Research Paper

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Antioxidant Profiling of Some Important Medicinal Plants

Botany

KEYWORDS : Ascorbate peroxidase, Catalase, Glutathione reductase, Guaiacol peroxidase, Reactive oxygen species, Superoxide dismutase

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ABSTRACT

The study was aimed to understand the homeostatic balance of antioxidant enzymes (APX, GPX, CAT, GR and SOD) and metabolites among some important medicinal plants. In this study it was proved that plants maintain a "balancing strategy" in their antioxidant defense system by up regulating the activities of some antioxidant enzymes and metabolites and down regulating the others. For example, in Murraya koenigii Spreng., superoxide dismutase activity was lower then other enzyme activities while in Abutilon indicum (Linn.) Sweet., Aegle marmelos Corr. and Aloe vera Linn., it was higher then other enzyme activities. These findings will help to understand the mechanism of controlling the levels of reactive oxygen species (ROS) and how do the antioxidant enzymes and metabolites protect the cells from injury, by scavenging reactive oxygen species in an up regulating and a vice versa system.

Introduction

Free radicals exert dual effect on plant metabolism as they help to defend the plants from attack by pathogens, but on the other hand, they can react with biological molecules to generate Reactive Oxygen Species (ROS) (Rio et al., 2006). Plants are carrying weapons to fight with these different type of ROS, with primary (Antioxidant enzymes and plant metbolies like proline etc) and secondary defense systems (phytochemicals like flavanoids). The generation of these metabolites and phytochemicals is always regulated and monitored on the basis of external as well as internal cues (Foyer & Noctor, 2000).

Medicinal plants are considered to be a chemical factory as they contain multitude of chemical compounds. The defense system of plants consists of low molecular weight antioxidants such as ascorbate, glutathione, α-tocopherol, and β-carotenoids, as well as several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacole peroxidase (GPX), ascorbic acid peroxidase (APX) and glutathione reductase (GR). Phytochemicals with antioxidant activity: allyl sulfides (onions, leeks, garlic), carotenoids (fruits, carrots), flavonoids (fruits, vegetables), polyphenols (tea, grapes).

Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX, GPX, and CAT subsequently detoxify H2O2. In contrast to CAT, APX requires an ascorbate and GSH regeneration system, the ascorbate-glutathione cycle. Detoxifiying H2O2 to H2O by APX occurs by oxidation of ascorbate to malondialdehyde (MDA), which can be regenerated by MDA reductase (MDAR) using NAD(P)H as reducing equivalents (Asada & Takahashi, 1987). Small molecules such as vitamin C and vitamin E that can donate or receive an electron to stop free radicals to generate more radicals in a chain reaction that otherwise is the situation when a free radical reacts with another molecule, as in the lipid peroxidation process.

Occurring naturally in foods and beverages from plant sources such as fruits, vegetables, berries, tea, and wine, flavonoids are polyphenolic compounds that provide an important dietary source of antioxidants (Kahkonen *et al., 2001). In plants, proline constitutes <5% of the total* pool of free amino acids under normal conditions. After stress this level can increase to up to 80% of the amino acid pool (Aspinall and Paleg, 1981). Certain sulfated, acetylated, and phophorylated derivatives of carrageenan oligosaccharides exhibited significant antioxidant activities in *in vitro experiments, in some cases higher than the activity of polysaccharides and oligosaccharides. The effect of molecular mass was not obvious as both poly-and oligosaccharides showed similar antioxidant activity (Yuen et al., 2005).*

Today there is growing interest in chemical composition of plant based medicines. Several bioactive constituents (phytochemicals) have been isolated and studied for pharmacological activity. Aim of the present study was to establish a classical approach for well define antioxidant system in medicinal plants. For this a strategy was followed, in which first, endogenous level of antioxidants, comprising enzymatic and non-enzymatic antioxidant defense system (antioxidant enzymes and metabolites), was measured. Later 'balancing strategy' for the formation of these enzymes and metabolites into the plant system in the influence of different internal and external cues was established by comparing the up regulation and down regulation mechanisms.

Materials and Methods

The plants which were selected to evaluate their antioxidant potential are : *Aegle marmelos, Abutilon indicum, Eclipta alba, Asparagus racemosus, Tinospora cardifolia, Aloe vera, and Murraya koenigii.* Leaf tissues (1g each) was ground with 2ml of 50mM EDTA, 1 Mm L-Ascorbic acid, 2% (w/v)PVP and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar. The homogenate was centrifuged at 10,000 g for 10 min at 4° C and the supernatants were collected and subjected to various enzymatic and non-enymatic assays as described below:

(a) Glutathione reductase (GR, EC 1.6.4.2): Glutathione reductase activity was measured according to Carlberg and Mannervik (1985). Activity staining of GR was performed by Rao et al. (1996) method.

(b) Catalase (CAT, EC 1.11.1.6): Catalase activity was assayed by Aebi (1983) method. Catalase activity staining was done by procedure of Thorup et al. (1961).

(c) Guaiacol peroxidase (GPX, EC 1.11.1.7): The guaiacol peroxidase activity was assayed by guaiacol and hydrogen peroxide (Rucusen and Foote, 1965). Guaiacol peroxidase isoenzymes were separated by Siegel and Galston (1967) method.

(d) Ascorbate peroxidase (APX, EC 1.11.1.11): Ascorbate peroxidase (APX) was assayed as described by Nakano and Asada (1981). Activity staining of APX was done by a modified method of Chen and Asada (1989).

(e) Superoxide dismutase (SOD, EC 1.15.1.1): The assay of superoxide dismutase was done by the method of Beyer and Fridovich (1987). SOD in the gels was visualized by the activity staining procedure of Beauchamp and Fridovich (1971).

Antioxidant metabolite analyses:

(a) **Ascorbic Acid:** The ascorbic acid (AA) concentration was measured by using the photometric method of Guri (1983).

(b) **Carotenoids:** Total carotenoids were measured according to Jenson (1978).

(c) **Chlorophyll:** Extraction and estimation of chlorophyll was done by Arnon's method (1949).

(d) **Flavanoids:** The total flavanoid concentration was measured using a colorimetric assay developed by (Zhishen *et al., 1999).*

(e) **Soluble carbohydrate (Fructose content):** Soluble carbohydrates were quantified by Halhoul and Kleinberg, (1972) method.
(f) **Total phenolics: T**otal phenolics estimation was carried out with Follin-ciocalteu reagent (FCR) (Bray ***et al.,*** *1954).*
(g) **Total polysaccharide:** Total polysaccharide content was measured by alcohol insoluble hexose test method (Dubois *et al. 1956).*
(h) **Proline:** Extraction and determination of free proline was carried out from freeze dried material (Bates *et al., 1973).*
(i) **Soluble proteins:** Bradford method (1976) was used for the estimation of soluble proteins.
(j) α-**Tocopherol:** High performance liquid chromatography (HPLC) was used for estimation of α-tocopherol using the method of Schmieden and Wild (1994).
(k) Data analyses were done by one way ANOVA and Tukey's test for the comparison of results

Results and Discussions

The lifetime of active oxygen species within the cellular environment is determined by the antioxidative system, which provides crucial protection against oxidative damage (Foyer and Noctor, 2000). In all seven medicinal plants studied here, considerable amount of antioxidant metabolites and enzymes activities were detected. Basically the aim was to establish a balance of such metabolites and enzyme activities in such system.

For example in Murraya koenigii higher activities of ascorbate peroxidase and guaiacol peroxidase were balanced by moderate activity of catalase and lower activity of superoxide dismutase activity and glutathione reductase as well as in Asparagus racemosus CAT works as major scavenger (Figure1a-e; Table1). This gave an indication that H_2O_2 detoxification in Murraya koenigii and Asparagus racemosus is mainly done by peroxidases rather than the superoxide dismutase, the strategy previously reported by Jim´enez et al., 1997; Yamaguchi et al., 1995. In Tinospora cordifolia CAT activity is higher than other peroxidases and scavenging enzymes like SOD and GR (Figure1a-e; Table1) which was previously stated by Willekens (1997). But in Aegle marmelos and Aloe vera, superoxisde dismutase and glutathione reductase showed their maximum activities among the plant group and their is enzyme patterns also showed the higher activity, this gave an indication that SOD and GR works as major antioxidant enzymes in both the plants and the activities of other peroxidases (APX, CAT and GPX) were suppressed (Figure1a-e; Table1) which was in agreement to the study of differential regulation of superoxide dismutases in plants exposed to environmental stress by Tsang et al. (1991) and suggested by Noctor et al. (2002) in wheat leaves. Similarly enhanced activity of SOD in Abutilon indicum and APX in Eclipta alba decreased the activities of other enzymes (Figure1a-e; Table1). Similar studies were reveled for wheat seedlings by Keles¸ and Öncel (2002). In the extreme climatic conditions of areas like Rajasthan, high irradiance, high temperature, and rapid temperature change create unfavorable conditions for photosynthesis. Like other plants, medicinal plants are also affected by the high light intensity. Streb et al. (1997) suggested that desert plants employ different strategies to protect themselves against high light intensity and temperature. Some plants have high antioxidant concentrations while others have very low concentrations of antioxidant compounds as shown here in the present study. In Murraya koenigii and Eclipta alba ascorbic acid (Figure1f; Table1), carotenoid (Figure1g; Table1), flavanoid (Figure1i; Table1) and phenolics work as major antioxidants. On the contrary, in Aegle marmelos and Abutilon indicum fructose (Figure 1j; Table1) and chlorophylls (Figure1h; Table1) were the major antioxidants. In Aloe vera ascorbic acid (Figure1f; Table1) and proline (Figure1m; Table1) detoxify the reactive oxygen species. Our investigation also supported the antioxidant nature of polysaccharides of Tinospora cordifolia and Asparagus racemosus (Figure1l; Table1).

Table 1: Activities of certain enzymes, metabolite contents and pigment contents of antioxidant scavenging system of selected medicinal plants. Values represent mean ± standard error (n=3).

PARAMETERS	Aloe vera	Aegle marmelos	Abutilon indicum	Asparagus racemosus	Tinospora cordifolia	Eclipta alba	Murraya koenigii	$F_{4,16}$ **	P value
Catalase	0.549 ± 0.12^{a*}	2.839 ± 0.19^{b}	2.654 ± 0.13^{b}	2.068 ± 0.17^{c}	3.170 ± 0.08^{b}	2.590 ± 0.05^{b}	2.910 ± 0.11^{b}	43.14	< 0.001
Ascorbate peroxidase	0.756 ± 0.02^{a}	0.415 ± 0.06^{a}	0.606 ± 0.03^{a}	0.345 ± 0.03^{a}	0.570 ± 0.07^{a}	1.760 ± 0.12^{b}	2.90 ± 0.10^{c}	165.25	< 0.001
Guaiacol peroxidase	0.081 ± 0.005^{a}	0.420 ± 0.004^{b}	0.432 ± 0.003^{b}	0.322 ± 0.015^{c}	0.504 ± 0.02^{d}	0.415 ± 0.009^{b}	0.463 ± 0.009^{b}	159.43	<0.001
Superoxide dismutase	0.089 ± 0.001^{a}	0.108 ± 0.005^{b}	0.094 ± 0.003^{a}	0.023 ± 0.001^{c}	0.052 ± 0.003^{d}	0.065 ± 0.001^{e}	0.021 ± 0.001^{c}	152.3	<0.001
Glutathione reductase	0.102 ± 0.003^{a}	0.077 ± 0.002^{b}	0.022 ± 0.001^{c}	0.044 ± 0.001^{d}	0.031 ± 0.001^{e}	0.025 ± 0.001^{c}	0.052 ± 0.001^{d}	213.69	<0.001
Chlorophyll-a	0.2233 ± 0.01^{a*}	1.2533 ± 0.01^{b}	1.3100 ± 0.01^{b}	0.5300 ± 0.06^{c}	0.5766 ± 0.01^{c}	1.2866 ± 0.01^{b}	1.1190 ± 0.001^{d}	290.43	< 0.001
Chlorophyll-b	0.1923 ± 0.03^{a}	1.0533 ± 0.02^{b}	1.4100 ± 0.04^{c}	0.2386 ± 0.002^{d}	0.3573 ± 0.001^{e}	1.6800 ± 0.02^{f}	1.1703 ± 0.001^{g}	998.02	< 0.001
Total Chlorophyll	0.4156 ± 0.01^{a}	2.4366 ± 0.12^{b}	2.7200 ± 0.03^{c}	0.7676 ± 0.07^{d}	0.9340 ± 0.01^{e}	2.9666 ± 0.021^{c}	2.2866 ± 0.003^{b}	387.88	< 0.001
Carotenoids	0.0514 ±0.0003^{a}	0.1070 ± 0.002^{b}	0.1102 ± 0.0015^{b}	0.1129 ± 0.004^{b}	0.0947 ± 0.003^{c}	0.1095 ± 0.0003^{b}	0.1223 ± 0.009^{d}	96.74	< 0.001
Flavonoids	0.1596 ± 0.0009^{a}	2.1900 ± 0.116^{b}	0.7393 ± 0.003^{c}	1.6833 ± 0.039^{d}	0.7233 ± 0.009^{e}	2.3000 ± 0.056^{b}	2.3900 ± 0.03^{b}	297.53	< 0.001
Phenolic compounds	0.087± 0.01^{a*}	0.366± 0.02^{b}	0.256± 0.01^{c}	0.186± 0.01^{c}	0.276± 0.02^{c}	0.536± 0.05^{d}	0.436± 0.01^{b}	45.64	< 0.001
Proline	0.456 ± 0.02^{a}	0.330 ± 0.005^{b}	0.246 ± 0.09^{c}	0.336 ± 0.09^{b}	0.206 ± 0.003^{c}	0.436 ± 0.09^{a}	0.263 ± 0.02^{d}	47.87	< 0.001
Ascorbic acid	0.573 ± 0.09^{a}	0.423 ± 0.02^{b}	0.544 ± 0.02^{a}	0.441 ± 0.02^{b}	0.520 ±0.005^{a}	0.563 ± 0.03^{a}	0.606 ± 0.01^{a}	11.35	< 0.001
Fructose	33.00 ± 2.08^{a}	66.16 ± 0.60^{b}	60.66 ± 0.66^{b}	72.33 ± 0.33^{c}	16.33 ± 0.20^{d}	45.53 ± 0.31^{e}	64.00 ± 2.11^{b}	297.96	< 0.001
Polysaccharides	7.08 ± 0.02^{a}	3.27 ± 0.06^{b}	8.51 ± 0.07^{c}	14.77±0.12^{d}	17.19 ± 0.41^{e}	6.76 ±0.352^{f}	8.6 ± 0.15^{c}	474.24	< 0.001
Soluble Protein	1.56 ± 0.01^{a}	7.24 ± 0.05^{b}	5.56 ± 0.09^{c}	2.79 ± 0.12^{d}	4.58 ± 0.11^{e}	5.54 ± 0.09^{c}	6.19 ± 0.02^{f}	563.62	< 0.001

* Values in the same column with similar letters in superscript indicates no significant difference according to Tukey's multiple comparison procedure at P< 0.05.

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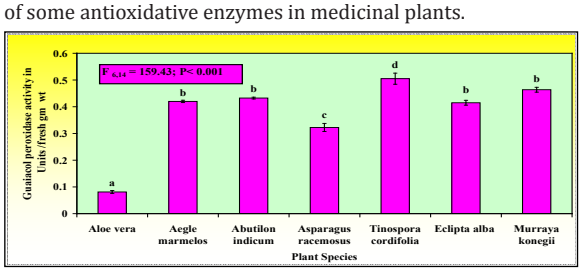
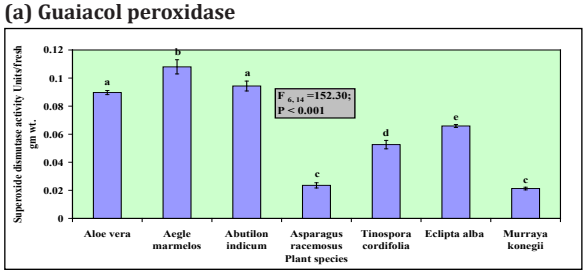
** Dark columns represent one-way analysis of variance at P< 0.05 for the corresponding columns.

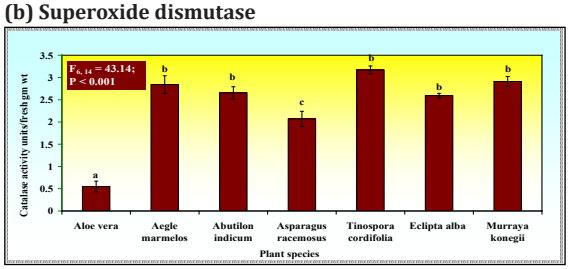
Presence of different isoenzymes of these enzymes in varying intensities in different plants also supported the balancing mechanism. For example higher band-intensity of GR was detected in Aloe vera (Figure 2e) while five different isoforms of APX were detected in Eclipta alba (figure 2d). Previously five APX forms were suggested by Sano et al., 2001 in unicellular red alga Galdieria partita. All ten major isoforms of SOD (Figure 2a) were detected in seven medicinal plants. It represented an overview of the functional interrelationship of these enzymes and metabolites in selected plants. In Murraya koenigii all three isoforms of catalase was observed (Figure 2c). Previously it was reported that there are three main isoforms of catalase: CAT1, CAT2, and CAT3 (Willekens et al., 1997; Havir and McHale, 1989; Ni and Trelease, 1991; Havir, 1992; Willekens et al., 1994; Ni, Turley and Trelease, 1990). Guaiacol specific peroxidase (GPX) activity has been used as an indicator for stresses like high temperature, salinity and drought. Maximum activity was found in Aloe vera in which all the ten bands of isoperoxidases were visible (Figure 2b). Further, our results clearly showed a differential extent of alterations of various isoforms of CAT, GPX, and SOD levels and antioxidative metabolites. Further, the present study is novel in that it characterizes the various isoforms of some antioxidative enzymes in medicinal plants.



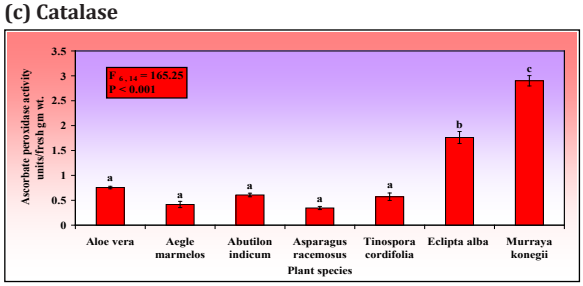
(a) Guaiacol peroxidase



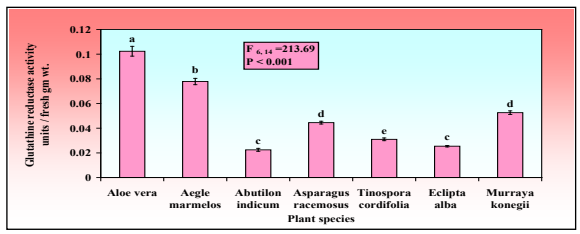
(b) Superoxide dismutase



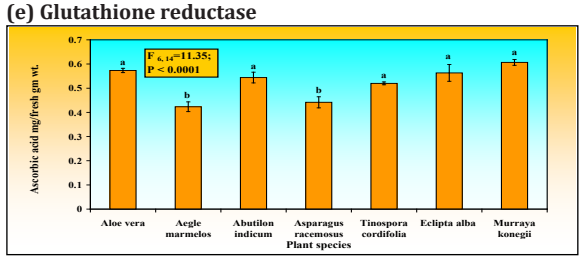
(c) Catalase



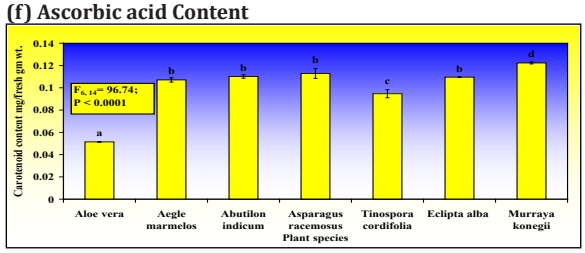
(d) Ascorbate peroxi



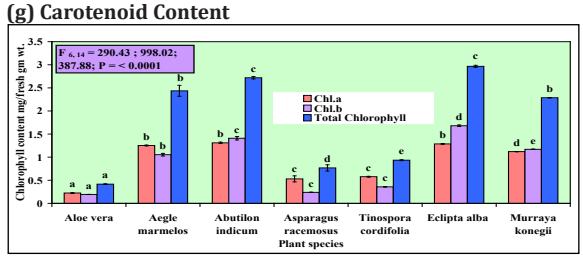
(e) Glutathione reductase



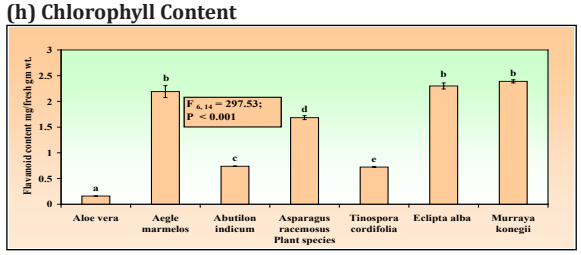
(f) Ascorbic acid Content



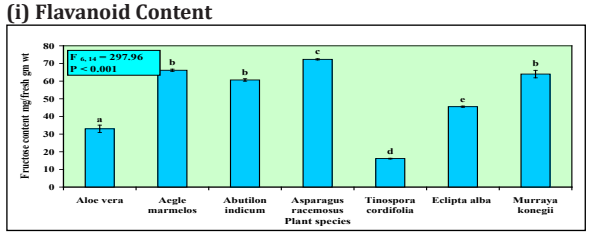
(g) Carotenoid Content



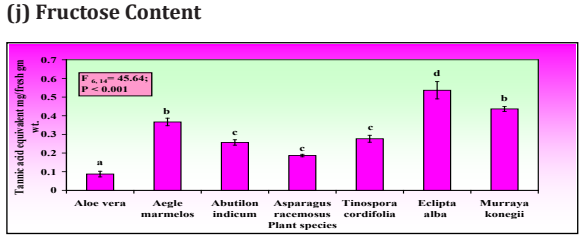
(h) Chlorophyll Content



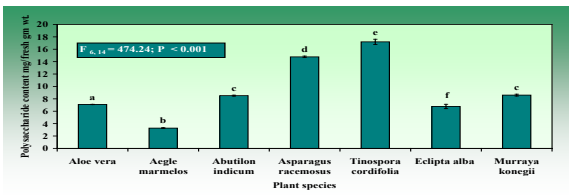
(i) Flavanoid Content



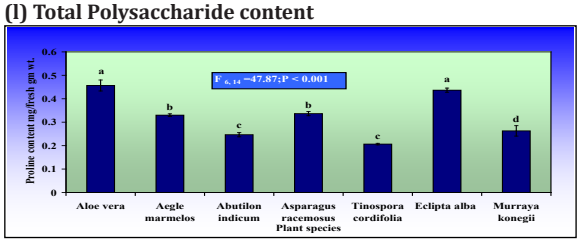
(j) Fructose Content



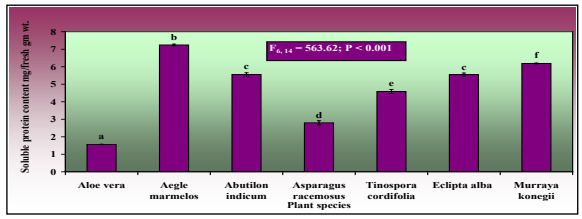
(k) Total phenolic Content



(l) Total Polysaccharide content



(m) Proline content



(n) Soluble Protein Content

Figure1: Columns represent mean value and vertical bars indicate ± s.e. (n=3). Similar letters above bars represent no significant difference according to Tukey's multiple comparison procedure at P< 0.05. Box in the chart area represents one way analysis of variance at P<0.05.

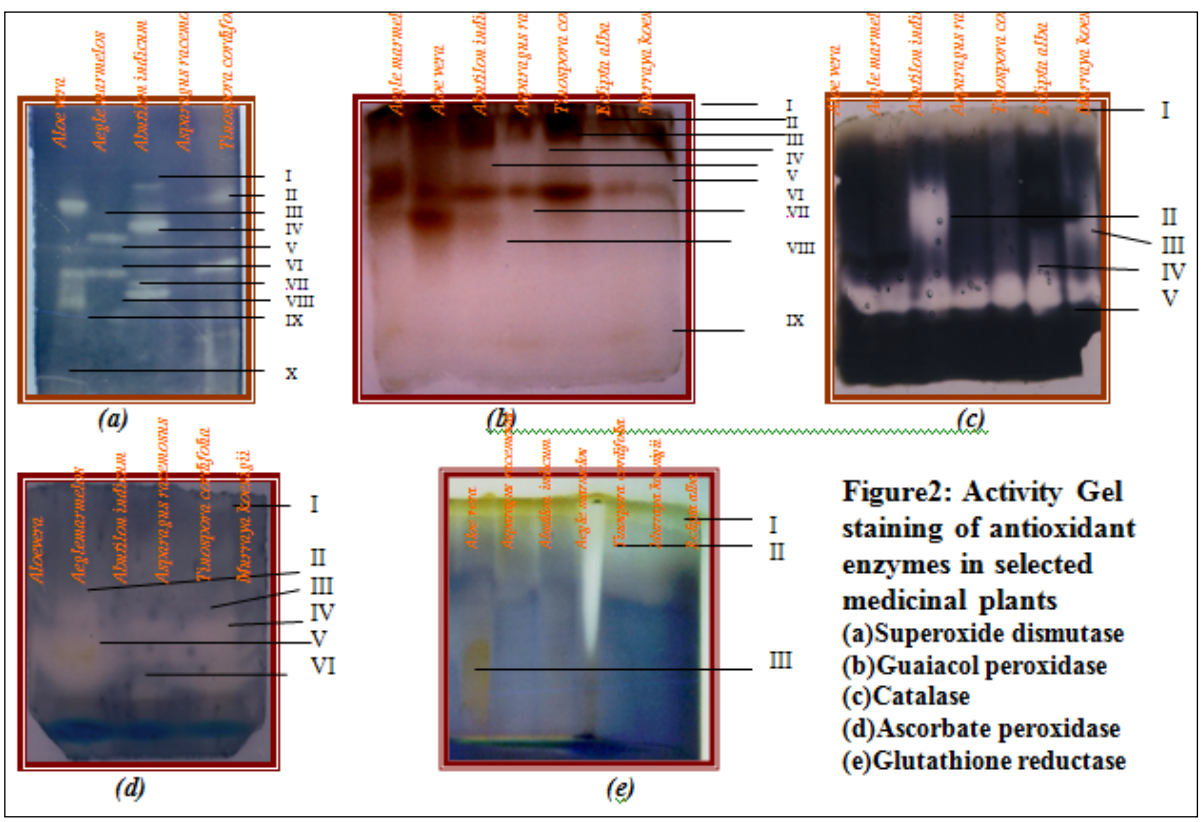


Figure2: Activity Gel staining of antioxidant enzymes in selected medicinal plants
(a)Superoxide dismutase
(b)Guaiacol peroxidase
(c)Catalase
(d)Ascorbate peroxidase
(e)Glutathione reductase

Conclusions

Thus our finding emphasized a "balance strategy" about these enzymes and metabolites which helps to maintain a homeostatic balance in the plant cell (Vranová and Van, 2002). The extent of oxidative stress in a cell is determined by the amounts of superoxide, H2O2, and hydroxyl radicals. Therefore, the balance of SOD, APX, and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms. For example, when CAT activity was higher in plants, scavenging enzymes such as APX and GPX were down regulated. Unexpected effects can also occur. So our study leaves an open question about changing the pattern of theses antioxidant enzymes in the plant cell by inducing several stress mechanisms.

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