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ORIGINAL ARTICLE

Chemical Fingerprint Based Involvement of Plant Metabolites and Osmoregulatory Solutes in Providing Abiotic Stress Tolerance to Invasive Plant *Lantana camara*

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Background: Invasive alien species (IAS) are broadly distributed all over the world by disturbing or reducing the growth and development of native vegetation. *Lantana camara* is basically a noxious weed and have a potential to invade a region's indigenous vegetation. Even after knowing all its harmful effects, there has been a little research on various mechanisms followed by this plant to harm the plant species.

Results: Random sampling was performed to take plant samples without any bias, to study various mechanisms carried out in plant species. Chemical fingerprinting of samples were then performed in various abiotic stress conditions (cold and hot) to study changes in *L. camara* under these stressful conditions in order to find the reason behind the invasiveness of this plant species. Stress indicator like malondialdehyde (MDA)/lipid peroxidation was also performed and increased lipid peroxidation during both extremities showed that plant is experiencing oxidative stress. ROS imaging was performed on the leaves of *L. camara*, which also showed rise in ROS staining during extreme conditions. Elevated peaks (major and minor) and detection of secondary metabolites like glycine betaine in chemical fingerprinting observed under stressful conditions showed that plant may produce some increased level of metabolites in stress conditions that might play a role in minimizing the oxidative stress that plant is facing.

Conclusions: On the behalf of obtained results, it can be assumed that *Lantana camara* has the capability to survive in or tolerate extreme environmental abiotic stress conditions by producing or agglomerating various stress-reducing metabolites.

Key words: Chemical fingerprinting, Chromatograph, Invasiveness, Lantana camara, Lipid peroxidation, Secondary metabolites

Invasive alien species are the plants which have been accidentally escaped from their natural habitat and introduced into the ecosystems which are non-native to them. They have broad distribution all over the world in natural and agricultural areas, and are trying to create its own dominating world by displacing native vegetation (Henkel et al. 2009). Out of many plants being introduced to a new habitat, only a few become invasive which suggests that there is existence of some special characters or traits which enable invasiveness in particular plant. If we talk about various threats to global biodiversity, invasion is one of them majorly and is very widespread. There are certain definitions of invasive species known yet, which are given by different organizations which are engaged in invasion research. It is basically the potential of invasive species that make them so strong invaders and colonizers of the environment, whether introduced with will or by chance. It is still a matter of debate that why the invasive alien species are so invasive.

Various environmental factors are there which adversely affect plant's growth and limits plant production, drought, nutrient imbalance, high temperature, etc. are the major factors among them. Changes in temperature occur rapidly as compared to other changes in stress such as salinity, drought, etc. With the help of nature, plants have developed various strategies to tackle certain stress conditions. It is already known that the acquisition of large number of natural products entirely depends on their growing conditions like temperature, light and nutrient supply (Falk et al. 2007; Ballhorn et al. 2011). Different stressful conditions will also influence the metabolic pathways responsible for the acquisition of various secondary plant products, that further leads to biochemical changes in plants (Scheel et al. 2016). Variety of experiments have shown that plants under stressful conditions accumulate high level of secondary metabolites like in complex phenols, numerous terpenes (Selmar et al. 2013). Therefore, there is no doubt that onset of various stresses enhances the level of secondary plant products.

The fingerprint techniques are one of the modern approaches for analyzing several metabolites (Grauwet et al. 2014; Esslinger et al. 2014). Chemical portrait given by fingerprinting is representative of ecosphere where plant grows, evidencing secondary metabolites pattern being driven in quantity and amount of compounds by the sensitivity of plants to ecological variables. The plant fingerprints might help in bio-monitoring and biological research. Chemical fingerprinting of plant's various secondary metabolites could be performed with the help of different methods, that provide a graphical representation of the qualitative changes in secondary metabolites. Chromatograph approaches are obviously the most useful tool to reach the fingerprint target in terms of sensitivity, and even they are fast with high information content. The advantage of using this technique is to study the changes in chromatographic pattern without knowing the components of an unknown compound (Dunn and Ellis 2005). Chemical fingerprinting using chromatography is an important tool to study different patterns of plant metabolic variations in different conditions. Chemical fingerprinting coupled with chromatography separation is used for analyzing various components, that are existing in number of traditional medicines and various medicinal plants (Sermakkani et al. 2012).

Recently, biological insights have also been obtained from fingerprinting analysis of abiotic stress (Bailey 2003). These techniques are proved to be useful for analysis of the metabolic profile of plants under various environmental stress like conditions. Kuleff et al. (2003), also made a mention that by using chemical fingerprinting, plants can be demarcated on the basis of their species and geographical strain. It has various applications:(a) evaluation of different chemical components in different stress conditions; (b) the possibility to compare different groups of plants.

Lantana camara is a noxious and ornamental weed form family *Verbenaceae* and it is commonly known as red sage or wild sage is an evergreen strong smelling shrub (Thamotharan 2010) native to the American tropics. It has been expanding in various regions of the world and also includes India. There is very little knowledge about the various mechanisms involved in the tolerance of *Lantana camara* to stressful environmental conditions. Knowledge of these mechanisms of this invasive plant may give us idea

about its growth and development in variety of areas under stressful conditions which can help us in controlling the growth of *L. camara*. Thus, the present study was carried out in order to know the possible cause of vast adaptability and invasiveness of the plant *L. camara*. It was hypothesized that there is a production of various cell metabolites in these plants in response to stress conditions which provide adaptation against stress. Therefore, we have studied the changes in chemical fingerprint profile using AKTA prime Plus chromatography system to obtain chemical fingerprints of Lantana under different temperature conditions in their natural environment.

MATERIALS AND METHODS

Plant material

The samples (leaves/flowers, in 10 batches) of *Lantana camara*: pink and violet that were at reproductive stage and growing in natural conditions, were collected from four different areas of Jalandhar. Varieties of the plant species can be differentiated by its flower color. Area of Jalandhar experiences three different type of seasons in year i.e. low temperature (February), moderate temperature (March), high temperature (April) (Table.1). This region experiences extremities of cold and hot. Jalandhar is located at a latitude of 71°31' east and longitude of 30°33' north. The experiments were conducted from February 2018 to April 2018. The temperature of the days was recorded (given in table). Random sampling was then conducted so that the study remain unbiased. Plant samples were pooled together for analysis. The sampling sites were marked previously so that no bias could occur in our study.

Extraction

The collected tissues of *Lantana camara* (leaves and flowers, 5g) were homogenized in 15ml of each of the solutions (1,2-Dichloroethane/Acetone/Ethanol/ Methanol/Water). The crude extracts were then centrifuged for 15min at 10,000rpm at room temperature. The supernatant was collected. An appropriate aliquot of each extract was filtered in 0.2µm (PALL Life Sciences) syringe filters, stored in 2 ml storage vials at -20°C prior to further analysis.

Chemical fingerprinting

Metabolic fingerprint analysis above filtered supernatants was carried out using AKTA prime PLUS system (GE healthcare, Sweden), equipped with gradient pump, auto sampler, and Prime view 5.0 software for data acquisition and processing. The analysis was carried out using HiTrap™ column (5 x 5 ml)with flow rate 0.5 $mLmin^{-1}$, operating pressure, 1MPa (145 psi). The fingerprints were registered at wavelength λ = 254 nm and an aliquot of 1 ml was injected for acquiring chromatograms. A pre-equilibration period of 10 min with 20% ethanol was used between individual runs.

Identification and Quantization

The eight chemical standards — Rutin (in methanol), Phenylalanine (in distilled water), Glutathione Reduced (in distilled water), Glutathione Oxidized (in distilled water), Tannic acid (in ethanol), Glycine Betaine (in ethanol), Proline (in ethanol) and Vanillic Acid (in methanol) were prepared in appropriate solvents. The concentrations of standards used for calibration were 5, 10, 15, 20, and 25 (mg/ml). The standard curves were performed by analyzing standard reference solutions in triplicate and calibrated using linear regression equation derived from the peak areas. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte at S/N of 3 and 10, respectively. All the solutions were filtered through 0.2µm filters prior to analysis. Identification of analytes namely Glycine Betaine, Rutin, Phenylalanine, Glutathione Reduced, Glutathione Oxidized, Tannic Acid, Proline and Vanillic Acid was conducted by comparing their retention times with those of the extracts. Quantization was established by preparing calibration curves with pure analytes.

Lipid Peroxidation (LP) and ROS staining

LP was estimated by measuring malondialdehyde (MDA) level. MDA content was calculated by given equation: MDA (μMgm^{-1} FW) = $[(A_{532} - A_{600}) / 156] \times 10^3$ x dilution factor. Histochemical detection of ROS (H_2O_2) was done by using 3,3'-Diaminobenzidine (DAB) as the chromogenic substrate. Plant material was collected at random (n=10). The leaves were immersed in DAB (1mg/ml) staining solution for detection of H_2O_2 The petri plates were wrapped in aluminium foil and kept

overnight at room temperature. H_2O_2 was visualized as reddish brown stains formed on the leaves by the reaction of DAB with the endogenous H_2O_2.

RESULTS AND DISCUSSION

Invasive alien plants have encroached various ecosystem worldwide and affected biodiversity and crop productions at large. However, their biological mechanism to tolerate abiotic conditions under natural environment is still a matter of conjuncture. It is well known that major environmental stresses like heat, cold, drought dramatically affect metabolite synthesis through the synthesis of physiological and biochemical responses in plants. For plants, any external factor that exerts negative influence and produces changes and responses may be considered stressful. Being naturally present in plant (leaves and flowers), secondary metabolites and derivatives can be analyzed and used as stress indicators. In this study the effect of abiotic stress like cold and heat under natural conditions on changes in metabolic profiles by chemical fingerprint was studied in invasive alien plant *Lantana camara*.

Lipid peroxidation and ROS imaging

Lipid peroxidation in the cell membranes is said to be one of the most challenging and detrimental effect of water stress in the membranes of all the cells exposed to varied degree of stress (Thankamani et al. 2003). The degree of lipid peroxidation measured in terms of MDA content which is one of the determinants indicates the severity of stress experienced by any plant. In present study, exposure to both the temperature extremities-high temperature (April) and cold temperature conditions (February) caused increased levels of lipid peroxidation in leaves of both the varieties (pink and violet) and in flowers of pink variety of plant as compared to moderate temperature (Fig. 1), indicated that plants have experienced oxidative stress being caused by abiotic stress factors like cold and heat. MDA is generally considered as sensitive marker to assess oxidative stress induced membrane peroxidation (Goel and Sheoran 2003). Similar results were obtained by Sharma and Mamik (2017) in *Parthenium hysterophorus* and Lu et al. (2008) in *Eupatorium sp*. Histochemical studies also validated the occurrence of oxidative stress as enhanced level of ROS was detected after staining leaves of both varieties (Fig. 2). ROS are important molecules regulating numerous physiological and biochemical processes in the cells and can cause lipid peroxidation (Vranova et al. 2002). As with every mechanism involved in both normal functioning and upon the occurrence of stress, strategies to counteract ROS must take into account their critical importance in the normal functioning. ROS production particularly H2O2 in different cell compartments in response to environmental stress is well established (Apel & Hirt 2004). This ROS could be expected to be involved in stress signalling as well as in other important metabolic pathways conferring stress resistance to the plant.

Chemical fingerprint analysis

In the present study, Chemical Fingerprinting Profile of flowers and leaves of both the varieties of *Lantana camara* under different temperature regimes were studied in four different solvents: 1,2-Dichloroethane, Acetone, Ethanol, Methanol. As the three solvents, Acetone, Ethanol & Methanol did not give much information about the chemical fingerprint profile of this plant; we have not proceeded with these three solvents. Therefore, our whole study of Chemical Fingerprinting is based on the solvent - 1,2-Dichloroethane (Fig.3-4). Chemical fingerprint analysis of *L. camara* extracts (both leaves and flowers) revealed substantial amount of significant secondary metabolites as evident from chromatogram peaks (Fig. 3, Table 2) under adverse abiotic conditions. Results also indicated that the contents of secondary metabolites varied greatly among *Lantana* varieties samples collected in different environmental conditions. Different fingerprint profiles among both varieties were quite expected. It is plausible that plants collected at different times may considerably differ in chemical components, thereby, resulting in different profiles. It is generally believed that these differences are essentially caused by various environmental conditions where the plants are grown and harvested. In case of pink leaves, number of chromatographic peaks (major and minor) was detected in environmental condition dependent manner (Fig. 2). For instance, under both adverse conditions as compared to moderate temperature, although numbers of peaks were less, however, total area under peak was substantially higher by many folds (Table 1).

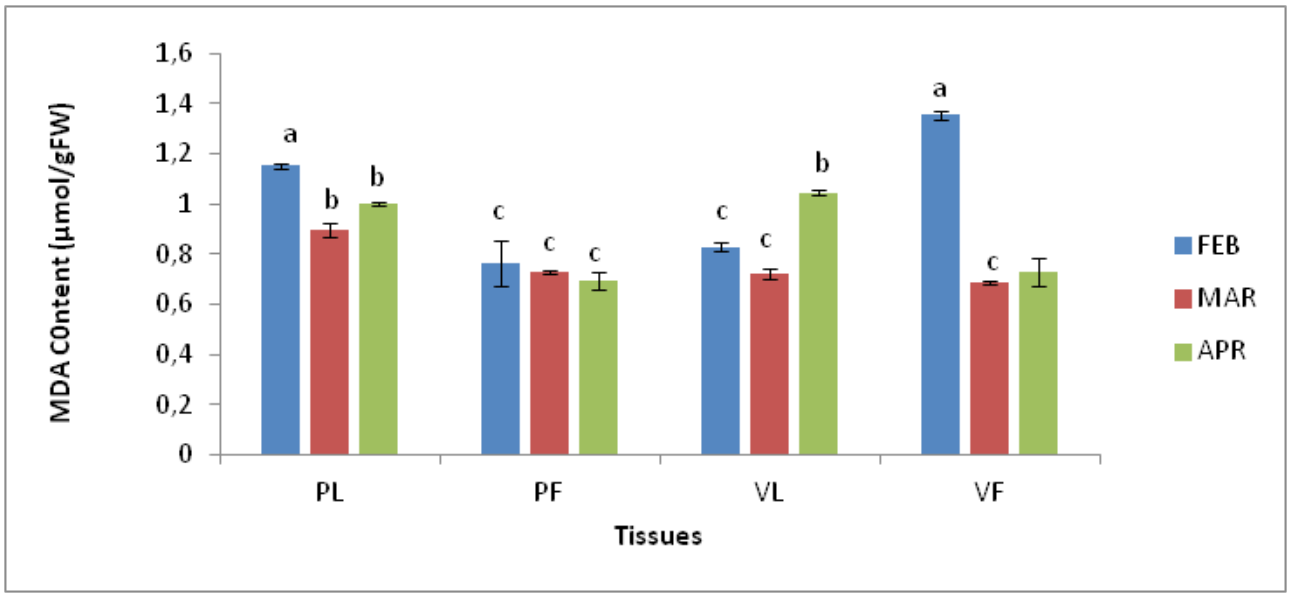

Figure 1: MDA Content in leaves and flowers of *Lantana camara* under conditions of different temperature regimes. Symbols used PL=Pink leaves, PF=Pink flowers, VL=Violet leaves, VF=Violet flowers. Values are means of three replicates±SE. Means with different letters are significantly different at P<0.05 using Tukey's multiple range test.

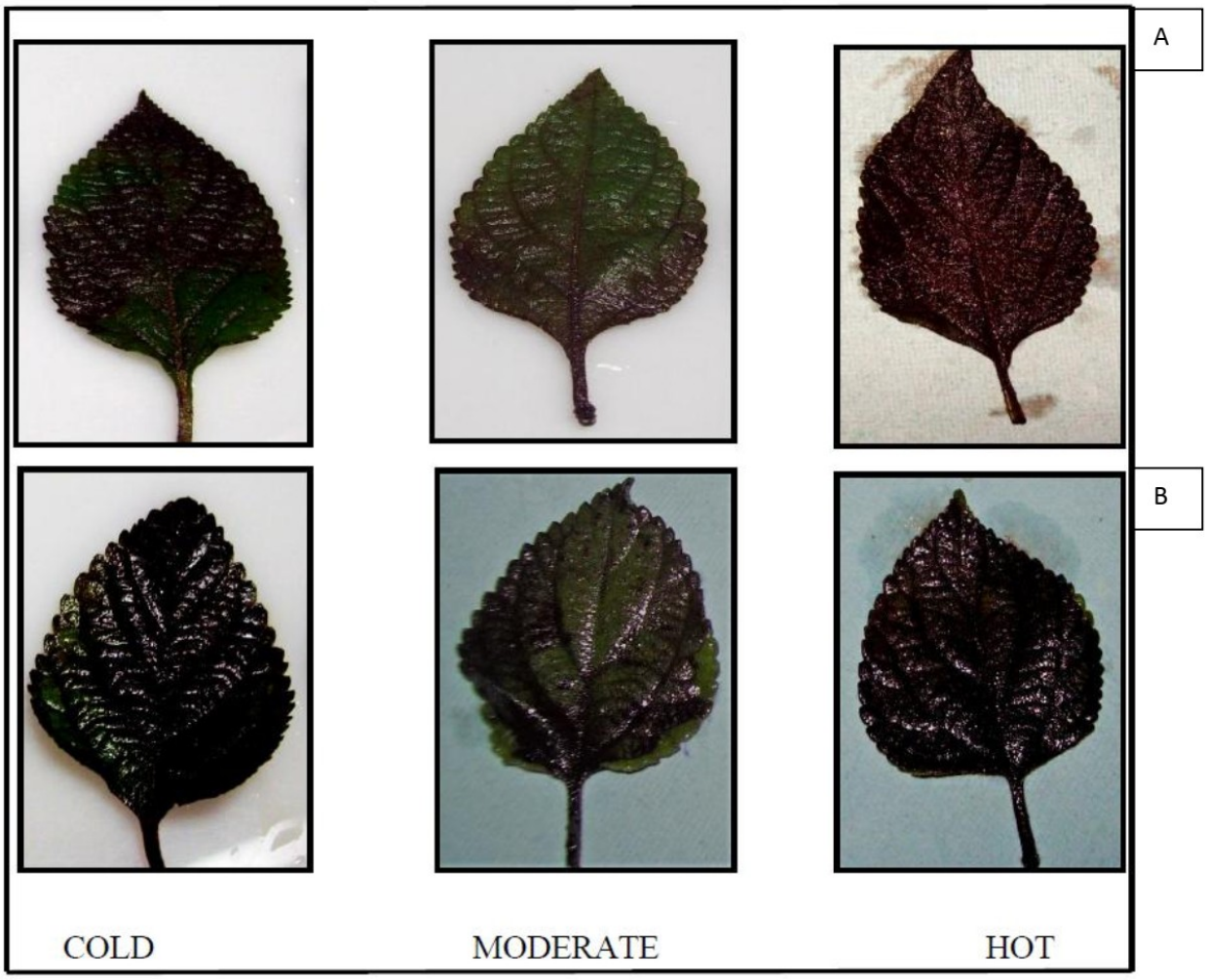

Figure 2: Representative image of ROS histochemical staning of leaves of *Lantana camara* Pink (A) and Violoet varieties (B). [n=10]

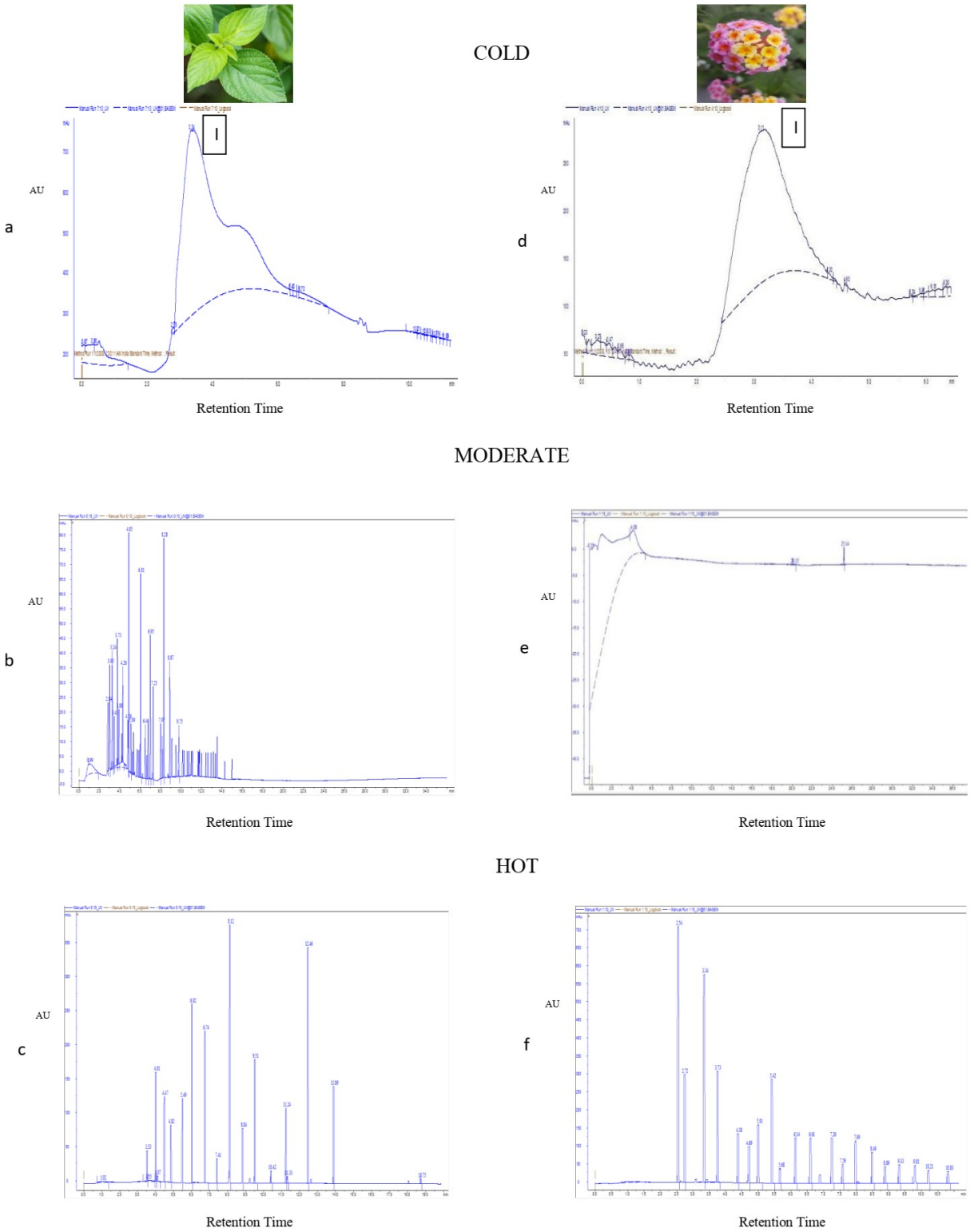

Figure 3: Chemical Fingerprint Analysis of Pink leaves (a, b, c) and flowers (d, e, f) extracted in 1,2-Dichloroethane in the three different temperatures, where 'I' represents Glycine betain (having content 8mg/gFW in pink leaves and 9.7mg/gFW in pink flowers).

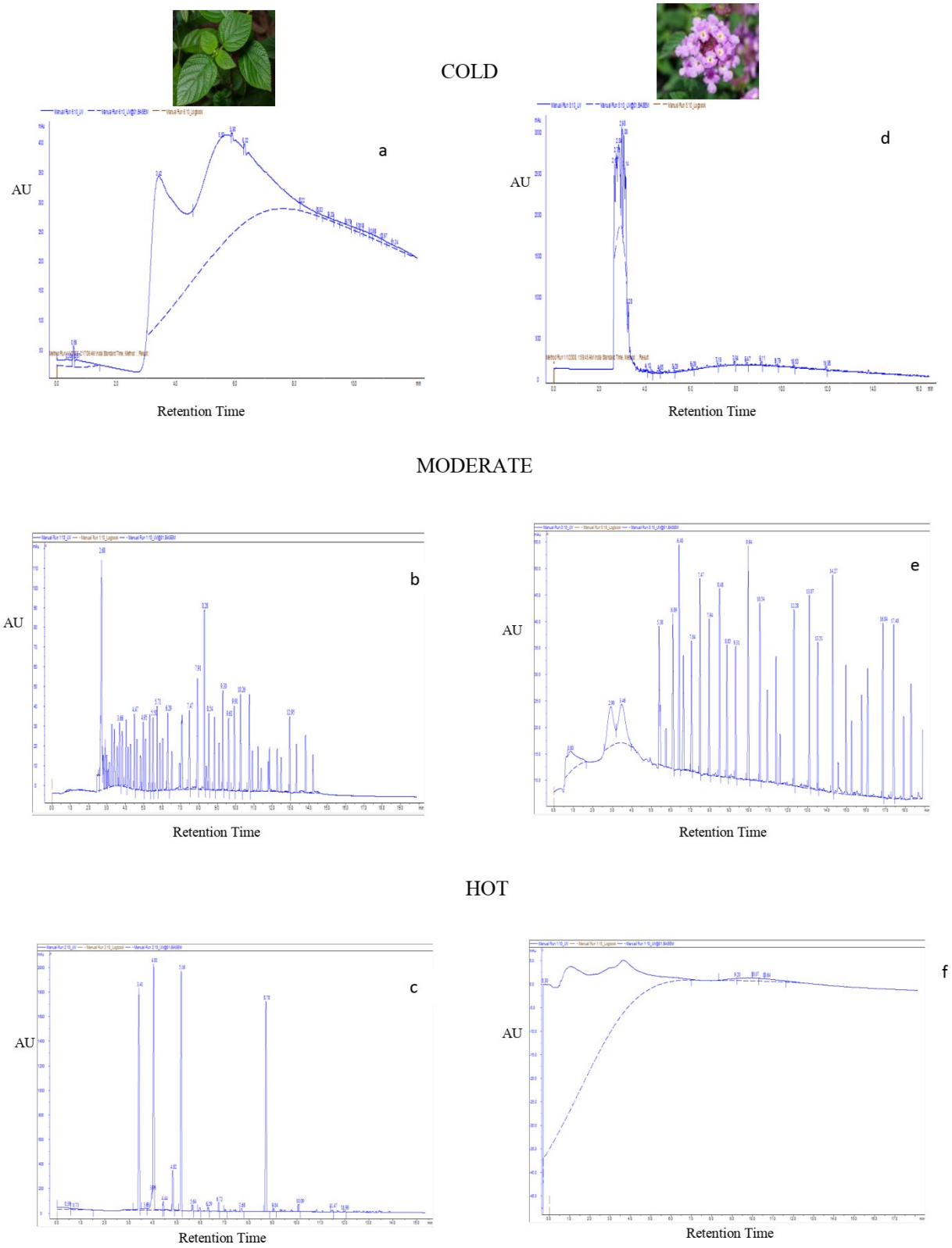

Figure 4: Chemical Fingerprint Analysis of Violet leaves (a, b, c) and flowers (d, e, f) extracted in 1,2-Dichloroethane in the three different temperatures.

Table 1: Temperature and day length during different months in Jalandhar area.

Month	Max. Temperature (C)	Min. Temperature (C)	Day Length
February	19°	5°	9h 47min
March	25°	14°	12h 13min
April	40°	26°	13h 18min

Table. 2: Chemical fingerprinting data of two varieties (pink & violet) of *Lantana camara* extracted in 1,2-Dichloroethane

S.No.	Variety	Temperature	Total no. of detected peaks	Total Area (mAu*min)	Ratio (Peak Area/Total Area)
1.	Pink Leaves	Cold	20	710.05	0.9994
		Moderate	241	48.90	0.7097
		Hot	158	113.61	0.9832
2.	Pink Flowers	Cold	20	166.99	0.9998
		Moderate	225	68.61	0.9818
		Hot	89	167.96	0.9734
3.	Violet Leaves	Cold	32	669.80	0.9941
		Moderate	167	84.72	0.5786
		Hot	95	435.89	0.9627
4.	Violet Flowers	Cold	178	525.85	0.8987
		Moderate	148	46.47	0.7372
		Hot	131	655.53	0.9696

Notably, under cold conditions, one major peak was detected with very large height and area which was absent in moderate temperature, indicating enhanced accumulation of metabolites under stress conditions. In pink flowers, as compared to moderate temperature, many minor and a very few major peaks were detected in adverse conditions (Fig. 3). Notably, under cold conditions, major peak having high peak area was detected which was totally absent in sample being collected under moderate temperature conditions. Similarly, in hot conditions, many minor peaks with high peak area were detected. Fingerprint data of violet leaves and flowers also revealed similar data with many minor and major peaks. Under adverse conditions, although number of peaks decreased, however, total area increased dramatically along with emergence of few major peaks. Notably, under cold conditions, two large peaks with very high area and height were detected which was absent in moderate conditions (Fig. 4).

The chromatographic fingerprints showed abundant diversity of chemical constituents which were further analyzed by identification and quantization in tissues. So eight analytes viz. Glycine Betaine , Rutin, Phenylalanine, Glutathione Reduced, Glutathione Oxidized, Tannic Acid, Proline and Vanillic acid were unambiguously recognized by comparing their retention times with those of the extracts. Notably only one analyte i.e. Glycine Betaine was detected under only under cold conditions (Fig 3). Guerrini and Sacchetti (2014) have also made a mention that type of plant fingerprint, that allow to better understand the grown capacity of a plant species, is dependent on a particular environment to which plant is exposed. It was cited that genetic and environmental factors influence metabolic pathway and produce different pattern chemical composition thereby causing variation in fingerprint. Therefore, on the basis of above findings, it could be speculated that more number of secondary metabolites are producing in cold and hot conditions in *L. camara* which showed that these metabolites favored the growth of this plant in both adverse conditions. Similar results were obtained by Lin et al. (2014), when studied the chemical fingerprint analysis of *Phyla nodiflora* extract

prepared in methanol, where they found that contents of active compounds varied greatly among the samples collected at different periods, especially, the contents being higher in samples collected in the summer.

CONCLUSION

It can be concluded from the results that malondialdehyde (MDA), coupled with ROS accumulation, is an oxidative stress indicator that plant is experiencing in unfavorable conditions.. Large number of major and minor peaks that were observed in chromatograph of *L. camara* showed the presence or production of certain metabolites in plant species during extreme conditions as compared to cold condition, therefore, suggesting the role of these metabolites in providing protection or adaptation in plant species against certain extremities.

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DISCLOSURE CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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