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Assessment of Enzymic, Non-enzymic Antioxidants and *In vitro* Free Radical Scavenging Activities of Different Extracts of Leaves and Roots of *Eclipta alba*

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Authors' contributions

This work was carried out in collaboration between both authors. Author AK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AK and SGD managed the analyses of the study. Author SGD managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

To obtain the scientific evident for antioxidant properties of *Eclipta alba* by using enzymic, non-enzymic antioxidant activity and *in vitro* free radical scavenging activity. This study was conducted on different organic solvents and hot aqueous extracts of leaves and root of *Eclipta alba* were tested for enzymic, non-enzymic antioxidant activity and *in vitro* free radical scavenging activity. Among the enzymic antioxidants tested interestingly, in leaves and roots, glutathione reductase showed potent activity of 178.2±4.5 U/mg, 120±9.0 U/mg respectively. Carotenoids expressed 305.5±1.70 mg/g being the most effective among the other non- enzymic antioxidants tested. All the extracts of leaves and roots exhibited a potent inhibition against DPPH free radical, hydrogen peroxide, lipid peroxidation, nitric oxide and superoxide generation. The data obtained in the *in vitro* tests clearly established the antioxidant potency of all extracts. Hence, overall results suggested that

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E. alba is a promising source of bioactive compounds that can be exploited as antioxidants in food products as well as in pharmaceutical therapeutic and cosmetic industry use.

Keywords: Eclipta alba; enzymic and non-enzymic antioxidant activity; In vitro free radical scavenging activity.

1. INTRODUCTION

Free radicals are molecules with unpaired electron in their outer orbit. They have very important role in origin of life and biological evolution, leaving beneficial and harmful effects on the organisms [1]. Reactive oxygen species (ROS) include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and non-free-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) [2]. The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cells [3].

Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers [4]. Some vitamins, many herbs and spices and plant extracts contain antioxidant components as well. Natural phenolic antioxidants, such as synthetics, can effectively scavenge free radicals, absorb light in the ultraviolet region (100 to 400 nm) and chelate transition metals, thus stopping progressive autoxidative damage and production of off-odors and off-tastes [5].

These antioxidants plays an important role in delaying, intercepting or preventing oxidative reactions catalyzed by free radicals, which may mainly be due to the presence of phenolic components such as flavonoids, phenolic acids and phenolic diterpenes [6]. Numerous studies have shown that aromatic and medicinal plants are source of diverse nutrient and non-nutrient molecules, many of which display antioxidant and antimicrobial properties which can protect the human body against both cellular oxidation reaction and pathogens. Thus it is important to characterize different types of medicinal plants for their antioxidant potential [7].

In this context, plants possess a complex battery of enzymatic and non-enzymatic antioxidative defense systems that can protect cells from oxidative damage. The enzymatic systems include set of gene products such as superoxide dismutases (SOD), catalase (CAT), ascorbate peroxidases (APX), glutathione peroxidases (GPX), and glutathione reductases (GR) [8]. Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical induced tissue damages.

Eclipta alba (L.) Hassk is traditionally known for its various medicinal properties. It is belonging to the family Astereaceae. Several compounds have been isolated and reported from this plant [9]. Due to presence of bioactive compounds in this plant, the objective of the present study is to evaluate the enzymic and non-enzymatic antioxidant status and to analyze the *in vitro* free radical scavenging effects of *Eclipta alba*.

2. MATERIALS AND METHODS

2.1 Collection of Sample

The plant *E. alba* was freshly collected from local market at Coimbatore. The plant was duly authenticated by Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore. A voucher specimen was deposited in the Department of Biochemistry, Avinashilingam Deemed University for Women. Coimbatore. TamilNadu. India.

2.2 Preparation of Extracts

The collected leaves and roots of the plant were washed and air dried in the shade at room temperature for complete drying. The dried sample was crushed with the aid of mechanical grinder to make powder form. The powder obtained was extracted with selected solvents based on polarity using soxhlet apparatus in 6 hours intervals. The solvents used in sequential order were respectively petroleum ether, benzene, chloroform, acetone and methanol.

2.3 Preparation of Hot Aqueous Extract

5 grams of dried leaves and root powder were boiled with 100mL of water until it becomes half

of the volumes. The extracts were kept as such in the room temperature for evaporating until it become semi solid and all of the dried extracts were dissolved in dimethylsulfoxide (DMSO), stored in the refrigeration until required for use.

2.4 Determination of the Enzymic and Non-enzymic Antioxidants

Enzymic and non-enzymic potential of *Eclipta alba* was determined by protocol of catalase [10], superoxide dismutase [11], glutathione reductase [12], glutathione-S-transferase [13], glutathione peroxidase [14], carotenoids [15], flavonoids [16], vitamin C [17], vitamin E [18], polyphenols [19] and reduced glutathione [20].

2.5 Determination of *In vitro* Free Radical Scavenging Activity

Determination of *in vitro* free radical scavenging activity of leaves and root extracts of *Eclipta alba* was performed by following regular protocols DPPH [21], hydrogen peroxide [22], superoxide [23], *in vitro* lipid peroxidation [24] nitric oxide [25].

2.6 Statistical Analysis

Values are given in the mean ± SD and the differences between values were determined by the student's t-test. Values of $P < 0.01$ were considered significant.

3. RESULTS

3.1 Assessment of Enzymic Antioxidant Status

The activity of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione – S – transferase (GST), glutathione peroxidase (GPx) were assessed in leaves and root of *Eclipta alba* and the results are presented in the Table 1. In this study leaves and root of *E. alba* showed 44.3±2.3 U/mg and 62.3±4.5 U/mg of catalase activity respectively. The maximum activity of SOD was found to be higher in roots (6.6±0.05 U/mg) than that of leaves (2.1±0.1 U/mg).The activity of GR was exhibited higher in leaves (178.2±4.5 U/mg) than that of the root (120.0±9.9 U/mg). A marked increase in the GR activity expressed in mg of protein was observed in the leaves of *Eclipta alba.* The results showed that the *Eclipta alba* has considerable GR activity in the leaves and roots which indicated that they are rich in GR enzyme. Thus the present study proves that the activity of GR might neutralize the free radicals and prevent the cells from damage.

The activity of GST was found to be 2.0±0.05 U/mg in the leaves and 1.97±0.09 U/mg in the root of *Eclipta alba*. Similarly the leaves have higher activity of GPx (2.00±0.8 U/mg) than the root 1.70±0.1 U/mg. Both the leaves and root of *Eclipta alba* are not a very good source of GST, GPx and SOD, but accumulation of GR and CAT present in the leaves and root of *Eclipta alba* was high. Since *Eclipta alba* contains significant activity of all the enzymes analyzed, it may reduce the risk of serious diseases caused by reactive oxygen species like cancer, cardiovascular diseases, hepatocellular damage, diabetes mellitus and aging.

3.2 Assessment of Non-enzymic Antioxidant Status

Table 2 revealed that *Eclipta alba* is a moderate source of non enzymic antioxidants assessed. The present study reveals that the leaves and root has 305.5±1.70 mg/g and 61.3±1.30 mg /g of carotenoids content respectively. The leaves were found to have high levels of carotenoids than the roots. The flavonoid content in leaves and root were expressed as 1.6±0.60, 4.6±0.23 mg/g of dry plant material respectively. The results showed very low level of vitamin C in both root and leaves of *Eclipta alba.* The root has a higher level of vitamin C (0.7±0.20 mg/g) than the leaves (0.2±0.03 mg/g). The leaves and roots has 1.4±0.50 mg /g and 1.7±0.20 mg/g of vitamin E content respectively. The polyphenol content in root 4.0±0.90 mg/g is higher than that of the leaves (3.0±0.79 mg/g).

The maximum content of the reduced glutathione was found to be in the leaves of *Eclipta alba.* The leaves (446.8±22.30 nm/g) and root (318.0±10.40 nm/g) have appreciable level of reduced glutathione. Since, *Eclipta alba* was found to contain moderate levels of these non enzymic antioxidants; it may be used for treating various diseases which are associated with free radical damage.

3.3 *In vitro* Free Radical Scavenging Activities

The *in vitro* free radical scavenging potential of *E. alba* extracts were assayed using DPPH scavenging activity, hydrogen peroxide

scavenging activity, inhibition of *in vitro* lipid peroxidation, inhibition of superoxide generation and inhibition of nitric oxide generation.

3.4 DPPH Radical Scavenging Activity

As shown in Fig. 1, it is clear that the methanol and acetone extract of leaves showed highest inhibition against DPPH free radicals 72% and 56% respectively. The order of DPPH radicals scavenging abilities of different extracts of leaves was methanol > acetone > aqueous > chloroform > benzene > petroleum ether and the root was acetone > methanol > aqueous >chloroform >benzene > petroleum ether respectively.

3.5 Hydrogen Peroxide Scavenging Activity

Like a result of DPPH free radical scavenging activity, methanol extract of leaf expressed the better scavenging activity (70%) for H_2O_2 (Fig. 2). The order of hydrogen peroxide scavenging abilities of different extracts of leaves was methanol > acetone > aqueous > chloroform > petroleum ether > benzene and the root was methanol > acetone> aqueous > chloroform > benzene > petroleum ether.

3.6 Inhibition of Superoxide Generation

In the case of inhibition of superoxide generation, interestingly the aqueous extract of root was found to be most effective (74%) than the methanol extract of leaves (73%). Except for the chloroform extract of leaves and root, all the other extracts have demonstrated an appreciable inhibition of superoxide generation (Fig. 3). The percent inhibition of superoxide generation in leaves and root was observed as follows, leaves showed the following order methanol > acetone > aqueous > petroleum ether > benzene > chloroform and the percent inhibition of different extracts of root showed following order aqueous > methanol > acetone > petroleum ether > chloroform > benzene.

Table 1. Activities of enzymic antioxidant in leaves and root of *Eclipta alba*

Enzymic antioxidants	Activity (U/mg of protein)		t-test
	Leaves	Root	
Catalase[1]	44.3±2.3	62.3±4.5	7.60*
Superoxide dismutase[2]	2.1±0.1	6.6±0.05	3.83*
Glutathione reductase[3]	178.2±4.5	120.0±9.0	9.40*
Glutathione- S – transferase[4]	2.0±0.05	1.97±0.09	0.45ns
Gluathione peroxidase[5]	2.0±0.8	1.70±0.1	12.29*

*Values are mean± SD of triplicates, * - significant at P < 0.01 value ns - not significant*

1. Amount of enzyme that brings about decrease in absorbance of 0.05 at 240 nm, 2. Amount of SOD that cause 50% reduction in the extent of NBT oxidation, 3. Millimoles of NADPH oxidized /min /g sample, 4. Millimoles of CDNB - GSH conjugate /min/g, 5. Millimoles of GSH utilized /min

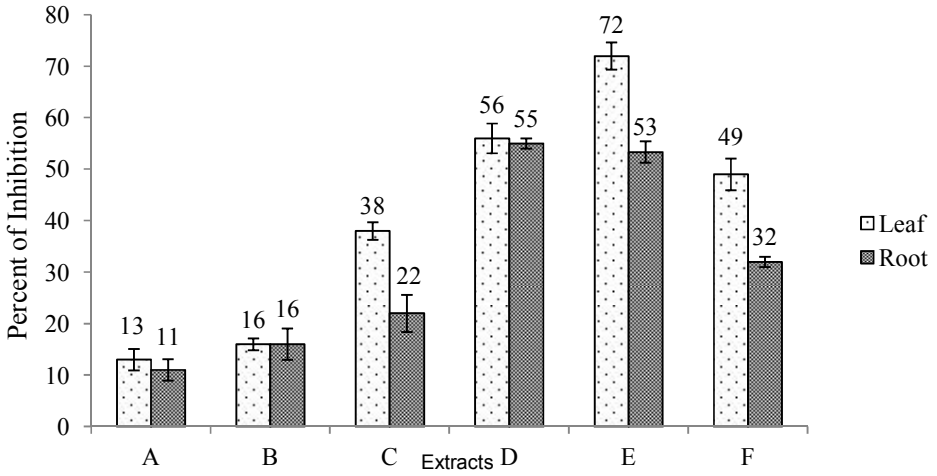

Fig. 1. DPPH radical scavenging activity of *Eclipta alba*

A - Petroleum ether, B – Benzene, C - Chloroform, D – Acetone, E – Methanol, F – Aqueous

Table 2. Levels of non enzymic antioxidants in *Eclipta alba*

Non-enzymic antioxidants	Quantity		
	Leaves	Root	t value
Carotenoids (mg/g)	305.5±1.70	61.3±1.30	54.627*
Flavonoids (mg/g)	1.6±0.60	4.6±0.23	6.717*
Vitamin – C (mg/g)	0.2±0.03	0.7±0.20	5.522*
Vitamin - E (mg/g)	1.4±0.50	1.7±0.20	0.401 ns
Polyphenols (mg/g)	3.0±0.70	4.0±0.90	0.526 ns
Reduced glutathione (nm/g)	446.8±22.30	318.0±10.40	8.483*

*Values are mean ± SD of triplicates, ns – not significant, * - significant at P<0.01 value*

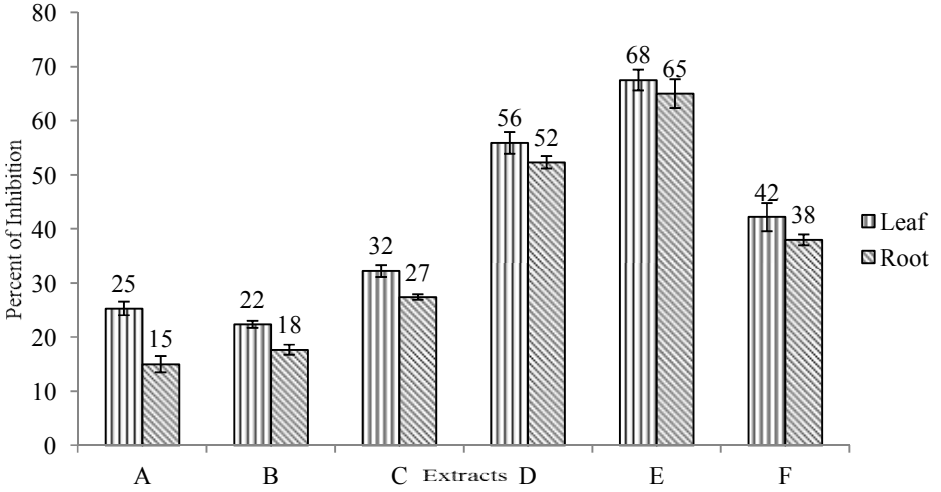

Fig. 2. Hydrogen peroxide scavenging activity of *Eclipta alba*
A - Petroleum ether, B – Benzene, C - Chloroform, D – Acetone, E – Methanol, F – Aqueous

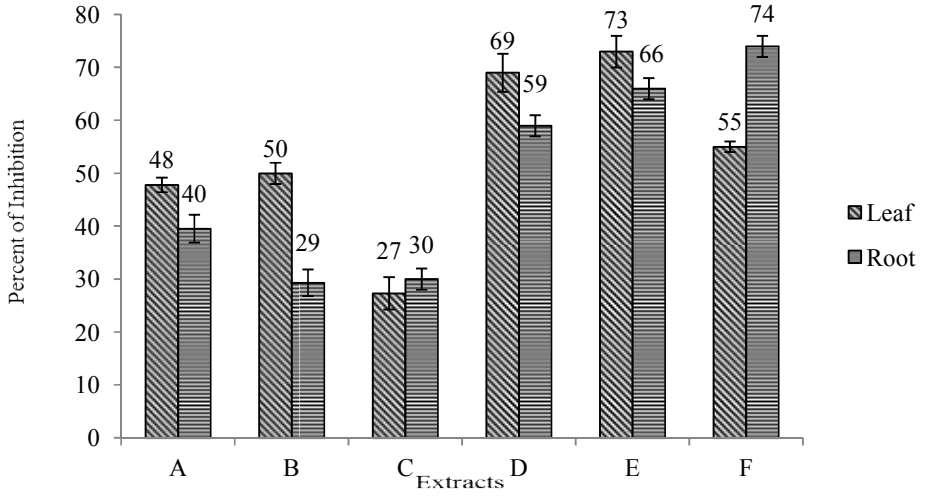

Fig. 3. Inhibition of superoxide generation of *Eclipta alba*
A - Petroleum ether, B – Benzene, C - Chloroform, D – Acetone, E – Methanol, F – Aqueous

3.7 Inhibition of *In vitro* Lipid Peroxidation

Once again the aqueous extract of leaves expressed the best scavenging activity as 66% (Fig. 4). The percent inhibition of different extracts of leaves has showed the following order aqueous > methanol > acetone > benzene > chloroform > petroleum ether and the root showed the following order acetone > aqueous > methanol > chloroform > benzene > petroleum ether.

3.8 Inhibition of Nitricoxide Generation

In the present study, the leaves and root extracts of *Eclipta alba* was checked for its inhibitory effect on nitric oxide production. Unlike other *in vitro* free radical scavenging activities, almost all the extracts demonstrated a weak NO scavenging activity. In fact the acetone extract was the only potent NO scavenger (67%). All other extracts of leaves and root showed a scavenging activity lesser than 50% (Fig. 5). The percent inhibition of different extracts of leaves of *E. alba* showed the following order acetone > methanol > aqueous > benzene > petroleum ether > chloroform and the root showed the following order acetone > petroleum ether > benzene > methanol > chloroform > aqueous. *Eclipta alba* was also found to possess significant inhibition of nitric oxide generation.

4. DISCUSSION

In this paper we studied the enzymic and non - enzymic antioxidant activity and *in vitro* free radical scavenging efficiency of several extracts of leaves and root of *Eclipta alba*. *Eclipta alba* plays the most significant role in antioxidant activity by up regulating natural antioxidant enzyme activities like glutathione reductase, catalase and superoxide dismutase. Antioxidant enzymes are rich in leaves compared to root. CAT is the class of enzymes that catalyze the conversion of hydrogen peroxide to oxygen and water. They include the classical Fe heme enzymes [26]. Catalase in leaves and root shows moderate level. Glutathione and glutathione-S-transferase are important detoxification enzymes vital to prevent the skin from photo oxidative stress due to their abilities to maintain the redox state [27]. In leaves, level of glutathione reductase was found to be increased. The level of glutathione reductase exhibited significant higher activity than other enzymes reported in enzymic activity.

The non-enzymic profile of *Eclipta alba* also seems to be good. The main antioxidant property of carotenoids is due to singlet oxygen quenching which results in excited carotenoids that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species [28]. The leaves of *Eclipta alba* might contain higher amount of carotenoids and reduced glutathione, which could react with free radicals to stabilize and block radical chain reactions. Reduced glutathione (GSH) is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects

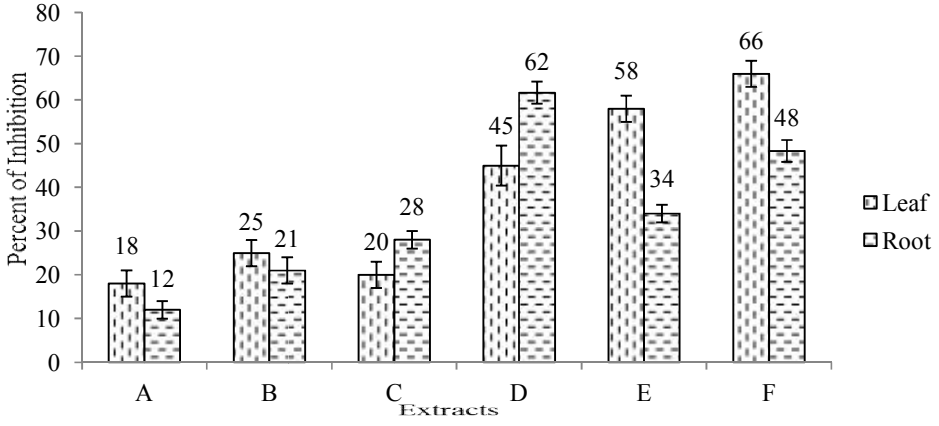

Fig. 4. Inhibition of *In vitro* lipid peroxidation of *Eclipta alba*
A - Petroleum ether, B – Benzene, C - Chloroform, D – Acetone, E – Methanol, F – Aqueous

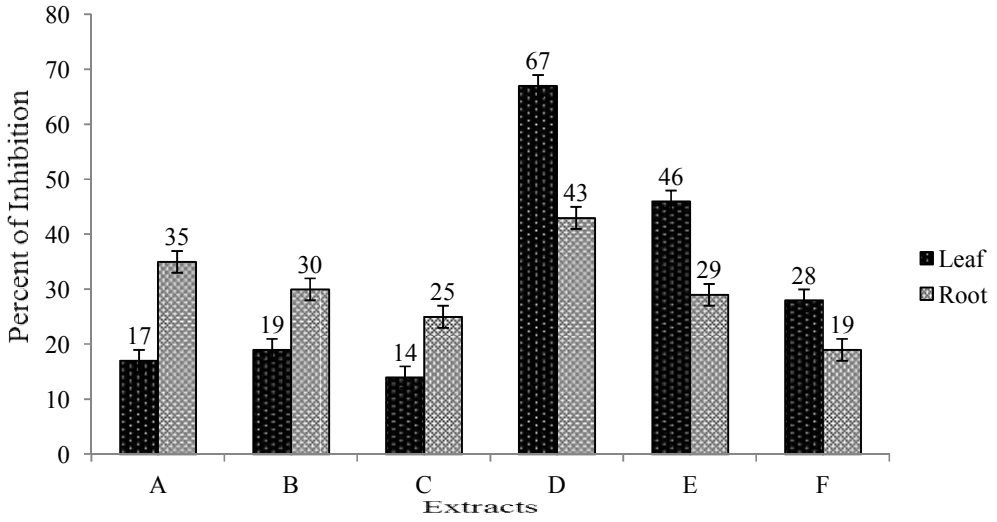

Fig. 5. Inhibition of nitric oxide generation of *Eclipta alba*
A - Petroleum ether, B – Benzene, C - Chloroform, D – Acetone, E – Methanol, F-Aqueous

cells against free radicals, peroxides and other toxic compounds. Deficiency of GSH in the lens leads to cataract formation [29]. Vitamin – C (Ascorbic acid) is believed to represent the first line of defense against potentially damaging external oxidants and it protects critical macromolecules from oxidative damage [30]. The result showed very less level of vitamin C in both leaves and roots. However, leaves of *Eclipta alba* are rich in enzymic antioxidants but non-enzymic antioxidants are prominent in roots.Hence, this data shows the capability of both the leaves and root of *Eclipta alba* able to scavenge the free radicals. Therefore *Eclipta alba* might be a good source of free radical scavengers that provide beneficial effects against oxidative damage.

DPPH test, which is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants, is a direct and reliable method for determining radical scavenging action [31]. Our result on the scavenging of DPPH free radicals shows that methanol extracts of leaf has significant effect in neutralizing harmful free radical. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells. Thus, the eradication of H_2O_2 is very important for antioxidant defense in cell or food systems [32]. The ability of methanol extract of leaves to remove H_2O_2 may be due to the presence of active components which determine their electron donating abilities to H_2O_2 thus neutralize it into water.

Superoxide radical is produced in human body by various oxidative enzymes in the form of one electron reduction of molecular oxygen. Xanthine oxidase is one of the major oxidative enzymes to produce superoxide radical as a result in tissue injury. *In vitro* superoxide radical was generated by xanthine oxidase during the reaction; Nitro blue tetrazolium (NBT) undergoes oxidation and leads to water-soluble blue formazan. The decrease in blue color formation after adding the solvent fractions in the reaction mixture was measured as superoxide radical scavenging [33]. The highest inhibiting effect was observed from root aqueous extracts of *Eclipta alba*, which was very active in inhibiting superoxide radical compared to all other extracts. Thus the present study showed that *Eclipta alba* is a potent scavenger of superoxide radicals.

Lipid peroxidation involves the reaction between the hydroxyl radicals and unsaturated fatty acid side chains of lipids and phospholipids, catalyzed by transition-metal ions. Thus the *E. alba* extracts may be able to prevent the initiation and propagation of free radical mediated chain reaction by stabilizing reactive species via electron or hydrogen donating before such deleterious reactions can occur. Interestingly, the

inhibition of lipid peroxidation corresponds to the highly quenching activity of OH by aqueous extract of leaves. Unusually among the other solvent test extracts, aqueous extract has shown a strong inhibition of lipid peroxidation. Thus the present finding indicated that *Eclipta alba* has very good potential to inhibit *in vitro* lipid peroxidation and thereby could prevent protein and DNA damage.

Nitric oxide ($NO^{\cdot}$) have also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. Despite the possible beneficial effects of $NO^{\cdot}$, its contribution to oxidative damage is also reported. This is due to the fact that $NO^{\cdot}$ can react with superoxide to form the peroxynitrite anion, which is a potential oxidant that can decompose to produce $OH^{\cdot}$ and NO. The procedure of NO estimation is based on the principle that, sodium nitro-prusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of $NO^{\cdot}$ may lead to tissue damage [34]. Except acetone extract of leaves, remaining extracts of either leaves or root show a weak inhibition activity which is less than 50 per cent.

5. CONCLUSION

In the present investigation, *Eclipta alba,* a valuable medicinal plant was found to possess *in vitro* free radical scavenging activity based on several assays. Overall, results of this study indicate that a non-polar solvent petroleum ether and low polarity solvents such benzene, chloroform extracts of both leaf and root have gained less attention, due to their weak ability to quench free radicals like DPPH, H_2O_2, nitric oxide and fail to inhibit *in vitro* lipid peroxidation. The bioactive phytochemical constituents may establish their contribution to quench free radicals by donating a hydrogen atom or an electron to them. Based on the data of this study, further research has to carry out to know the mechanism of plant extract and bio assayed fraction of crude extract in antioxidant activity, which will provide information to extend a research for studying anti-diabetic, anti-cancer activity. It can be concluded from the research work that *E.alba,* as potent antioxidative supplementary for food and therapeutic purpose.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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