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Profiling Of Enzymatic Antioxidants In Leaf Extracts Of Different Bael Varieties/Accessions.

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ABSTRACT

Reactive oxygen species are produced aerobically as a consequence of various metabolic pathways. Hence, to keep their concentration minimal plants are well endowed with antioxidants and ROS scavenging enzymes under favourable conditions of growth. However, production of reactive oxygen species may increase under certain unfavourable environmental conditions resulting in oxidative stress in many plant species. For protection against these toxic intermediates, plants and animals possess several detoxifying enzymatic systems. In this paper, we discussed the role of ROS, its generation and sites of production and as well as the cellular antioxidative defense mechanisms for scavenging the excess ROS production. The present study has therefore, been focused on the enzymatic profiles of Glutathione reductase, Guaiacol peroxidase, Polyphenol oxidase and Ascorbate peroxidase in the crude extract of different Bael varieties in order to gain insight about this plant's antioxidant potential. The enzyme activities of the crude extract were measured by using spectrophotometric method. Conclusively, our results showed that crude extracts of different varieties and accessions of *Aegle marmelos* possessed significant activity for all the enzymatic procedures tested.
Keywords: Bael (*Aegle marmelos*), Reactive oxygen species, oxidative stress, enzymatic antioxidants.

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INTRODUCTION

Plants have an extremely active antioxidant system which provides them adequate stability and inhibits free radical processes [1, 2]. The higher is the antioxidant activity, the more resistant is the species toward the stress conditions and elements [3]. The protective antioxidant system comprises of high and low molecular substances [2]. Enzymes such as peroxidase and catalase are high-molecular, which are capable of eliminating the hydrogen peroxide formed during non-enzymatic or enzymatic dismutation [4].

The natural outcomes of the aerobic metabolism are Reactive Oxygen Species (ROS) and plants have mechanisms to deal with them in normal circumstances. Cell homeostasis is disrupted under stress conditions, thereby increasing ROS production which in turn puts a heavy load on the antioxidative mechanisms to remove the excess ROS [5].

OXIDATIVE STRESS AND ROS PRODUCTION

Oxidative stress can be defined as the physiological modifications resulting in the production of excess quantities of reactive oxygen species (ROS) [6]. Furthur, the increase in ROS levels induces a metabolic response in the plant for their elimination which is dependent on the type of plant species, growth stage, and duration of the stress. ROS are produced by all aerobic organisms and generally, the equilibrium is maintained by the antioxidative mechanisms present in all living beings. In addition to the above important role, ROS concentration must be carefully controlled through adequate pathways since they play an essential role in signaling in plants [7, 8, 9]. ROS can be produced during normal aerobic metabolic processes viz. photosynthesis and respiration and thus, the majority of ROS are produced in the mitochondria, chloroplast, peroxisomes, plasma membrane and apoplast [10, 11]. Other sources of ROS production are NADPH oxidases, amine oxidases and cell-wall peroxidases [9]. The production of ROS increases under extreme biotic and abiotic stress conditions that exceed the capacity of the plant's defense mechanisms resulting in oxidative stress [12] as depicted in Fig.1

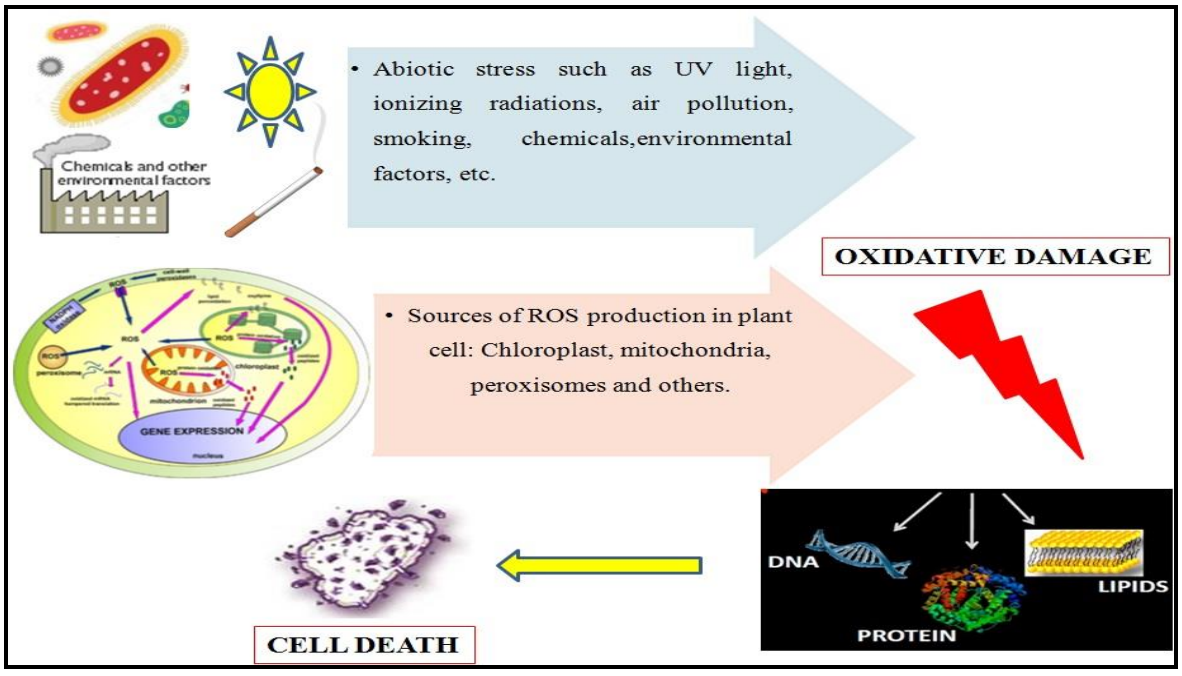

Fig.1 ROS production induced by abiotic stress resulting in cell death

In addition to the oxidative stress, certain environmental conditions such as temperature extremes, heavy metals, drought, water availability, air pollutants, nutrient deficiency, or salt stress when exposed to plants leads to an increase in production of ROS e.g., $^1O_2$, O_2^-, H_2O_2, and OH^-. To protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria, and

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peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses [13, 14, 15]. ROS are also produced continuously as byproducts of various metabolic pathways that are localized in different cellular compartments such as the chloroplast, mitochondria, and peroxisomes [16, 17] as shown in Fig.2.

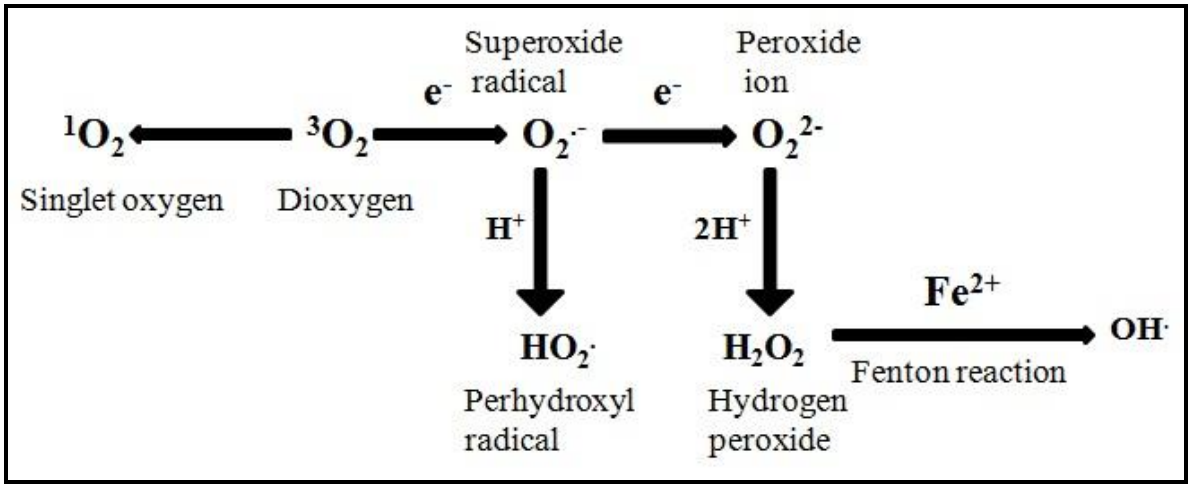

Fig.2 Generation of ROS by energy transfer

The superoxide radical is chiefly produced during photosynthesis and respiration both in the chloroplasts (photosystems I and II) and mitochondria and in peroxisomes [16, 18, 19], has a very short half-life (2-4 µs) and cannot able to pass phospholipid membranes [20]. Therefore, it is essential that the cells should have adequate *in situ* mechanism for scavenging of generated ROS. Superoxide dismutase can catalyze the conversion of this species into hydrogen peroxide. Superoxide radical can also be produced by NADPH oxidase in the plasma membrane [18].

Similarly, another ROS i.e. singlet oxygen ($^1O_2$) is a highly reactive species mainly produced in the chloroplasts at photosystem II [21] but may also result from lipoxygenase activity that can last for nearly 4 µs in water [22]. One of the other examples of ROS is hydrogen peroxide which is not a radical and can easily bypass plasma membranes diffusing across the cell and has a short half-life (~ 1 ms) [20]. It is mainly produced in peroxisomes [16] and also in mitochondria [19], and also results from the dismutation of superoxide.

Moreover, the produced ROS molecules are scavenged by several antioxidative defense mechanisms [23]. However, various biotic and abiotic stress factors e.g. salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks may be responsible for the imbalance between the production and the scavenging of ROS as shown in Fig.3. The imbalance in equilibrium leads to a sudden increase in intracellular levels of ROS causing significant damage to the cell [24]. It is noteworthy that ROS acts as damaging, protective or signaling components depends on the delicate equilibrium between ROS production and scavenging at the proper site and time [25]. Furthermore, the subcellular location for production of ROS is important for a highly reactive ROS, because it diffuses only a very short distance before reacting with a cellular molecule.

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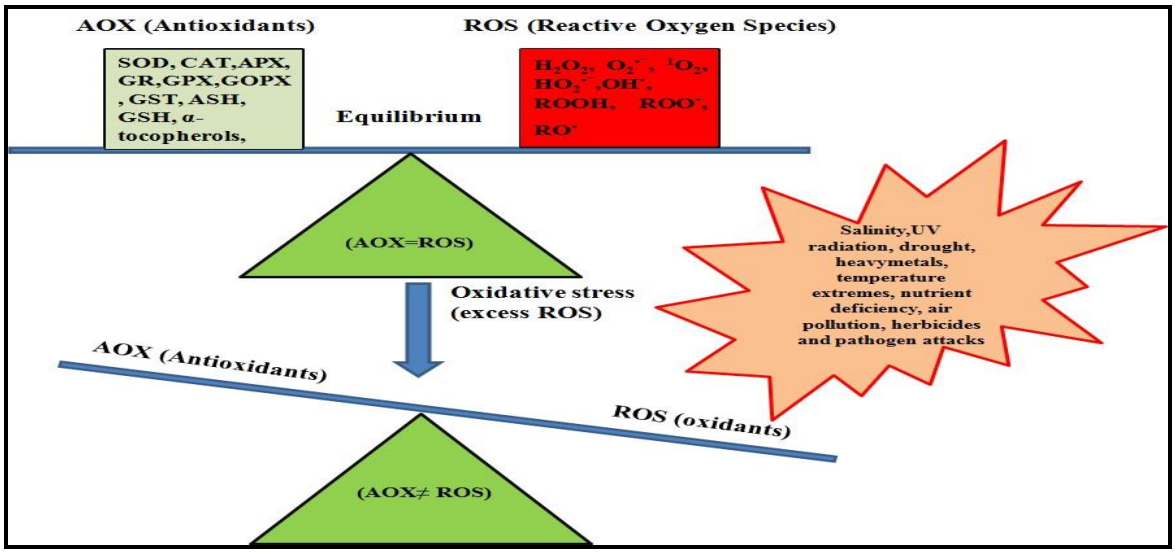

Fig.3 Equilibrium between Antioxidants and Reactive Oxygen Species

ROS scavenging antioxidant defense mechanism

The accumulation of ROS which is induced by stress is neutralized by enzymatic antioxidant systems including superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaiacol peroxidase, GOPX and glutathione-S- transferase, GST and non-enzymatic low molecular metabolites such as, ascorbic acid, ASH; glutathione, GSH; phenolic compounds, alkaloids, non-protein amino acids and α-tocopherols, carotenoids and flavonoids [5] as illustrated in Fig.4. Both antioxidant defense systems function in unison to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS.

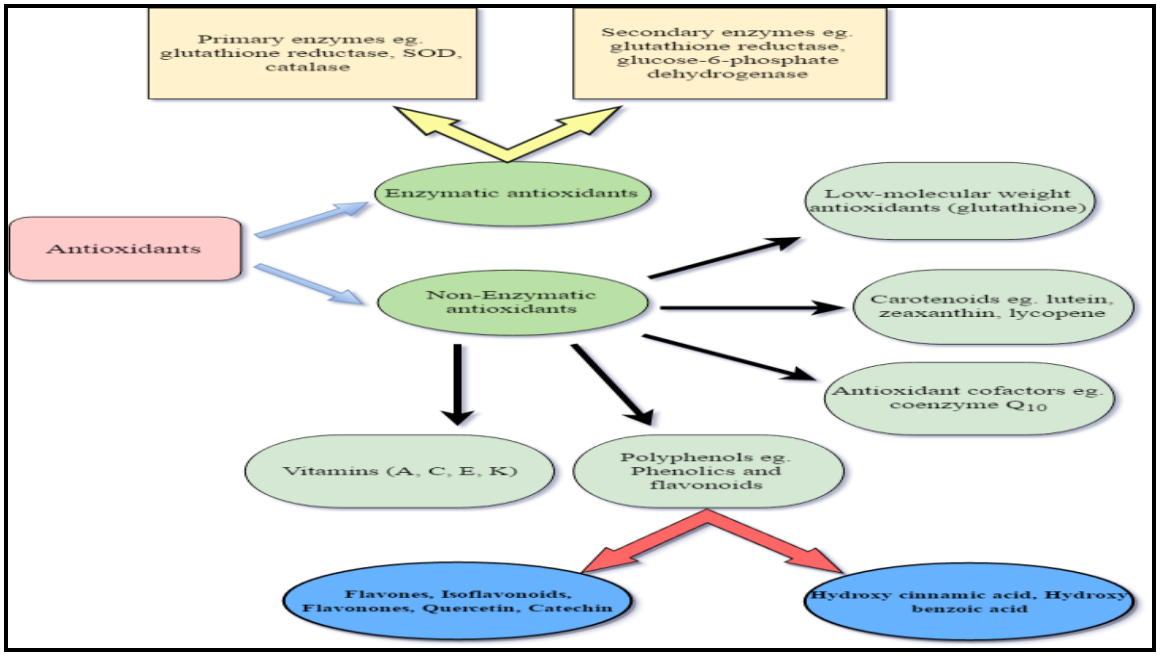

Fig.4 Enzymatic and non-enzymatic antioxidant defense systems

Superoxide dismutase (SOD)

It is well known that various environmental stresses lead to the increased production of ROS and SOD provide plant stress tolerance and the first line of defense against the toxic effects of elevated levels of ROS. SOD is ubiquitously present in all aerobic organisms and the most effective intracellular enzymatic antioxidant in all subcellular compartments. SOD catalyzes dismutation by removal of O_2^-, one O_2^- being reduced to H_2O_2 and another oxidized to O_2 as shown in Table 5.2. It removes O_2^- and hence decreases the risk of OH^- formation via the metal catalyzed the HabereWeiss-type reaction. The rate of this reaction is 10,000 fold faster than spontaneous dismutation. SODs are classified into three types on the basis of metal cofactors *viz*. copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD localized in different cellular compartments [9].

Catalases (CAT)

CATs are a heme-containing tetrameric protein having the potential to dismutate H_2O_2 into H_2O and O_2. It is essential for ROS detoxification under stress conditions [20]. CAT has highest turnover rates where one molecule of CAT can convert a million molecules of H_2O_2 to H_2O and O_2 per minute. CAT catalyzes the removal of H_2O_2 generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, and purine catabolism. The isozymes of CAT have been characterized comprehensively in higher plants [26] like 2 in *Hordeum vulgare* [27], 4 in *Helianthus annuus* cotyledons [28] and 12 isozymes in *Brassica* sp. [29]. Similarly, maize has 3 isoforms viz. CAT1, CAT2 and CAT3 present on separate chromosomes and exhibited independent regulation and differential expression [30]. CAT1 and CAT2 are localized in peroxisomes and the cytosol, whereas, CAT3 is mitochondrial.

Ascorbate peroxidase (APX)

The antioxidant enzyme APX is involved in scavenging ROS and protecting cells of higher plants, algae, euglena and other organisms. APX participates in scavenging of H_2O_2 in water-water and ASH-GSH (glutathione-ascorbate) cycles and utilizes ASH as the electron donor. The APX family comprises of various isoforms e.g. thylakoid (tAPX), glyoxisome membrane forms (gmAPX), chloroplast stromal soluble form (sAPX), cytosolic form (cAPX). APX possess a higher affinity for H_2O_2 (mM range) than CAT and POD (mM range) and it plays a vital role in the management of ROS during stress. Under Cd stress increased leaf APX activity has been reported in *Ceratophyllum demersum* [31], *Brassica juncea* [32], *Triticum aestivum* [33] and *Vigna mungo* [34]. An increase in APX activity was observed in *O. sativa* seedlings pretreated with H_2O_2 under non-heat shock conditions as reported by Hso and Kao [35] and also provides protection to rice seedlings from subsequent Cd stress.

Guaiacol peroxidase (GPOX)

Guaiacol peroxidase (GPOX) can be distinguished from APX in terms of differences in sequences and physiological functions. GPOX catalyzes the decomposition of indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defense against biotic stresses by consuming H_2O_2. The preferred substrates for GPOX are aromatic electron donors such as guaiacol and pyrogallol [36]. Depending upon plant species and stress, considerable variations are observed in the activity of GPOX.

Glutathione reductase (GR)

GR is a flavoprotein oxidoreductase ubiquitously present in both prokaryotes and eukaryotes [37] which is a potential enzyme of the ASH-GSH cycle and plays an important role in defense system against ROS by sustaining the reduced status of GSH. It is mostly localized in chloroplasts, however, a small amount of this enzyme has also been present in mitochondria and cytosol [38, 39]. GR catalyzes the reduction of GSH, which is involved in various metabolic, antioxidative and regulatory processes in plants catalyzing the NADPH-dependent reaction of disulfide bond of GSSG and thereby maintaining the GSH pool [40, 41]. GSSG comprises of two GSH linked by a disulfide bridge which can be converted back to GSH by GR (Table 1). GR also plays an important role in defense against oxidative stress, while GSH within the cell system, participating in the ASH-GSH cycle. Additionally, GR and GSH help in determining the plant tolerance under various stress conditions

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[41]. Therefore, the present study was focused on the quantitative evaluation of enzymatic antioxidants in different Bael varieties/accessions.

Table 1: Major ROS scavenging antioxidant enzymes

Enzymatic antioxidants	Enzyme code	Reactions catalyzed
Superoxide dismutase (SOD)	EC 1.15.1.1	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow 2H_2O_2 + O_2$
Catalase (CAT)	EC 1.11.1.6	$H_2O_2 \longrightarrow H_2O + {}^{1/2} O_2$
Ascorbate peroxidase (APX)	EC 1.11.1.11	$H_2O_2 + AA \longrightarrow 2H_2O + DHA$
Guaiacol peroxidase (GPOX)	EC 1.11.1.7	$H_2O_2 + GSH \longrightarrow H_2O + GSSG$
Monodehydroascorbate reductase (MDHAR)	EC 1.6.5.4	$MDHA + NAD(P)H \longrightarrow AA + NAD(P)^+$
Dehydroascorbate reductase (DHAR)	EC 1.8.5.1	$DHA + 2GSH \longrightarrow AA + GSSG$
Glutathione reductase (GR)	EC 1.6.4.2	$GSSH + NADPH \longrightarrow 2GSH + NAD(P)^+$

MATERIALS AND METHODS

Collection of plant material

The leaf samples of 18 varieties were collected from the orchard of Narendra Deva University of Agriculture and Technology Kumarganj Faizabad, India.

Chemicals

Potassium Phosphate, Monobasic, Dibasic, Trichloroacetic acid (TCA), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), sodium potassium tartrate, copper sulphate ($CuSO_4.5H_2O$), Folin's reagent, bovine serum albumin fraction V, EDTA (Ethylenediaminetetraacetic Acid), NADPH (Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form), hydrogen peroxide (H_2O_2), L-Glutathione (oxidised), Guaiacol, catechol, ascorbic acid, and all other chemicals used were purchased from Merck and Himedia. The UV spectrophotometer (Genesys 10S UV-VIS Spectrophotometer, Thermo scientific, USA) was used for the measurement of absorbance of different extracts under study.

CHARACTERIZATION OF ENZYMATIC ANTIOXIDANTS

Processing of plant samples

The leaves of the plant were properly washed in tap water and the rinsed in distilled water. The rinsed leaves were then shade dried. The dried leaves of each plant were pulverized using a mortar and pestle, to obtain a powdered form in liquid nitrogen. Finely powdered and lyophilized plant material was taken for experiments. The lyophilized form of these plants was stored in airtight falcon tubes in -20°C for various analysis of the sample.

Preparation of plant extract

Lyophilized leaf (0.20 gm) powders are homogenized in a mortar and pestle with 4 ml of ice-cold extraction buffer (100 mM potassium phosphate buffer, pH 7.0). The homogenate was filtered through muslin cloth and centrifuged at 16,000 g_n for 15 min. The supernatant fraction is used as a crude extract for enzyme activity assays. All operations are carried out at 4ºC.

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Determination of protein content by Lowry's method

500 µl of plant supernatant was taken in a microfuge tube and protein was precipitated with equal volume of ice-cold 20 % trichloroacetic acid (TCA) and kept at 4°C overnight. The pellet was recovered by centrifuging at 12,000 rpm for 5 mins at room temperature and decanting the supernatant. The pellet was washed with 0.1 ml ice-cold 10 % TCA and ice-cold acetone. Depending on the pellet size, it was dissolved in 0.5-1.0 ml of 0.1 N NaOH. The solution was subjected to heating for 5 min in boiling water bath and vortexed vigorously. The protein content was determined by Lowry's method [42]. For protein content determination, 0.5 ml of protein solution was taken in a test tube and 2.5 ml of the alkaline solution [prepared by mixing 2% Na_2CO_3 solution (in NaOH), 2% sodium potassium tartrate and 1% $CuSO_4.5H_2O$ in 100:1:1] was added. The contents were mixed well and the tubes were incubated at room temperature for 10 min. This was followed by addition of 0.25 ml of 1.0 N Folin's reagent. The contents of the tube were mixed thoroughly and after 10 min, absorbance at 660 nm against reagent blank was determined spectrophotometrically using bovine serum albumin fraction V as standard.

Glutathione reductase (GR) assay

Specific GR (EC 1.6.4.2) activity is assayed as described by Foyer & Halliwell (1976), with minor modifications [43]. The assay mixture consisted of 50 µL of the enzyme extract, 100 mM phosphate buffer (pH 7.8), 0.1 µM EDTA, 0.05 mM NADPH, and 3.0 mM oxidized glutathione in a total volume of 1.0 ml. NADPH oxidation rate is monitored by reading the absorbance at 340 nm at the moment of H_2O_2 addition and 1 min later. The difference in absorbance (ΔA340) is divided by the NADPH molar extinction coefficient (6.22 $mM^{-1}cm^{-1}$) and the enzyme activity expressed as mmol of NADPH min^{-1} mg^{-1} protein.

Guaiacol peroxidase (GPOX) assay

Peroxidase activity was assayed by the method described by Putter (1974), using guaiacol as the substrate [44]. The assay system consists of 50 mM sodium phosphate buffer (pH 7.0), 0.067% H_2O_2, 3.33 mM guaiacol and a suitable aliquot of enzyme in a final volume of 3 ml. The tetraguaiacol formation was monitored spectrophotometrically by measuring the increase in absorbance at 470 nm. The molar extinction coefficient of tetraguaiacol was taken as 6.39 cm^2 μmol^{-1}. One unit of enzyme activity is defined as the amount of enzyme catalyzing the production of one µmol of tetraguaiacol per minute at 30°C.

Polyphenol oxidase (PPO) assay

The assay mixture consisted of 10 mM catechol in 10 ml of 0.1 ml M potassium phosphate buffer (pH 6.0), 0.5 ml enzyme and 2.5 ml substrate in the buffer. The reaction was recorded as a change in absorbance/15secs at 420 nm. The enzyme activity is expressed as change in one OD/gm fresh wt. One enzyme unit is defined as the change in one OD/min/mg protein under experimental conditions.

Ascorbate peroxidase (APOX) assay

Ascorbate oxidase will be analyzed by the method of Nakano & Asada (1981) [45]. Rate of ascorbate oxidation is monitored by following the decrease in absorbance at 290 nm for 3 minutes in 3.0 ml of a reaction mixture containing 2.905 ml of sodium phosphate buffer (50mM, pH 7.0), 15µl of ascorbic acid (10mM) and 50µl of enzyme extract in which the reaction was triggered by the addition of 30µl of hydrogen peroxide (10mM). The enzyme activity is expressed as change in one OD/gm fresh wt. One enzyme unit is defined as the change in one OD/min/mg protein under experimental conditions.

Statistical Analysis

The results obtained were expressed as mean ±SD. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Tukey's multiple comparison test at a level of $P < 0.05$.

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RESULTS AND DISCUSSION

Estimation of Enzymatic Antioxidants

Free radicals or ROS are generated into the living system as a product of normal metabolism or from the environment. Naturally, occurring redox reactions are vital for maintaining the metabolic process of the living system. Since plants have evolved a well-regulated mechanism for scavenging ROS through the production of various antioxidative enzymes e.g. Superoxide dismutase, Peroxidase, Glutathione peroxidase, Ascorbate oxidase, Glucose 6- Phosphate-Dehydrogenase and Glutathione reductase. These enzymes are usually considered to be the most predominant ROS- scavenging in plant systems.

To the best of our knowledge, the result of the present study reveals first time the enzymatic antioxidant content present in different varieties/accessions of *Aegle marmelos*.

Proteins are macromolecules that act as a building block and alternate energy source when other energy sources are in short supply. The leaves of different varieties/accessions of *Aegle marmelos* were analyzed for its protein content and the results obtained are represented in Table 2. The AM-1 has been identified to contain a significant quantity of protein corresponding to 10.0 mg in one gram tissue followed by AM-6 which contained 8.8 mg of protein. A significant amount of protein was also observed in other cultivars such as AM-2, NB-4, Pant Shivani also contained an amount of (8.4 mg).

Table 2: Protein estimation in different Bael varieties/accessions by Lowry's method

S. No.	Different Bael varieties/accessions	Total protein content (mg/gm)
1.	**AM-1**	**10.0±0.031**
2.	**AM-2**	8.4±0.028
3.	**AM-3**	5.4±0.035
4.	**AM-4**	5.68±0.023
5.	**AM-6**	8.8±0.042
6.	**AM-7**	6.48±0.048
7.	**AM-8**	6.72±0.030
8.	**NB-1**	3.54±0.045
9.	**NB-4**	8.4±0.025
10.	**NB-5**	2.36±0.026
11.	**NB-7**	6.4±0.029
12.	**NB-9**	4.6±0.018
13.	**NB-16**	6.48±0.034
14.	**NB-17**	6.48±0.050
15.	**Pant Aparna**	7.08±0.036
16.	**Pant Sujata**	4.6±0.021
17.	**Pant Shivani**	8.4±0.043
18.	**Kaghzi**	2.96±0.015

The level of enzymatic antioxidants such as Glutathione reductase (GR), Guaiacol peroxidase GPOX, Polyphenol oxidase (PPO), Ascorbate peroxidase (APX) values is shown in Table 3, 4, 5 and 6. The difference between each pair of means has been depicted graphically in Fig. 5, 6, 7, 8 through Tukey's Multiple Comparison Test. The test compares GR activity was found to be maximum in NB-4 with a value of 13.39 mmol of NADPH consumed min^{-1} followed by AM-3 i.e. 11.61 mmol of NADPH consumed min^{-1}. In this study, GPOX level was found to be 85.33 µmoles per min in AM-3 followed by AM-1 exhibiting 66.4 µmoles/min activity. The activity of PPO in a fresh sample of *Aegle marmelos* was found to be in the range of 3.92 as a change in O.D. per gm fresh wt. in AM-2 to 0.336 as observed in Pant Shivani. Similarly, ascorbate peroxidase (APOX) activity was found to be 9.95 in NB-4 to 1.38 ΔO.D/gm fr.wt. in NB-17 in fresh tissues of Bael.

In one of the studies, the polyphenol oxidase level was found to be 2.19±0.127 µmoles/g tissue in a fresh sample of *Tylophora pauciflora* while ascorbate oxidase activity was found to be 27.23±0.57 unit/g

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sample. The activity of PPO was evaluated in the tubers of *Amorphophallus commutatus* having significant activity (0.8 u/g tissue) followed by young leaves (0.45u/g tissue) and mature leaves (0.23 u/g tissue).

The level of the active antioxidant enzyme guaiacol peroxidase of the leaf samples of plant *Curcuma zedoaria* (Christm.) was reported to be 7.21± 1.8U/mg. Similarly, guaiacol peroxidase activities of rhizome in *Curcuma longa* has also been reported [46], which was found to be 8.21± 0.09 U/mg.

A significant higher POD activity was observed in the mature leaves of *Amorphophallus commutatus* (1.9 U/g tissue) followed by young leaves (1.77U/g) and tuber (0.38 U/g tissue) [47]. Similarly, young leaves (1.3 U/g tissue) also have a considerably higher glutathione reductase activity (1.3 u/g tissue) compared to the tuber (0.65 u/g tissue) and the mature leaves (0.323 u/g tissue). GR is a ubiquitous NADPH-dependent enzyme and may be a rate limiting enzyme for defense against active oxygen toxicity [48].

Table 3: Glutathione reductase activity in different Bael varieties/accessions

S. No.	Different Bael varieties/accessions	Enzyme activity (mmol of NADPH consumed min^{-1})	Specific activity (U/mg protein)
1.	**AM-1**	7.47± 0.16	0.747
2.	**AM-2**	5.0± 0.16	0.595
3.	**AM-3**	11.61± 1.49	2.15
4.	**AM-4**	11.18± 0.30	1.968
5.	**AM-6**	6.32±1.84	0.718
6.	**AM-7**	4.99±0.32	0.77
7.	**AM-8**	7.82±0.20	1.163
8.	**NB-1**	3.39± 0.23	0.957
9.	**NB-4**	**13.39± 0.88**	1.59
10.	**NB-5**	9.11± 0.70	3.86
11.	**NB-7**	8.22± 0.13	1.28
12.	**NB-9**	7.67± 0.10	1.66
13.	**NB-16**	4.13± 0.18	0.637
14.	**NB-17**	3.15± 0.64	0.486
15.	**Pant Aparna**	5.4± 0.61	0.762
16.	**Pant Sujata**	3.14± 1.2	0.682
17.	**Pant Shivani**	6.05± 0.15	0.72
18.	**Kaghzi**	9.31± 0.20	3.14

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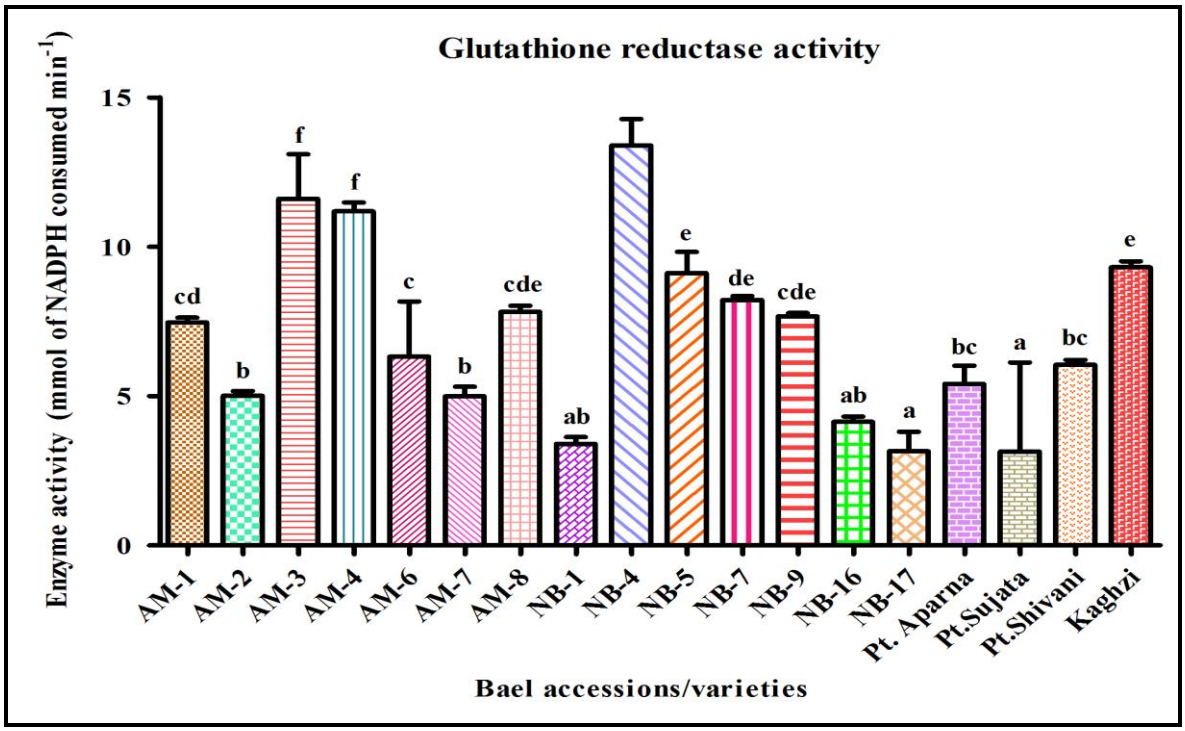

Fig.5 Glutathione reductase activity determination in different Bael accessions/ varieties. Values are expressed as means ± SD (n = 3) and are representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $P < 0.05$ as determined by one-way ANOVA (Tukey's Multiple Comparison Test)

Table 4: Guaiacol peroxidase (GPOX) activity in different Bael varieties/accessions

S. No.	Different Bael varieties/accessions	Enzyme activity (µmol/min)	Specific activity (U/mg protein)
1.	**AM-1**	66.4±1.00	6.64
2.	**AM-2**	59.36±0.56	7.06
3.	**AM-3**	**85.33±0.30**	15.8
4.	**AM-4**	61.13±0.23	10.76
5.	**AM-6**	6.83±0.65	0.776
6.	**AM-7**	7.66±1.04	1.18
7.	**AM-8**	22.5±0.27	3.34
8.	**NB-1**	24.86±2.40	7.02
9.	**NB-4**	35.26±1.26	4.19
10.	**NB-5**	12.43±1.25	5.26
11.	**NB-7**	11.86±0.80	1.85
12.	**NB-9**	32.43±1.92	7.04
13.	**NB-16**	29.33±1.71	4.52
14.	**NB-17**	10.06±0.98	1.55
15.	**Pant Aparna**	13.8±1.49	1.94
16.	**Pant Sujata**	19.36±1.41	4.2
17.	**Pant Shivani**	18.23±1.09	2.17
18.	**Kaghzi**	32.6±2.8	11.01

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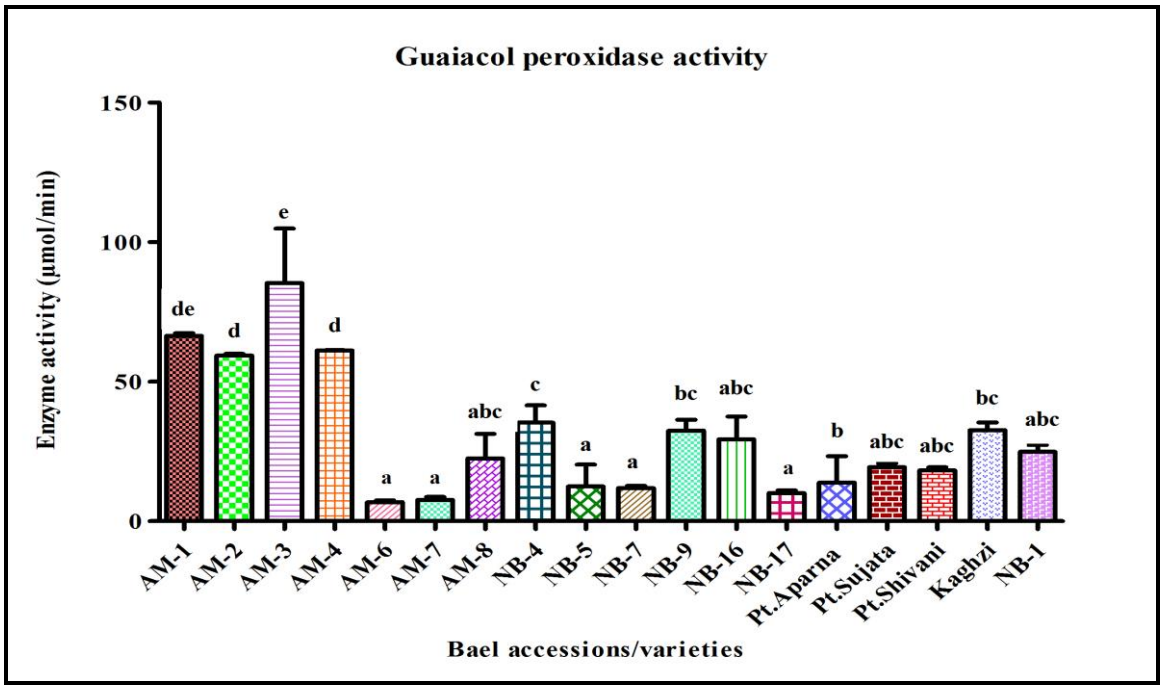

Fig.6 Estimation of Guaiacol peroxidase activity in different Bael accessions/varieties. Values are expressed as means ± SD (n=3) and are representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $P < 0.05$ as determined by one-way ANOVA (Tukey's Multiple Comparison Test)

Table 5: Polyphenol oxidase (PPO) activity in different Bael varieties/accessions

S. No.	Different Bael varieties/accessions	Enzyme activity (ΔOD/gm fresh wt.)	Specific activity (U/mg protein)
1.	**AM-1**	0.96±0.17	0.096
2.	**AM-2**	**3.92±0.072**	0.466
3.	**AM-3**	0.646±0.042	0.119
4.	**AM-4**	1.04±0.087	0.183
5.	**AM-6**	2.93±0.61	0.332
6.	**AM-7**	1.62±0.27	0.25
7.	**AM-8**	0.833±0.22	0.124
8.	**NB-1**	2.21±0.057	0.624
9.	**NB-4**	1.32±0.032	0.157
10.	**NB-5**	0.71±0.052	0.3
11.	**NB-7**	0.556±0.032	0.086
12.	**NB-9**	1.94±0.194	0.413
13.	**NB-16**	2.59±0.065	0.399
14.	**NB-17**	1.73±0.037	0.267
15.	**Pant Aparna**	0.546±0.233	0.077
16.	**Pant Sujata**	0.646±0.066	0.14
17.	**Pant Shivani**	0.336±0.11	0.04
18.	**Kaghzi**	1.36±0.081	0.459

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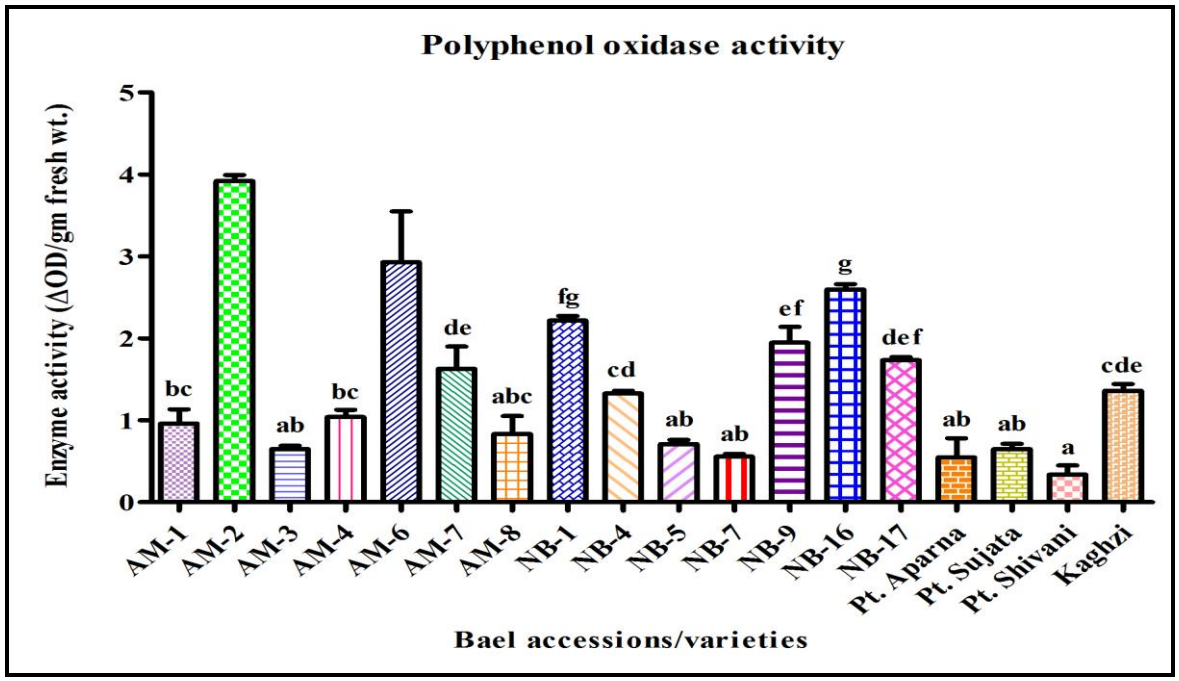

Fig.7 Evaluation of Polyphenol oxidase activity in different Bael accessions/varieties. Values are expressed as means ± SD (n=3) and are representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $P < 0.05$ as determined by one-way ANOVA (Tukey's Multiple Comparison Test)

Table 6: Ascorbate peroxidase (APOX) activity in different Bael varieties/accessions

S. No.	Different Bael varieties/accessions	Enzyme activity (ΔOD/gm fresh wt.)	Specific activity (U/mg protein)
1.	**AM-1**	5.59±0.10	0.559
2.	**AM-2**	3.41±0.04	0.405
3.	**AM-3**	7.56±0.04	1.4
4.	**AM-4**	2.44±0.13	0.429
5.	**AM-6**	6.39±0.05	0.726
6.	**AM-7**	2.6±0.19	0.401
7.	**AM-8**	5.19±0.08	0.772
8.	**NB-1**	7.38±0.07	2.084
9.	**NB-4**	**9.95±0.09**	1.184
10.	**NB-5**	6.15±0.12	2.6
11.	**NB-7**	7.21±0.10	1.126
12.	**NB-9**	5.96±0.10	1.295
13.	**NB-16**	8.35±0.06	1.288
14.	**NB-17**	1.38±0.13	0.267
15.	**Pant Aparna**	8.4±0.09	0.213
16.	**Pant Sujata**	4.58±0.04	0.995
17.	**Pant Shivani**	5.42±0.03	0.645
18.	**Kaghzi**	4.21±0.06	1.42

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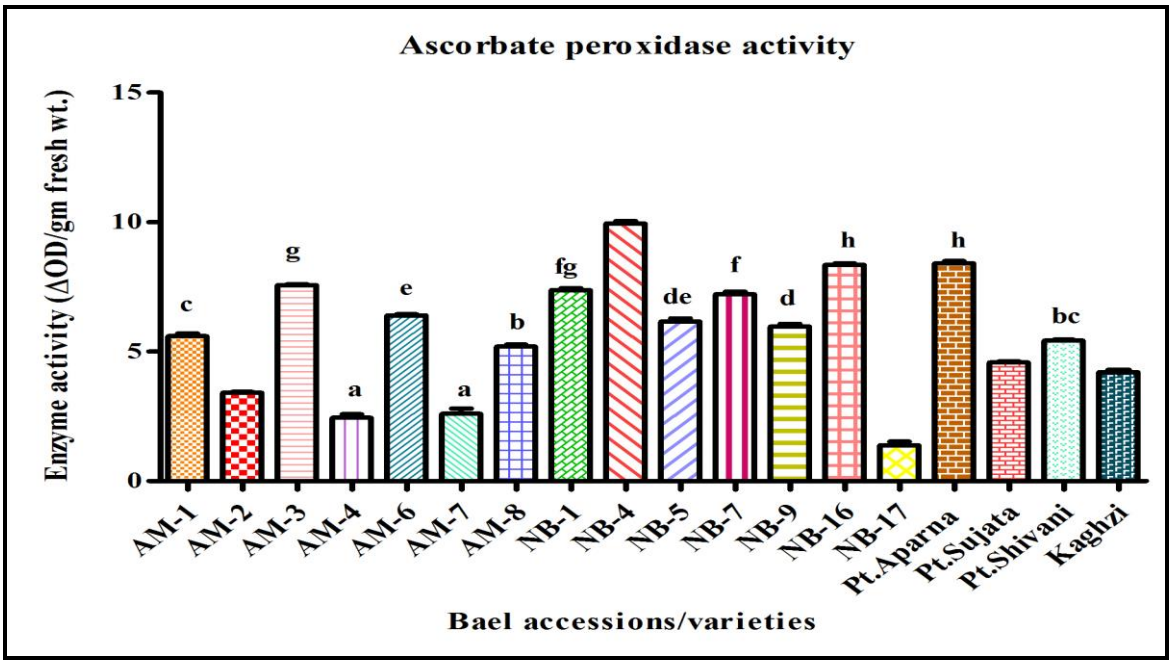

Fig.8 Determination of Ascorbate peroxidase activity in eighteen Bael accessions/varieties. Values are expressed as means ± SD (n=3) and are representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $P < 0.05$ as determined by one-way ANOVA (Tukey's Multiple Comparison Test)

Analysis of above results indicated that the leaves of different varieties/accessions of *Aegle marmelos* exhibited differential antioxidant profile. The leaves exhibited significantly greater activities of Guaiacol peroxidases, polyphenol oxidase, ascorbate peroxidase and glutathione reductase. The activity of Glutathione reductase was found to be significant in the leaves indicating the conversion of oxidized glutathione (G-S-S-G) to reduced (GSH). Moreover, the damaging effects of free radicals can be scavenged by the intracellular antioxidant enzymatic system, Guaiacol Peroxidase, and Glutathione reductase minimizes or removes cellular reactive radical cascades and decrease cytotoxic oxidative damage in cells. Guaiacol peroxidases are able to catalyze the reduction of lipid hydroperoxides to hydroxides during the oxidation of reduced Glutathione (GSH). Eventually, Glutathione reductase regenerates GSH and provides reducing power for various coupled thiol transferase and peroxidase. Furthermore, compounds decreasing free radicals and inducing antioxidative enzymes levels reduce intracellular oxidative stress and DNA damage resulting into a decrease in mutation production and cancer initiation.

CONCLUSION

The present study reports for the first time the innate enzymatic antioxidant potential of the plant *Aegle marmelos* an important medicinal plant. The different varieties/accessions exhibited significant GR, GPOX, PPO and APOX activity. The leaves revealed the presence of significant GSH content and harbor peroxidases revealing the scavenging of H_2O_2. *Aegle marmelos* contain all the antioxidative enzymes which can regulate the free radical activity and can reduce the generation of free radicals and can prevent cellular and tissue damage in the human body.

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