). Both products such as MetHb and O_2 from the oxidation are dangerous for the cells. Therefore, melatonin can reduce damage to biologically important molecules by decreasing the level autoxidation of ROS of the MetHb. According to the information presented in Table 2, the effect of melatonin on red blood cell damage was evaluated as a function of percentage he-

molysis factor. Sample A1 does not indicate the presence of any form of hemoglobin distortion, which determines the safe aspect of human use as compared to A2 and A3. Percentage hemolysis at 20 µg/mL; 8.1 %, 15 µg/mL; 3.3 %, 10 µg/mL; 0.09 %, 5 µg/mL; 0.1 % hemolysis. From the literature, Gilad and Zisapel (1995) demonstrated that melatonin is linked to hemoglobin and not directly to the heme group, which support the result of our study. In their results, they proposed that melatonin was connected with HbO_2 status. As a result, the efficiency of the erythrocyte defense system decreased. Oxidation can cause permanent defects that ultimately lead to the lysis of blood cells in in vitro conditions.

Table 2: Percent hemolysis activity of red blood cells of I. rugosus extract

Sr. No	Media (Sucrose 30g/l)	Combination of PCRs concentration (mg/l) control	Elicitor concentration (µm)	Nature of explant	Dry biomass after weeks (g)	Callus efficay (%)	Cailus color	Morphology of callus	Rate of callus formation
1	MS	TDZ 1.0+ NAA 1.3	1Mela	Stem	0.18	91	LG	C	++++
2	MS	TDZ 1.0+ NAA 1.3	2Mela	Stem	0.192	92	LG	C	++++
3	MS	TDZ 1.0+ NAA 1.3	3Mela	Stem	0.241	93	LG	C	++++
4	MS	TDZ 1.0+ NAA 1.3	4Mela	Stem	0.252	94	LG	C	++++
5	MS	TDZ 1.0+ NAA 1.3	5Mela	Stem	0.421	95	LG	C	++++
6	MS	TDZ 1.0+ NAA 1.3	10Mela	Stem	0.127	70	LG	C	++
7	MS	TDZ 1.0+ NAA 1.3	50Mela	Stem	0.108	70	LG	C	++
8	MS	TDZ 1.0+ NAA 1.3	100Mela	Stem	0.097	70	LG	C	++
9	MS	Control	---	Stem	----	---	---	---	--

A1; 5µMela, A2; pre-optimized concentration TDZ/NAA (1:3 mg/L), A3; whole plant extract

Conclusion

The findings of this study guesstimated the melatonin role in prompting phytoconstituents and therapeutic potential of I. rogosus in vitro cultures. Melatonin has been found to support callus induction and proliferation in stem explants which instead increase biosynthesis and overall metabolic activity when used within a moderate range. Immediately, enhanced phytochemical accumulation (TPC & TFC) was examined by HPLC, where predominantly rosmarinic acid, caffeic acid, plectranthoic acid, betulinic and oleanolic acid. Most likely, the cultures exhibited maximal free radical scavenging activities such as DPPH, FRAP (750.37 ± 12.98 µM TEAC) and ABTS (551.75 ± 13.43 µM TEAC), on the other hand, TRP (251.81± 0.59 µM AEAC) and TAC (60.33± 1.78 µM AEAC) contained optimal antioxidant enzymes at the same concentration. Furthermore, the therapeutic potential of enriched I. rogosus extracts was also evaluated. We hypothesized the inhibitory activity of polarized compounds showing α-amylase activity at 5 µM (42.50%). Among all the isolated metabolites, rosmarinic acid (IC50 µg / ml) can be considered a strong inhibitor against AChE and BChE at a low concentration of melatonin found in Alzheimer disease, for example. 4 Mela (µM) AChE 56.986 ± 1.12 and 5 Mela (µM) BChE 40.18852 ± 0.47. Furthermore, the therapeutic potential of enriched I.

rogosus extracts was also evaluated. Rosmarinic acid (IC50 μg/mL) among all the isolated metabolites was found as potent inhibitor against cholinesterases (AChE and BChE) found in Alzheimer disease. Besides, the raised cytotoxicity in HepG2 cell lines, reduced RBC hemolysis and alpha amylases were recorded when melatonin treated extracts were used. Overall, an effective and competent strategy was developed; expressing strong association of melatonin in both plant as well as human defensive responses. Thus, the present manuscript emphasized the role of the antioxidant capacity of melatonin in the reduction of diseases related to oxidative stress induced by free radicals.

Such decrease in antioxidant activities may due to the decline of antioxidant active components contents (i.e. total phenolics, total flavonoids and total flavanols) of grapes and wine

From a practical standpoint, this study was carried out to provide sufficient experimental evidence to prove such decrease in cytotoxicity and antioxidant activities may due to the decline of antioxidant active components contents result from the exogenously applied melatonin growth regulators

wine colour and flavour, and making fresh wine by means of improving cultivation practice and application of.

Conflicts of Interest

All authors of this document do not declare any conflict of interest.

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