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Determination of the Quantitative Phytochemical Screening and Antioxidant Activity of *Impatiens balsamina* L. Leaves and Flower under Heavy Metal Stress (Nickel)

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ABSTRACT

Secondary metabolites serve as a pivotal role in plant adaptation to the environment and recovery from stress and as well as provide therapeutically important metabolites for human health. Plants that are the -rapeutically important are known to be affected by various biotic and abiotic stress factors. Heavy metal is regarded as one of the most undesirable, among the various types of stresses, since it causes economic loss and as well as effects human health. Plants growing in heavy metal environments, on the other hand, create larger levels of active chemicals such as antioxidants that protect them from free radicals and reactive oxygen species and protect the photosynthetic process. This study was conducted to establish the effect of heavy metal (Nickel) on the *Impatiens balsamina* (a medicinal plant)antioxidant potential to nickel stress. Phenolics, alkaloids and flavonoids content in our study increased as compared to those in the control by two times. Presence of nickel increased the oxidative stress in the plant tissue up to 80%. Such biochemical responses to heavy metal stress in the leaf and flower of *I. balsamina* can be a source of novel metabolites that can be further explored for its therapeutic uses.

Keywords Heavy metal, *I. balsamina* L., Phytochemical, Antioxidant.

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INTRODUCTION

Heavy metal concentrations in the environment have increased as a result of industrial expansion and agricultural intensification. Heavy metals are only able to accumulate in ecosystems in increasing proportions due to their difficulty to biodegrade, high toxicity and ability to alter food chain movement, posing an ecological danger (Rai *et al.* 2019). Heavy metals in the environment can come from metallurgical and mining effluents, industrial wastewaters, motor transportation, fossil fuel burning, pesticides and fertilizers, among other things (Zwolak *et al.* 2019). According to Thakur *et al*. (2016) many plants have evolved mechanisms to mitigate the impact of such abiotic stresses in the environment and just 0.2% of plant species can store heavy metals, with high metal concentrations above ground and in the low soil content. Heavy metals that we are exposed to in our daily life may cause the excessive production of free radicals and cellular toxicity (Ceylan *et al*. 2019). Kausar

et al. (2018) showed that plant produced certain metabolites such as flavonoids, terpenoids, tannins and phenolic compound that work as potential compounds in eliminating stress and such compounds are important for the antioxidant activity. Plants have mechanisms for translocating and storing heavy metals that serve as micronutrients, for example, certain plants store Ni and Cu in their roots (Nematshahi *et al.* 2012) metals in bulk may also be found in stems and leaves (Rafati *et al.* 2011). It was reported that the heavy metals also effect the seedling stage, yield and induces reactive oxygen species (ROS)(Amari *et al.* 2017). Studies have been published on the effects of heavy metals on the formation of plant secondary metabolites (Behnam *et al.* 2017, Borges *et al.* 2017, Mousavi and Razavizadeh 2021).

But these plant secondary metabolites are known to improve the growth, especially in adverse environments (Zandalinas *et al.* 2017). There are reported studies that when plants get exposed to abiotic and biotic stresses such as salinity, UV radiation, heavy metals, drought, herbicides and pathogenesis alter the morphology, biochemical and antioxidant activity of the plants. In response to these stresses, plants also create alternative processes that operate as constraint tools (Bano *et al.* 2017, Fardiyah *et al.* 2020, Hosseini *et al*. 2021). According to a study by Fan *et al.* (2018) multiple genes are involved in the accumulation of metals like 'Cd' and 'As' in plants. Helena *et al.* (2020) found that when the *Cynara cardunculus* L. was exposed to abiotic factors such as salinity, drought, heavy metal and others, the synthesis of physiologically essential compounds like polyphenolic compounds and inulin was increased. Salt stress was reported to enhance the production of carotenoids in the plants *Daucus carota* and *Bixa orellana* (Sankari *et al.* 2019). There have been an increased levels of tannic acid, flavonoids and gossypol in cotton plants when exposed to salinity (Wang *et al.* 2016). Drought conditions also increase oxidative stress in Willow leaves, resulting in higher levels of flavonoids and phenolic acids (Larson 2018). Senescence was shown to be triggered by heavy metals by increasing ethylene production, followed by the jasmonic acid signalling (Keunen *et al.* 2016).According to Singla and Garg (2017) certain agent, such as UV-radiation, operate as elicitors and stress factors, resulting in increased synthesis of a variety of secondary metabolites.

Impatiens balsamina L., often known as Garden Balsam or Rose Balsam, is a member of the Balsaminaceae family (Kang *et al.* 2013). *I. balsamina* showed cadmium (Cd) and lead (Pb) tolerance and as well as their accumulation ability, indicating that it has potential for heavy metal phytoremediation (Wang 2005). This plant was also reported to remove several polychlorinated chemicals (Liu *et al*. 2020). Various plant parts of *I. balsamnia* were reported to be used in traditional medicine, for example aerial portions including leaves and flowers are utilized to treat articular rheumatism, abscesses and tumours (Imam *et al.* 2012, Li *et al.* 2015). Pharmacological studies have proven antipruritic, antidermatitic and antinociceptive effects as well as cyclooxygenase-2 inhibitory activity of the *I. balsamnia* flower extract (Oku and Ishiguro 2002, Kim *et al.* 2017) several studies have reported the antioxidant activity of this plant (Shivakumara *et al.* 2014). Previous phytochemical investigations of this plant identified different structural compounds including naphthoquinones, coumarins, phenolic acids, flavonoids, anthocyanidins, steroids and peptides (Thevissen *et al.* 2005, Bartomeus *et al.* 2010, Skálová Jarosik 2013).

The focus of this research was to look into the plant's response mechanism to heavy metal stress. This experiment was carried out not only with the goal of increasing plant secondary metabolite synthesis and antioxidant capacity, but also to gain essential bioactive chemicals that may be employed in medicine.

MATERIALS AND METHODS

Sample preparation

The leaves and flowers of *I. balsamina* were collected from the plant grown with the treatment of heavy metal and without heavy metal as mentioned in our previous study (Pandya and Nallanchakravarthula 2022) in publication. The seeds of the plants were treated with the heavy metal i.e., Nickel as in form of $NiCl_2$ termed as treated plant (T) and plant without inoculation of heavy metal termed as control plants

Table 1. Quantitative phytochemical analysis of secondary metabolites.

Extracts	Phytochemical test: Flavonoids content€ (µg/mg) Leaves	Flower	Akaloids content¥ (µg/mg) Leaves	Flower	Phenol content £ (µg/mg) Leaves	Flower
MC	29.33 ± 1.77[b]	27.24 ± 0.59[b]	271.25 ± 1.58[b]	262.64 ± 5.97[b]	11.74 ± 0.15[c]	17.1 ± 1.53[c]
MT	56.22 ± 0.89[a]	58.13 ± 1.64[a]	508.72 ± 1.09[a]	520.75 ± 6.01[a]	67.11 ± 0.62[a]	70.1 ± 5.03[a]
HC	12.44 ± 1.16[d]	25.72 ± 1.10[b]	66.23 ± 6.89[d]	196.45 ± 2.66[d]	11.82 ± 0.18[c]	15.55 ± 0.18[c]
HT	6.18 ± 0.71[c]	27.13 ± 0.71[b]	213.56 ± 2.66[c]	237.27 ± 6.89[c]	24.9 ± 0.65[b]	29.46 ± 1.87[b]

(C). The concentration of metal is 100 mg/kg of the dry weight of soil is added according to Gopal *et al.* (2014). The leaves and flowers of *I. balsamina* were washed with water and dried at room temperature, later the dried leaves and flower were powdered with pestle and mortar.

Solvent extraction

Powdered plant material (flower and leaf) was extracted with different solvents (hexane and methanol) using Soxhlet extraction in accordance with Alara *et al.* (2018) with some modifications. Leaves and flower of *I. balsamina* were weighed and the respective solvents were used in feed-to-solvent ratio (1:1, i.e., 100 g of plant material was suspended with 100 ml of their respective solvents and was placed in the extraction apparatus). Then, the extract was filtered through a filter paper (Table 1) and concentrated to dryness using a rotary evaporator. The extracts were stored in a refrigerator at 4°C until further analysis.

Quantitative analysis of secondary metabolites

Total flavonoid content (TFC)

It was performed according to Sathish kumar *et al.* (2013) with some modifications. Total flavonoid content was estimated and expressed as mg Quercetin / g of plant tissue. For flavonoid estimation 1mg/ml of the plant extract was taken and 0.3 ml of 5% sodium nitrite ($NaNO_2$) was added. 3 ml of 10% aluminium chloride ($AlCl_3$) was added, it was shaken well for 5 minutes. 2 ml of 1M sodium hydroxide (NaOH) was also added and after 6 minutes the absorbance was recorded at 510 nm. The absorbance of the mixture was measured using UV-Spectrophotometer against blank.

Total phenolic content (TPC)

Total phenolic content (TPC) determination was carried out following the protocol described by Ainsworth and Gillespie (2007). 100 µL of plant extracts were mixed with 200 µL of 10% (v/v) Folin-Ciocalteu reagent and incubated for 2 min at room temperature in the dark. Next, 800 µL of 0.7 M sodium carbonate was added and the samples were incubated for 2 h at 25°C in the dark. The absorbance was measured at 765 nm against a blank and a calibration curve was performed by using Gallic acid as standard. Results were expressed as Gallic acid equivalents (GAE) in mg/g dry weight. All experiments were carried out in triplicates.

Alkaloid content

The total alkaloid content was determined according to UV Spectrophotometer method (Manjunath *et al.* 2012). This method is based on the reaction between alkaloid and bromocresol green. The extract from the plant parts (flower and leaves) were dissolved in 2 N HCl and then filtered. 1 ml of this solution was transferred to a separate tube and washed with 10 ml chloroform. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. 1 ml of this solution was transferred to a separate tube and then 5 ml of bromocresol solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was fractioned with chloroform by vigorous shaking. The fractions were collected in a tube and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. All experiments were performed

in triplicate; the results were averaged and reported in the form of mean ± SEm (Standard Error Mean).

***In vitro* anti-oxidant activity of plant extract**

Reducing antioxidant power

The reducing power of *I. balsamina* leaf and flower were determined using the protocol of Díaz *et al.* (2011) with some modifications. Different concentrations of the leaf extract, ranging from 0.2mg to 1mg mixed with 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of potassium ferricyanide. The mixture was incubated at 50˚C for 20 min and centrifuged at 10,000 rpm for 10 min. The supernatant was separated out and mixed with 1.5 ml distilled water and 0.3 ml (0.1%) ferric chloride. 10% Acetic acid was used as a positive control, blank solution was prepared with all reagents, except the plant extract. The absorbance of the mixture was measured using UV-Spectrophotometer against blank.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Assay

The antioxidant activity of plant extracts was measured using 1, 1- diphenyl, 2-picryl hydrazyl (DPPH) (Veeru *et al*. 2009). DPPH of 0.1 mM solution was prepared in the methanol, then 1 ml of DPPH stock solution was mixed with 1ml of plant extract solution of different concentrations (100, 250, 500, 750 and 1000 µg/ml). The mixture of 1ml methanol and 1 ml DPPH stock solution was used as control. Ascorbic acid was used as the standard reference compound with same concentration. The reaction was incubated at room temperature and absorbance was measure by UV-Spectrophotometer at 517 nm. The inhibition percentage was calculated using following formula:

DPPH scavenging effect (%) = ((Abs control − Abs sample)/Abs control) X 100

Abs C- Absorbance of control

Abs S- Absorbance of test sample

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

The antioxidant capacity was estimated using the technique described by Re *et al.* (1999). ABTS radical cation (ABTS•+) was produced by reacting ABTS solution (7 mM) with ammonium persulfate (2.45 mM) and the mixture was maintained in the dark at room temperature for 12–16 h. Different concentrations of each extract (methanol and hexane) (100–1000 µg/ ml) were assayed. Quercetin (0.5–5 µg/ml; Sigma-Aldrich) were employed as positive controls. The solvent was used as a negative control. The reactive mixture was allowed to stand at room temperature and absorbance was recorded at 734 nm. The results were expressed in milligram equivalents of quercetin per milligram of dry weight.

% ABTS radical scavenging = $((A_C - A_T) / (A_C)) \times 100\%$

A_C- Absorbance of control

A_T- Absorbance of test sample

RESULTS AND DISCUSSION

Quantitative analysis of secondary metabolites

The effects of heavy metals on medicinal plant associated phytochemicals are less studied when compared with crop plant species (Ahmadi *et al.* 2020, Ammar *et al.* 2017). The phenolics and flavonoids of plants were reported to have antibacterial and anti-inflammatory effects and are widely used in medicine (Petukhov *et al.* 2021, Edward *et al.* 2020, Hadadi *et al.* 2020). Total phenolic, flavonoid and alkaloids content (*In vitro*) was determined for methanol and hexane extracts of the aerial parts (leaves and flower) of *I. balsamina*.

The presence of 'Ni' has increased (5-473%) the phytochemicals evaluated in the present study except flavonoids in leaf extract (50% decrease). There was an effect of solvent on the phytochemicals, for e.g., there was a 50% decrease in the flavonoid content of hexane leaf extracts in comparison with an increase of more than 90% in methanolic extracts.

The phenolics in the methanolic leaf extracts was shown to increase by 473% due to the presence of 'Ni', followed by 309% same solvent extract from

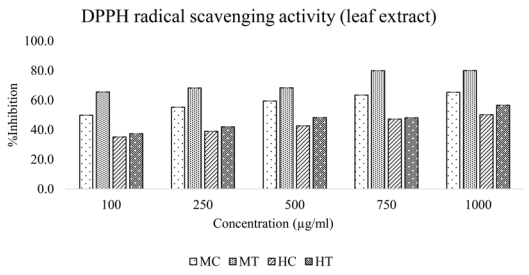

Fig. 1. DPPH radical scavenging activity of leaf extract. Data were expressed as the mean±SE of three independent experiments (n=3) and were analyzed by one-way ANOVA ($p<0.05$).

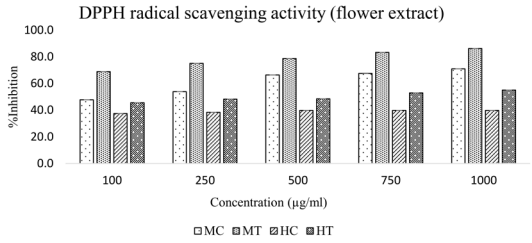

Fig. 2. DPPH radical scavenging activity of flower extract. Data are expressed as the Mean±SE of three independent experiments (n=3) and were analyzed by one-way ANOVA ($p<0.05$).

flower. There was also negative effect for e.g., there was a 50% decrease in the flavonoid content of hexane leaf extracts.When the results of control and treated plant extract (flower and leaf) are compared, the difference between control and treatment is considerable. However, there is no significant difference between control and treatment in flavonoid concentration of flower extract of hexane extract.Singh *et al.* (2016) has shown that the highest amount of phenol was obtained in the ethyl acetate extract of *Impatiens sulcata* when compared with extracts (petroleum ether and methanol). There was also evidence that phenolic metabolism induction occurs as a response to metal stress and phenolic compounds have powerful antioxidative activities in the heavy metals tressed plant (Dunja *et al.* 2021, Palistha *et al.* 2021). In the chemical structure of *I. balsamina*, the antioxidant action is established mainly (Singh and Malik 2011). Król *et al.* (2015) showed that tomato plants exposed to the cold stress, production of the phenolic content get decreased. Petridis *et al.* (2012)also showed that the production of the secondary metabolites was cultivar dependent as well as duration of cultivation. Dursun *et al.* (2019) showed in an experimental study that the production of major phenolic compounds gets decreased when a tomato plant was exposed to the Cd and Pb stress.

Flavonoids possess a wide range of bioactivities including antioxidant activity. The presence of hydroxyl groups in the chemical structure of flavonoids is responsible for their antioxidant activity (Lijun *et al.* 2011). Kang *et al.* (2013) has shown that in *I. balsamina* the number of flavonoids and phenol is higher in the leaves than that of the stem. Both phenolic and flavonoid compounds from *I. balsamina* L. are known to have diverse biological activities and may also be responsible for the radical-linked antioxidant effects of *I. balsamina* L.. Therefore, these results indicate that high flavonoids and phenolic compounds in leaf extracts may account for their strong antioxidant and antimicrobial activities (Reanmongkol *et al.* 2003). *Hypericum perforatum* physiological responses to Lanthanum and Cadmium excess in different tissues (shoots and roots) were recorded and the results showed a general raise in some phenolic acids (e.g., ferulic acid) and on the contrary, a decrease of flavonoids (e.g., epicatechin and procyanidin) were reported (Babula *et al.* 2015). The present study revealed that the presence of Ni increased the alkaloid amount in the plant parts and the highest value is found in the methanolic extracts of the flower. Cetin *et al.* (2014) showed that in plant *Vitis vinifera*, alkaloids increased in the presence of Cd stress. According to Tiong *et al.* (2013) the production of alkaloids increased in presence of heavy metal stress to protect the damaged caused by ROS which is due to antioxidant potential. Soleimani *et al.* (2019) showed that in plant *Narcissus tazetta* the exposure amount of Cd increased the production of alkaloid.

In vitro anti-oxidant activity of plant extract

Many phytochemicals have been discovered to exhibit antioxidant effects due to hydroxyl groups in their structural formulas, making plants the primary source

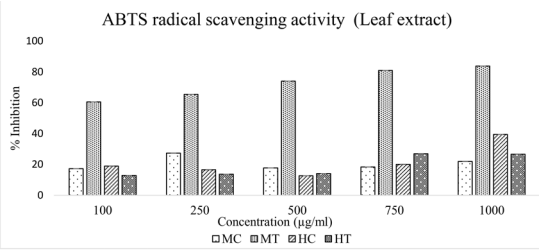

Fig. 3. ABTS radical scavenging activity of leaf extract. Data are expressed as the Mean±SE of three independent experiments (n=3) and were analyzed by one-way ANOVA ($p<0.05$).

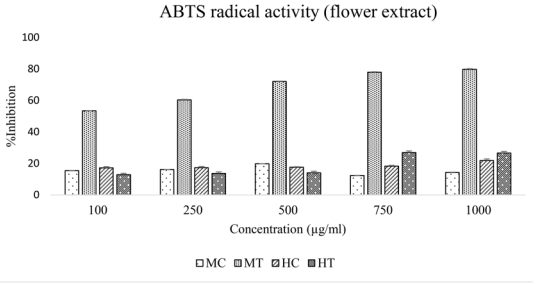

Fig. 4. ABTS radical scavenging activity of flower extract. Data are expressed as the Mean±SE of three independent experiments (n=3) and were analyzed by one-way ANOVA ($p<0.05$).

of natural antioxidants and their primary function is to protect the immune system from oxidative stress caused by free radicals (Abbas *et al.* 2014).

The DPPH radical has been widely used to test the free radical scavenging ability (FRSA) of various natural products and has been accepted as a model compound for free lipids-originating radicals (Da Porto *et al*. 2000).DPPH radical scavenging activity was used to measure the antioxidant activity of the I. balsamina leaf and flower methanolic extracts to the heavy metal treatments (Figs. 1 and 2). Ibrahim *et al.* (2017) depicted in their study that there was a positive correlation between antioxidant activity and secondary metabolites production as well as antimicrobial activity when plant is treated with single (Cadmium)heavy metal but if the combination of the heavy metals (Cadmium and Copper) were used the activity get decreased. According to Ali *et al.* (2018) the DPPH radical scavenging activity of the stressed plants may increase due to the production of some secondary metabolites like phenol, flavonoids. Our results are in the similar trend as of those obtained by (Taie *et al*. 2019, Zhao and Yang 2008) that DPPH radical scavenging activity was higher in Ni exposed plants as compared to control plants.Waliullah *et al.* (2019) showed that the plant *Datura alba*, methanolic extract of the stem exhibited the highest antioxidant activity due to the high amount of flavonoids presence at the 50µg/ml concentration of plant extract.

Figs. 3 and 4 depict the results for the ABTS radical assay for the methanolic as well hexane extract of the treated and control plants. For the leaf and flower exract the radical scavenging activity of the extract is increased for the treated plants sample in the concentration dependent manner. For leaf and flower of the treated plant, at the concentration of 1mg/ml, it shows inhibition up to 80%.In ABTS assay, antioxidant molecules quench the free radicals and decrease the intensity of color by donating an electron and providing hydrogen atoms (Patrikakou 2015). Shalini *et al.* (2020) showed that plant has high phenol content induced the high flavonoid content in the plant and confirmed the high ABTS activity. Tahir *et al.* (2021) has shown that in butanol extract of leaves of plant *Chromolaena odorata* L. the ABTS activity is found to be higher as the concentration is increased. In the study of Bari *et al.* (2021), results shown that leaf methanolic extract of *S. calendulacea* showed the ABTS scavenging activity up to 75%. Patel *et al.* (2019) shown in their study that husk of the Psyllium plant have ABTS radical scavenging activity ranging from 50.62 to 66.44%. According

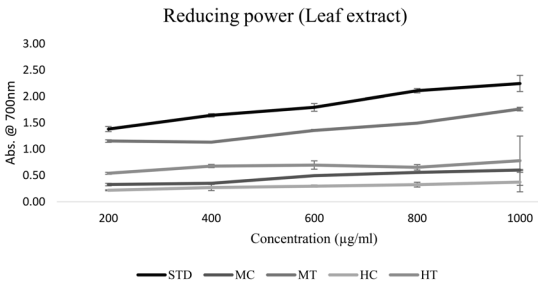

Fig. 5. Reducing power assay of leaf extract. Data are expressed as the Mean±SE of three independent experiments (n=3) and were analyzed by one-way ANOVA ($p<0.05$).

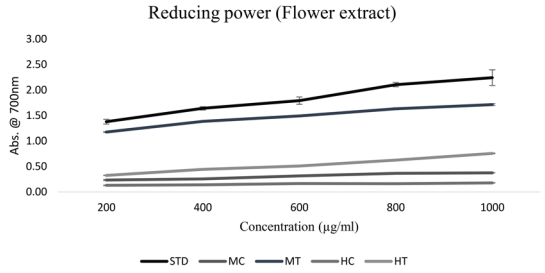

Fig. 6. Reducing power assay of flower extract. Data are expressed as the Mean±SE of three independent experiments (n=3) and were analyzed by one-way ANOVA ($p<0.05$).

to Insan *et al.* (2020) when plant extract is exposed to high temperature high ABTS radical scavenging activity was reported.

Stress exposed plant extractexhibited high reducing power activity. The reducing power activity is mainly due to redox properties and play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang 2001, Afolayan *et al.* 2007). The methanol extract of the treated plant showed the highest reducing power as compared to other extract. Highest reducing power activity was seen at 1000 µg/ml. Increasing activity indicates dose dependent properties of the extract. As the dose increases, antioxidant activity also increases.Shivakumara *et al.* (2014) showed that the extract from the seed of *I. balsamina* L. have highest reducing power at the concentration of 500 µg/ml. Result of reducing power presented in the Figs. 5 and 6 which illustrated that the heavy metal stress increased the reducing power capacity of the plant. Many studies have confirmed the intricacy of the reaction between reducing potential and some metabolites, particularly when the plant is grown under stress (Velarde *et al.* 2012, Pottosin *et al.* 2014). Sofidiya *et al.*(2008) showed that high amount of phenolic compound in the plant showed the high reducing power capacity.

CONCLUSION

This study was devoted to determining the effects of Ni on the secondary metabolites production and antioxidant activity in the medicinal plant *I. balsamina* to predict the effect on the efficacy of this plant. It was found that treatment of heavy metal (Ni) had the influence on the metabolites production as well on the antioxidant activities. The production of biologically important metabolites was observed to be increased compared to control plants under the exposure of the stress. The antioxidant potential was highest under the heavy metal exposure.

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Values are expressed as the means±SE of three replicates from three independent experiments. Where; MC-Methanol control, MT-Methanol treatment, HC-Hexane control and HT- Hexane treatment, '€'-mg Quercetin/gm of extract, ¥-mg Atropine/g of extract and £-mg Gallic acid/g of extract values with a different letter, the difference is statistically significant ($p<0.05$) from each other evaluated from DMRT.

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