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Research Article

ANALYSIS OF PHYTOCHEMICAL AND ANTIOXIDANT POTENTIAL OF *OCIMUM KILIMANDSCHARICUM* LINN

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ABSTRACT

The modulations in enzymatic and non-enzymatic antioxidants were clearly evident in *Ocimum kilimandscharicum* exposed to UV-B stress. Recovery on lipid peroxidation and alterations in the contents of free radicals in leaves was studied. Lipid peroxidation measured in terms of MDA level increased with UV-B doses and the ratio was higher with high dose of radiations. Recovered leaves showed lower MDA content and hydroxide radicals. Ascorbate, flavonoid and proline contents increased highly in leaves recovered from UV-B stress. Higher free radical scavenging capacity and more efficient antioxidant potential of *Ocimum kilimandscharicum* cultivars were proved *in vivo*, using the thiobarbituric acid assay in liver and muscle assay systems of ovarian models. The present findings suggested that UV-B doses have modulated the antioxidative machinery of *Ocimum* plants. Differences in responses were closely related to the differences in the activities of antioxidants and overall growth responses. The GC-MS profile showed the presence of chemical compound that are known for their antioxidant properties. A significant conclusion can be drawn from the findings that improved tolerance to UV-B stress may be accomplished by increased capacity of antioxidant system of plants and *O. kilimandscharicum* essential oils are most suitable for the liver comparative to the muscle during oxidative stress conditions.

Keywords: Lipid peroxidation, Malondialdehyde, UV-B, Glutathione, antioxidants

INTRODUCTION

Uttarakhand state is known for being one of the richest regions for biodiversity in India. It favors the luxuriant growth of diversified and rich vegetation that are used in many traditional medicines due to the great altitudinal variations and wide array of climatic zones[1]. Herbal crops have been evaluated from centuries for their flavouring characters and therapeautic properties. *Ocimum* is one of the most popular culinary herbal crops known for its medicinal properties. The genus of *Ocimum* belongs to the family Lamiaceae (Labiatae). It includes at least 60 species and numerous cultivars[2]. It has been attracted the attention of researchers due to its high contents of essential oil that is being used in food, perfumery and cosmetic industries[3]. A high degree of polymorphism in the genus *Ocimum* determines a large number of subspecies, different varieties and forms producing essential oils with varying chemical composition offering variable level of medicinal potential[4]. Essential oils extracted from *Ocimum* plants have been reported to possess an interesting spectrum of antifungal properties[5], antinociceptive property[6], anticonvulsant[7], antioxidant[8,9,10]). *Ocimum kilimandscharicum* is an aromatic under shrub with pubescent quadrangular branchlets. It is employed as indigenous medicine for a variety of ailments like cough, bronchitis, viral infections, foul ulcers, anorexia, mosquito repellant and for healing wounds[11,12]. *Ocimum* plants contain large amounts of antioxidants other then Vitamin C, Vitamin E, flavonoids and carotenoids. The presences of many pharmacologically active compounds in *Ocimum* species provide them protection against free radical induced oxidative damage of cellular components.

Numerous studies have been conducted to analyze the antioxidant potential of various *Ocimum* species[13] and alterations in their values due to various stresses[14,15]. Plants need to have special mechanisms for adjusting to the changed environment. Furthermore, many groups of stresses like heavy metals, pesticides and ultraviolet radiations are shown to generate singlet oxygen and other active oxygen species. Free radical-induced lipid peroxidation of cellular metabolites may produce oxidative stress in cells, affecting the growth of plants. Enhanced exposures of UV-radiations are reported to inhibit various plant processes such as photosynthesis, respiration etc producing free radicals[16,17]). Plant metabolize these free radicals by invoking an increased antioxidant machinery that include both enzymatic antioxidants such as superoxide dismutase, catalase, peroxidase IAA oxidase etc[18,19] and non-enzymatic antioxidants such as proline[20], flavonoids, ascorbate etc[21].

Ocimum species have been attracted the attention of several workers due to the high contents of natural antioxidants. Lipid peroxidation is a well established mechanism of cellular injury, in both plants and animals which is used as an indicator of oxidative stress in cells and tissues. MDA, a secondary product of lipid peroxidation is used as an indicator of tissue damage. MDA reacts with thiobarbituric acid and produces red colored products. Total antioxidant capacity can be assayed using this property of TBA. Considering the above facts, the authors had been using rapid quantifiable screening protocols to identify the extent of antioxidant potential of *Ocimum kilimandscharicum* and its role against UV-B stress in terms of variations in the total contents of protein, ascorbate, proline, Flavonoids, enzymatic antioxidants along with the comparative measurement of total antioxidant activities in liver and muscle assay systems of ovarian models.

MATERIALS AND METHODS

Plant materials and UV-B Treatments

The seeds collected from naturally growing wild plants from parts of the districts Dehradun & Chamoli (Uttarakhand) India, were surface sterilized in 0.25% $HgCl_2$ and transferred in growth chamber with an illumination of 150 μM $m^{-2}s^{-1}$ for 13 hours followed by 11 hours dark period and 70-75% relative humidity. The plantlets (4 weeks old) grown in soil extract liquid nutrient medium were exposed to Ultraviolet Light (0.4 Wm^{-2}) for different time intervals. UV- B irradiation (280-312nm) was provided by fluorescent tube with its main output at 312 nm and required intensity was obtained by adjusting the distance (30 cm in present case) between the source and cultured plantlets.

Estimation of total protein, ascorbate, flavonoid, proline and glutathione levels

Protein contents were determined using Folins-Lowry method using lysozyme as the standard[22]. Ascorbic acid content in leaf homogenates, in UV-B treated/untreated plants were estimated by

making slurry in sulfosalicylic acid[23]. Incubating reaction mixture (temp, 60^0C; time, 35 minutes) contained sodium molybdate (2%), 0.15 N H_2SO_4, 1.5 mM K_2HPO_4 and tissue extract. Absorbance of the supernatant was recorded at 660 nm and the amount was calculated by using L-Ascorbate as standard. Flavonoids were estimated in *Ocimum kilimandscharicum* leaves by keeping them in acidified methanol (methanol: water: HCl, 78: 20: 2, v/v) for 24h at 4^0C. The extracts were treated with few drops of 10% lead acetate solution for the confirmatory test of flavonoids. The formation of yellow precipitate confirmed the presence of Flavonoids. Total contents were expressed as absorbance g^{-1} fresh mass of tissue at 320 nm[24,25]. Proline contents were extracted and estimated using standard method and amounts were expressed as µg g^{-1} FW[26]. Glutathione was measured by its reaction with DTNB (5- 5'dithiobis, 2 nitrobenzoic acid) to give a yellow colored complex with its absorption maximum at 412 nm[23]. 500 µl of the sample was mixed with 125 µl of TCA (25%). The tubes were cooled on ice for 5 minutes, then added 600 µl of 5% TCA and centrifuged for 5 minutes. 300 µl of supernatant was taken for GSH estimation. The reaction mixture consists of 300 µl of supernatant and 700 µl of phosphate buffer and 2 ml of DTNB (0.6 mM). After an incubation of 10 minutes at 37^0C, the absorbance of the reaction mixture was measured at 412 nm. The amount of glutathione was expressed in terms of nM/mg protein.

Estimation of enzymatic antioxidants

In vivo catalase (EC 1.11.10.6) activity was determined by using the method of Egashira *et al.* (1989)[28]. Plant leaves were suspended in fresh 50 mM of phosphate buffer (pH 7.0). In each samples catalase activity was determined by recording O_2 evolution for 1 min after the addition of 5 ml of 50 mM phosphate buffer (pH 7.0) containing 50 mM H_2O_2. To this, 1 ml of cell suspension was added and in darkness O_2 evolution was monitored. SOD (EC 1.15.1.1) activity was assayed by the method of Giannopolitis and Ries (1977)[29]. The reaction mixture contained 1.3 µM riboflavin, 13 mM L- methionine, 0.05 M Na_2CO_3, (pH 10.2), 63 µM p– nitroblue tetrazolium chloride (NBT) and crude plant extract. Peroxidase (EC 1.11.1.7) was estimated by adding 0.1 M Phosphate buffer (pH 7.0) to homogenized leaf samples. The enzyme reaction mixture consisted of 0.1 M Phosphate buffer + 20 mM guaiacol + 12.5 mM H_2O_2 and plant extract[30]. IAA oxidase activity was assayed using the method of Byrant and Lane (1979)[31]. The enzyme reaction mixture contained: 0.071 M Phosphate buffer + 0.5 mM $MnCl_2$ + 0.05% paracoumaric acid + enzyme extract. After ½ hour incubation in dark 5 M perchloric acid and 0.1 M ferric nitrate solution was added. Optical density was measured at 535 nm, after incubation for 60 minutes in dark.

Estimation of % antioxidant index (TBARS ASSAY)

The essential oils of *Ocimum kilimandscharicum* were isolated by hydrodistillation using an essential oil distillation apparatus (Quick Fit). The dilutions of essential oils were made in 8.1% (w/v aq.) sodium dodecyl sulphate (SDS). All the experiments were conducted on liver and muscle of chickens. Organs were stored at -20^0C until required. Homogenate (10%) were prepared with KCl (1.15%, w/v) using a tissue homogenizer[32]. Three groups of experimental organisms were maintained:

Control (-): Tissue homogenate without essential oil

Control (+): Tissue homogenate **+** antioxidant of known property (L-Ascorbate)

Test samples: Tissue homogenate + essential oil in different concentrations.

In order for direct comparison to be made between all assays, the antioxidant index (AI) was expressed as value relative to control. It was calculated using following formula Aeschbach *et al.* (1994)[33] which was used to demonstrate the comparative protective properties of the essential oil in the respective assays.

% AI= (1-T/C) x 100

Where C is the absorbance value of fully oxidized control and T is the absorbance value of the test sample.

All the experiments were repeated three times with variable replicates (3 to 6) per repeat and data presented are mean + standard error (SE).

Gas chromatography-Mass spectrophotometric (GC-MS) analysis

The component analysis of essential oil was performed with The Perkin Elmer Clarus 500 (Turbomass software Ver.5.0.0) data handling system with manual injection/injector with mass detector, equipped with a Rtx®-5 capillary column (60 m X 0.32 mm ID X film thickness 0.25 µm) crossbond®5 % diphenyl siloxane. Analysis was carried out using helium as the carrier gas, with the flow rate at 1.0ml/min[34]. The column temperature was programmed from 60°C to 220°C at 3°C/min for 5 minutes. The sample size was 0.2µ1, the splitting ratio 1:50.The injection part temperature was 210°C. The solvent delay time was set for 5 minutes and moss range was m/z 40-500 a.m.u. The Kovat's indices were determined and the separated components were identified by matching with those of computer library search (*NIST/PFLEGER/WILEY*) and available authentic components. The quantitative determination was carried out based on peak area integration.

RESULTS AND DISCUSSION

Modulations in ascorbate, proline and flavonoid contents

In the present study, interesting spectrum of modulations in enzymatic and no-enzymatic antioxidants was observed that helped the plants to mitigate the free-radical induced toxicity. Enhanced exposure of ultraviolet radiations acted as stimulating agent for protein synthesis at low doses. It was observed that the % control increase in protein contents were 15.21, 29.55 & 43.38 at UV-B exposure for 20, 40 and 60 minutes, respectively (figure 1 a). Increase in protein synthesis rate may be correlated with the synthesis of enzymatic antioxidants that help plants in recovering from oxidative stress[35]. Ascorbate contents showed very little increase in the amounts (82.1 & 89.5 µg/g FW) with UV-B exposure for 20 and 40 minutes and the decrease at high doses (70.32 µg/g FW, after 60 minutes of exposure), compared to untreated samples (control = 75.47 µg/g FW), in *Ocimum kilimandscharicum* (figure 1 b). There are two possibilities regarding increase in ascorbic acid contents; either its synthesis has increased or its regeneration rate has increased (as observed in *Ulva fasciata*)[36]. Although, the enzymes involved in regeneration of ascorbic acid has not been estimated in the present study, several reports indicate considerable increase in ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase enzymes actively involved in regeneration of ascorbic acid in response to UV-B[37]. Proline contents were found to be accumulated at all the doses of UV-B in *Ocimum* plants. At the longest duration of exposure (60 minutes), the values were observed to be 27.1 µg g -1 FW, in comparison with control (10.75 µg g -1 FW) (figure 1 b). Similar to ascorbic acid, proline content has also been shown to be regulated in plants exposed to various stresses such as drought, salt and UV-B[38]. The accumulation and protective effect of proline has been observed in many higher plants and prokaryotes. Proline is a reliable indicator of the environmental stress imposed to plants[39].

The chemical evolution and significance of flavonoids has been assumed to play an important role in overcoming the oxidative stress in cells[40]. Evidences suggest that the presence of flavonoids in UV-B irradiated leaves could alter the perception or response of other defense mechanisms[41]. Presently, Flavonoid contents showed enhanced synthesis in UV-B treated *Ocimum kilimandscharicum* plants in comparison with untreated samples (control= 0.77 Absorbance/g FW). The values were found to be 0.82, 0.98 and 1.17 Absorbance/g FW (figure 1 c). Earlier findings showed remarkable alterations in the Flavonoids contents of the UV-B exposed soybean cultivars[42]. Flavonoids inhibit the enzymes responsible for superoxide anion production and AOS generation, such as xanthine oxidase[43], cyclo-oxygenase, lipoxygenase, glutathione s- transferase and mitochondrial succinic oxidase[44]. Remarkable alterations in protein, ascorbate, proline and flavonoid contents in UV-B treated plants (% control inhibition in the ascorbate contents was observed to be 9.31%, 14.99% and 17.81% while Flavonoids showed 20.65%,

17.65% and 6.98% reduction with respect to the controls) were clearly evident. Similar pattern of modulations were found in the contents of Proline also.

Lipid peroxidation and glutathione levels

The level of hydrogen peroxide radicals were enhanced in all the UV-B treated plant samples, in comparison with untreated controls. The values of peroxide radicals in *Ocimum kilimandscharicum* were observed to be 42.3, 52.5 and 68.5 µmol/g fresh weight at 20 minutes, 40 minutes and 60 minutes of UV-B exposure, respectively (figure 1 d), in comparison with control (28.9 µmol/g fresh weight). A % control increase of 62% in the contents proved a high level of peroxidation at 60 minutes of UV-B exposure.

Hydrogen peroxide radicals may be very damaging, since they can attack lipids in cell membrane, proteins in tissues or enzymes and DNA to induce oxidations, which cause membrane damage, protein modification and DNA damage[45]. UV-B induced lipid peroxidation of the cellular components in *Ocimum kilimandscharicum* was studied by estimating the level of MDA and the related data are depicted in the figure 2 (a).

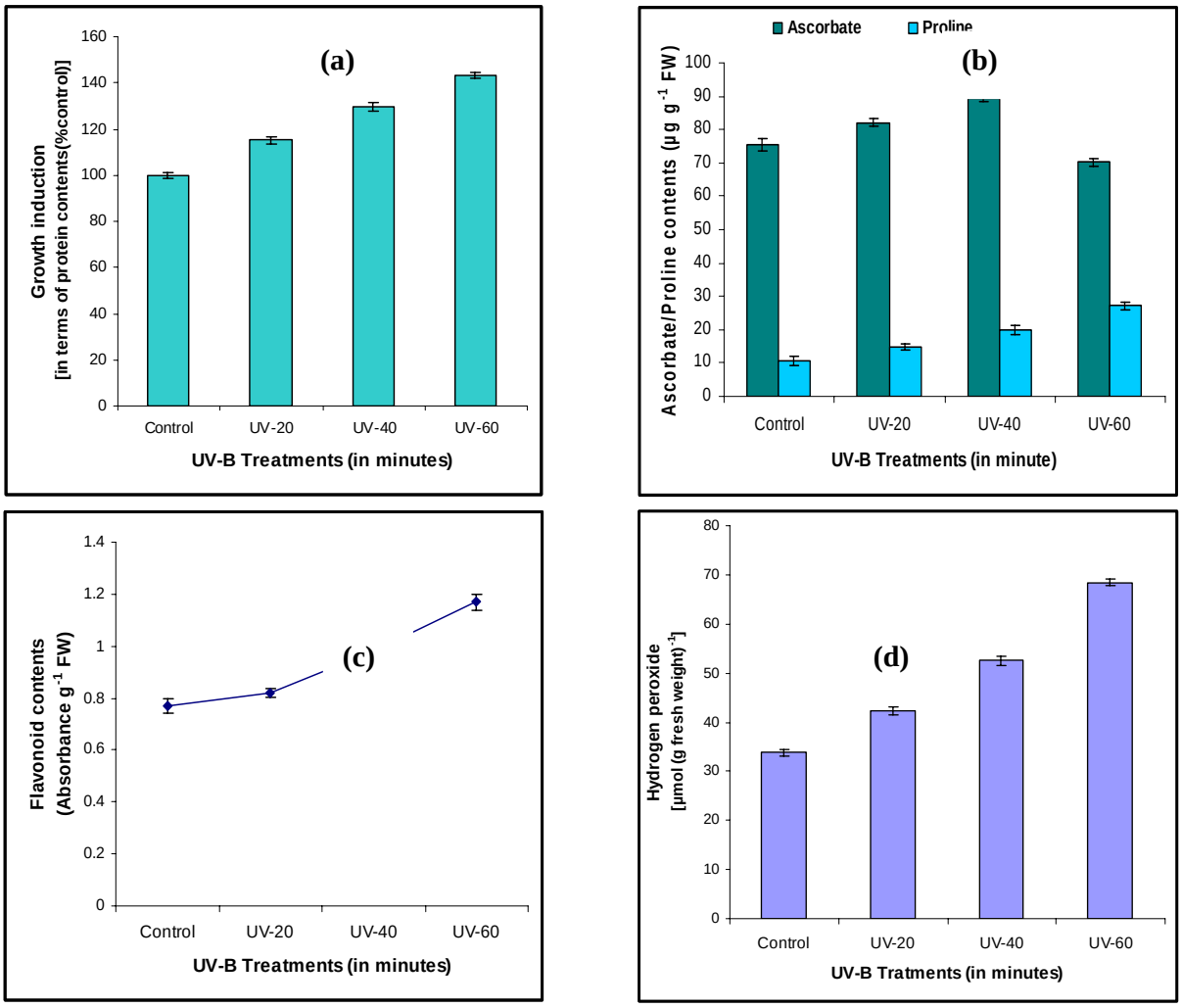

Fig. 1: UV-B induced alterations in (a) total protein (b) ascorbate and proline (c) flavonoids and (d) hydrogen peroxide radical contents, in leaves of *Ocimum kilimandscharicum*. The values are means + SE

Total MDA contents in untreated controls was 1.56 nmol MDA (mg fresh mass)$^{-1}$. It is clearly shown that the level of lipid peroxidation was increased in all the treated samples. UV-B exposure upto 60 minutes caused lipid peroxidation to increase by 59.32% in *Ocimum* plants. The results obtained here were in agreement with the works previously done[46]. Glutathione levels were observed to be decreasing with the increasing oil concentrations (figure 2 d). In liver homogenates, GSH activity was decreased to 13 and 39% with 50 and 100 µl of oil while in liver homogenates it reduced up to 3.2 & 28% with same amount of essential oils used. It proved that the glutathione production could be controlled using the antioxidant potential of *Ocimum* oils.

Enzymatic antioxidants

The activity of the superoxide dismutase in non-stressed plants was increased continuously (% control increase at 60 minutes of UV-B exposure was 27.8) in *Ocimum kilimandscharicum* (figure 2 b). It indicated that plant samples appeared to be more resistant against Superoxide radicals produced due to various kinds of stresses. The enhancement in the activity of SOD may be as a consequence of increased production of O_{2^-} radicals. SOD converts relatively less toxic O_{2^-} radicals to more toxic H_2O_2. Thus H_2O_2 scavenging activity is increased. The Peroxidase activity showed varied responses with UV-B treatments. Peroxidase activity was increased with low duration treatments and this was continued linearly but at the

higher intensities, their values were inhibited in all *Ocimum* species as compared to the untreated samples (figure 2 b). Increased activity of Peroxidase indicated more powerful mechanism of detoxification of overproduced H_2O_2.

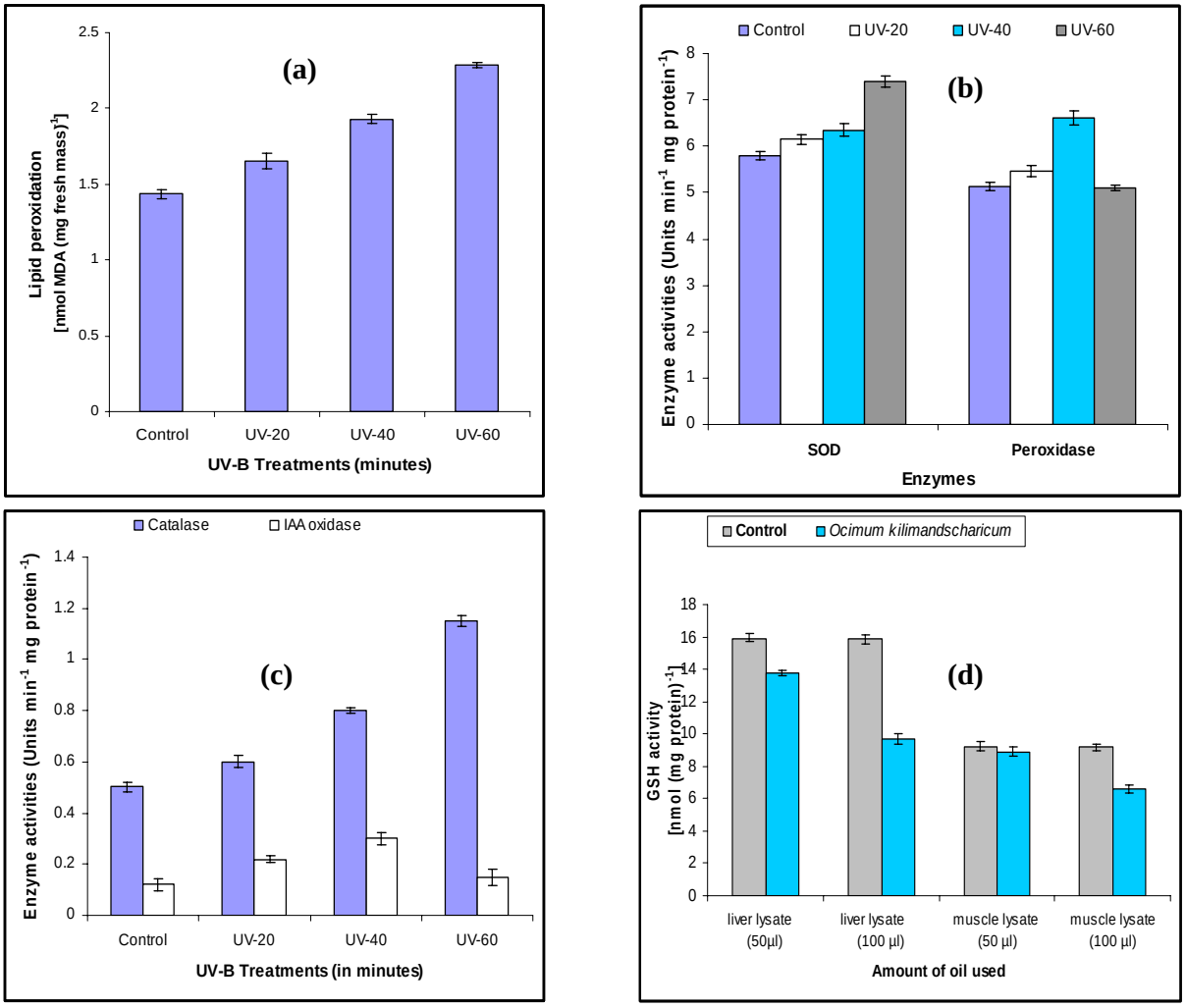

Fig. 2: UV-B induced formation of total MDA contents (a) & modulations in the activity of SOD, Peroxidase (b), catalase, IAA oxidase (c) and GSH (d)

It was observed that the catalase activity showed enhancement in plants exposed to 60 minutes of UV-B irradiation (1.15 Units min^{-1} mg $protein^{-1}$ as compared to the control value; 0.502 Units min^{-1} mg $protein^{-1}$). Treatments of shorter durations stimulated catalase activity a little but high duration exposures increased the enzymatic activity rapidly (figure 2 c). The increase in the activity of catalase might be due to the need to decompose H_2O_2 and to protect cell membranes. IAA oxidase activity increased initially showing an enhancement in the enzyme activity but it showed remarkable reduction in values at higher doses (% control alteration in the values at 20, 40 and 60 minutes of UV-B treatment were : 83%, 150% & 25.8%, respectively) (figure 2 c). The results were in accordance with the studies done in *Vigna radiata*[47].

Total antioxidant potential (Antioxidant index) and GC-MS.

The total antioxidant activity was measured in terms of antioxidant index (%) by using two bioassay systems (chicken liver and muscles) as depicted in the figures 3 (a) & (b). It was observed that both assay systems demonstrated an ability to reflect differing antioxidant capacities proportionately with dilutions of the oils. In chicken liver assay, the maximum antioxidant capacity was shown by the highest oil concentration used (AI= 92.47%) while the standard antioxidant L-Ascorbate showed an antioxidant index of 66.67% with the same concentration (100 ppm) of essential oil (figure 3 a). Even 1.5 ppm concentration of oils extracted from *Ocimum kilimandscharicum* was found to be more effective than the standard antioxidant (L- ascorbate) used in the present study. The results clearly indicated that the antioxidant potential of *Ocimum* species increased with the increase in oil concentrations. Similarly in chicken muscle assay, the increasing trends of oil concentrations were linearly correlated with the increasing % antioxidant index (Figure 3 b). It increased up to 78.56 from 10.44. The differences in both, qualitative and quantitative aspects of the lipids present in the tissues within the media clearly play a vital role in the variable responses seen in the assays. The experimental organs were obtained from sources with high degree of uniform lipid contents). Here, it is clearly evident that a distinctive inconsistency was found with various concentration of *Ocimum* oil in chicken muscle assay system, inspite of a number of repeats of the experiments. Overall dose responses for the liver and muscle systems in chickens significantly showed different responses with variations in organs. It is evident that *Ocimum kilimandscharicum* showed a significant level

of protection against lipid peroxidation from free radical induced damage, in both liver and muscle assay systems. In all the cases, oil achieved similar levels of lipid protection at the 100 ppm concentration of oils thus making it more suitable for screening for antioxidant properties. The different chemical composition of essential oils may help in explaining the observed differences in antioxidant activity. GC-MS analysis of *Ocimum kilimandscharicum* oil showed that the major component found was camphor (56.07%). Besides camphor, DL- limonene (13.56%), camphene (7.32%), 4-terpineol (3.50%), β- ocimene (2.00%), linalool (1.70%), β- myrcene (1.58%) and α-terpinolene (1.33%) were reported to be present. L-phellandrene was found in least concentration of about 0.26% (Table-1 and GC-MS chromatogram). Most of the above compounds have already been demonstrated to possess *in vitro* antioxidant properties.

The current work provides preliminary information and methodologies for rapid quantifiable screening of antioxidant potentials of *Ocimum kilimandscharicum*. It is widely used in many traditional medicines prescribed under different systems of medicine. It is, therefore, important to study the total antioxidant properties and chemical profiling of *Ocimum* species. Overall growth of the *Ocimum* species was found to be retarded by longer UV-B exposures. Slight enhancements in the contents of enzymatic and non-enzymatic antioxidants due to the low duration exposures may be the sign of recovery from oxidative stress condition. Antioxidants as Flavonoids, proline and ascorbate contents showed usually enhancements proving their potential for scavenging reactive oxygen species. *In vitro* screening of antioxidant activities in organ systems showed varied responses that might be explained on the basis of variable enzymatic contents in different organs. *Ocimum kilimandscharicum* oil was proved to be better antioxidant for liver than muscle systems in oxidative stresses. It was observed that there was an intimate relationship among the contents of natural antioxidant and recovery potential of plants from oxidative stress conditions in terms of antioxidant index.

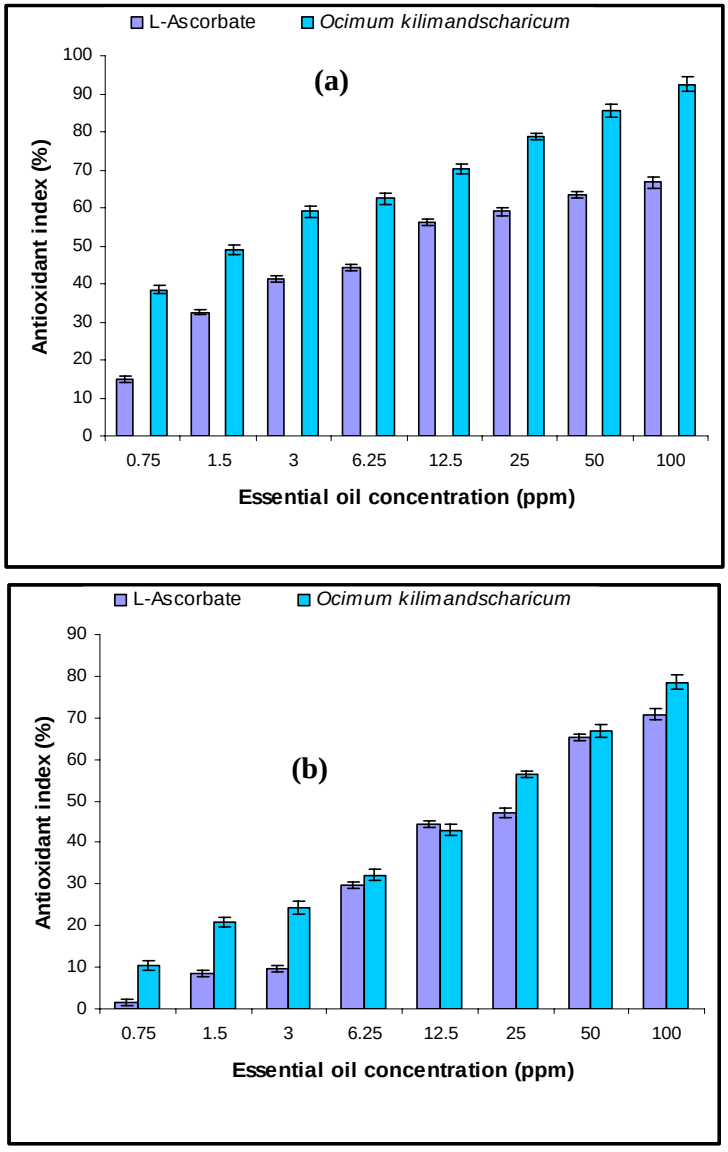

Fig. 3: Differential responses in Antioxidant Index to various dilutions of *Ocimum kilimandscharicum* essential oils using the chicken liver (a) and chicken muscle (b) assay systems. The values represented are mean + SE and are significantly different from their respective controls ($p<0.05$)

Table 1: Major essential oil components (GC-MS analysis)

S. No.	Components	Scan	Percentage
1.	α – Pinene	599	1.23
2.	Camphene	660	7.32
3.	β – myrcene	809	1.58
4.	Ethylamyl carbinol	837	0.88
5.	L – Phellandrene	876	0.26
6.	α – terpinene	927	0.33
7.	p – cymene	962	0.62
8.	DL – limonene	981	13.56
9.	1,8 –cineole	1005	0.85
10.	β – ocimene	1053	2.00
11.	γ – terpinene	1109	0.88
12.	Trans- sabinene hydrate	1162	0.49
13.	α – terpinolene	1242	1.33
14.	Linalool	1292	1.70
15.	cis – sabinene hydrate	1305	0.47
16.	Camphor	1531	56.07
17.	4 – terpineol	1660	3.50
18.	Myrtenol	1751	1.24
19.	Trans – caryophyllene	2712	0.33
20.	Germarcrene-D	2961	0.43

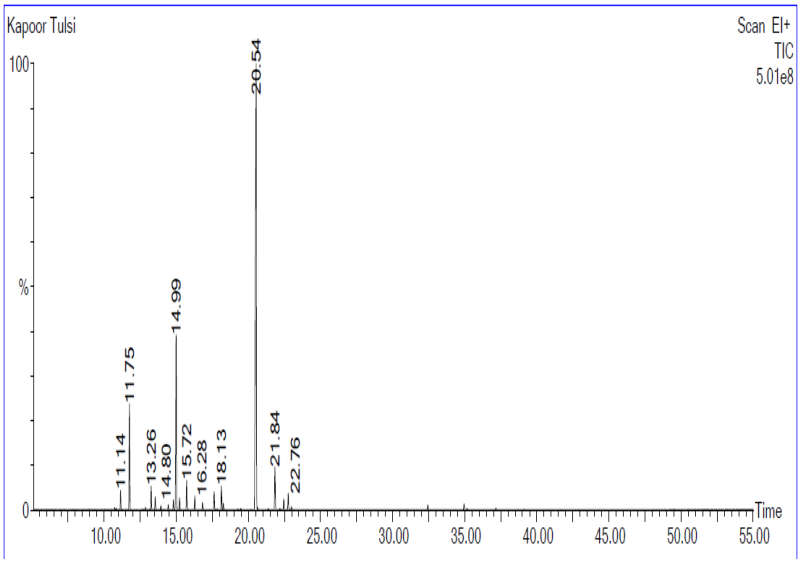

Fig. 4: GC-MS Chromatogram of *Ocimum kilimandscharicum* essential oil

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