African Journal of Biochemistry Research Vol. 6(13), pp. 165-171, November 2012
Available online at http://www.academicjournals.org/AJBR
DOI: 10.5897/AJBR12.013
ISSN 1996-0778 ©2012 Academic Journals

Full Length Research Paper

Antioxidant screening of ethanolic and aqueous extracts of *Organum vulgare* L. *in-vitro*

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Accepted 13 June, 2012

The present study was envisaged to assess the rationality for the use of traditionally invogue herb *Organum vulgare* as an anti-oxidant agent. Potential antioxidative activity of *O. vulgare* was evaluated using various *in vitro* methods including post mitochondrial supernatant (PMS) and microsomal lipid peroxidation, DNA sugar damage, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and FTC/TBA methods. Both ethanolic and aqueous extracts exhibited good antioxidant and radical scavenging properties but the effects were pronounced in ethanolic extracts of *O. vulgare*. The extracts strongly inhibited DNA damage. The extracts showed significantly remarkable scavenging effects in DPPH free radical scavenging assay and the activity indicated a marked correlation with phenolic content. From the results the extracts (that is, aqueous and ethanolic extracts of *O. vulgare* might be valuable antioxidative sources.

Key words: Antioxidant, radical scavenging, *Organum vulgare,* 2,2-diphenyl-1-picrylhydrazyl (DPPH).

INTRODUCTION

Organum vulgare L. is a sub-species of a widespread wild oregano found in Greece, Turkey and in South and East Asia - appears in almost all of Europe and Asia, Northwest Africa, the Iberian Peninsula and Macaronesian region (Castroviejo et al., 2010). In India, it grows at high altitudes in the Himalaya usually in rocky areas and in the Kashmir valley where it is properly known as (*watpan).*

Origanum Linn. (Labiatae)

A small genus of perennial herbs or undershrubs, Origanum is distributed in the Mediterranean region and extra tropical Asia. One species *O. vulgare* Linn. common or wild Marjoram. Hindi-Sathra; Punjab-Mirzanjosh; Kashmiri-Baber occurs in India. An aromatic, branched perennial herb 30-90 cm high, found in the temperate Himalayas from Kashmir to Sikkim, at altitudes of 1500-3600 m. Leaves broadly ovate, entire or rarely toothed; flowers purple or pink, in corymbose cymes; nutlets smooth, brown.

O. vulgare is very common in Shimla hills and in Kashmir valley. It is hardy and can be grown in all warm garden soils. It is propagated by seeds, cuttings, layers and root division. It can be sown during October in the plains and during March and April in the hills. The plant possesses aromatic, thyme like flavor. The leaves and tops cut prior to blooming are used to flavor foods in the same way as sweet Marjoram (*Majorana hortensis*). The plant is used in Punjab as a pot herb; it is eaten also as vegetable in Lahul. It was formerly employed to flavor ale and beer, before hops were introduced in the brewing industry.

The herb contains a volatile oil (0.15-0.40%), tannin (0.8%) and a bitter principle. The oil of European origin (specific gr.^{150}, 0.868-0.910; [α] D,-20° to -70°] possesses an aromatic, spicy, somewhat basil like odour and contains thymol (upto 7%). Carvacrol, free alcohols (C, 13%), esters (as geranyl acetate), free alcohols (2-3%) and a bicyclic sesquiterpene (12.5%). Steam distillation of the whole plant from Kashmir gave pale yellow oil (yield 0.2%) with a pleasant smell and the following characteristics:

Sp.gr.^{27}°, 0.8812; n^{27}° ,1.4795;[α]^{27}°^{D},-1.5°; acid value,

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2.5; ester value, 10.4 (after acetylation, 102.7); Phenol content, nil; free sol in 90% alcohol. It contained dl-pinene, dipentene, linalool, bi and tricyclic sesquiterpenes and palmitic acid. The oil called oil of origanum in trade is really thyme oil (from *Thymus vulgaris* Linn.).Oil of *O. vulgare* is often confused with sweet marjoram oil (from *Marjorana hortensis*) which is, however dextrarotatory (upto +40°).

The oil possesses carminative, stomachic, diuretic, diaphoretic and emmenagogue properties. It is given as a stimulant and tonic in colic and diarrhea. It is applied in chronic rheumatism, toothache and earache (Wealth of India). Due to the spasmolytic action of the oil, it is used in whooping cough and bronchitis. In homeopathy it is used for hysteric condition. It is used as an external application in healing lotions for wounds, usually in conjunction with other herbs. The oil has been employed in veterinary ointments. It is used in gargle and bath. It stimulates growth of hair. It is also used in cosmetic and soap industry. Its usage in the treatment of Rheumatic pain has evolved considerable interests in the recent past (Mukeerjee et al., 1940).

The whole herb of *O. vulgare* have been used for centuries in Indian system of medicine (Alves-Pereira and Fernandes-Ferreira, 1998) and the effects of herb on arthritis, diabetes and cancer (Wealth of India, 1966) have been reported. Chemical investigations have shown the presence of carvacrol, ursolic acid, caffeic acid, terpinolene, alpha pinene, camphene, myrecene etc. In view of the ethano botanical uses of *O. vulgare* as described above, the antioxidant potential of the extracts of plant together with their radical scavenging activities were studied. Phenolic content of the extracts were also determined.

MATERIALS AND METHODS

Plant material that is, *O. vulgare* was collected from different places of Kashmir valley and authenticated by a taxonomist. All the chemicals used in this study were of analytical grade and were procured from the standard commercial sources in India. Bovine serum albumin, hydrogenperoxide, tertiary butyl hydroperoxide (E.Merk), thiobarbituric acid, folins ciocalteus phenol reagent (CDH India), calf thymus DNA (Sigma Aldrich), ethanol (Bengal chemicals), ascorbic acid, ferric nitrate, trichloroacetic acid, sodium carbonate, sodium dihydrogen monophosphate, sodium hydrogen diphosphate, EDTA (Hi-media). Perchloric acid, sodium potassium tartarate and sodium hydroxide, were obtained from Thomas Baker India. 2, 2 diphenyl picryl hydrazyl (DPPH) was procured from Sigma Aldrich. Ammonium thiocyanate (Qualigens), ferrous chloride (BDH-Analar), Linoleic acid was procured from Sigma Aldrich.

Collection and preparation of extract

During collection the whole herb (*O. vulgare*) was authentically identified and collected from higher reaches of Kongdoori, Afarwath areas of Gulmarg and Thajwas glacier of Sonamarg in the month of May-June. The plants were identified by the courtesy of library and expert facilities of the KASH (Kashmir University Herbarium) in centre of plant taxonomy (COPT), University of Kashmir, Srinagar. The voucher specimen was deposited under herbarium no. Kash-bot/KU/org-Rb-001 at the Centre.

The authentically identified plant material (leaves and stem of *O. vulgare*) was shade dried. It was powdered and then subjected to different extraction procedures. The absolute ethanolic extract was prepared using soxhlet extractor. The extract was dried under reduced pressure using a rotary flash evaporator. The percentage yield of absolute ethanolic extract was 18 and 22 g.

The aqueous extract was prepared by decoction method. The powdered material suspended in just boiled distilled wate r(10 g/50 ml) and kept overnight (18-20 h). The extract was separated from the residue using a muslin cloth, and kept in autoclaved screw capped glass bottle in refrigerator. The residue was re-extracted in further 50 ml distilled water and the decoction was prepared as above. The supernatant was mixed with the earlier one and stored. The total volume of water used to prepare the decoction was thus 100 ml. The percentage yield for aqueous extract was 8-10 g. All the extracts were re-dissolved in autoclaved DDW and stored at 4°C for experimental use.

Preparation of post mitochondrial supernatant and microsomes

The post mitochondrial supernatant (PMS) and microsomes were prepared from the liver of the Male wistar rats. Liver from the freshly killed animals were perfused and kept in an ice cold normal saline 0.9% NaCl and extraneous material was removed. All operations were performed out in an ice at temperature of about 4°C. Tissue was blotted between the folds of a filter paper and weighed 10% (w/v), minced homogenate was prepared in Tris HCl buffer (50 mM) containing 0.15 KCl, pH 7.4. The homogenate was filtered through a muslin cloth and centrifuged at 6000 rpm for 10 min at 4°C to separate nuclear debris. The supernatant so obtained was centrifuged at 15000 rpm for 20 min at 4°C to get the PMS. The PMS fraction was then used as a model for lipid per-oxidation.

Microsomes were obtained by centrifuging a portion of PMS as obtained in an ultracentrifuge (Sorvall) at 105000X g for 60 min at 4°C. The pellet obtained was considered microsomes. The pellet was washed and dissolved in a phosphate buffer (0.1 M pH 7.4) containing KCl (1.17%).These were stored at 4°C for experimental use.

Lipid peroxidation assay (PMS and microsomes)

The assay for lipid peroxidation was done by using the method of Wright et al. (1981) with little modifications. The reaction mixture in a total volume of 1.0 ml contains 0.5 ml of PMS or microsomes (3 mg protein/ml), 0.1 ml of ferric nitrate (20 mM), 0.1 ml of ascorbic acid (500 mM). Various concentrations of the plant extracts were added wherever needed. The reaction was stopped by the addition of 25% trichloroacetic acid (TCA). Tubes were centrifuged at 5000 rpm for 5 min.To the supernatant 1 ml of 1.67% thiobarbutaric acid (TBA) was added to each tube. All the tubes were then immediately cooled. Amount of TBARS formed in each sample was assessed by measuring the absorbance at 535 nm using spectrophotometer (Shimadzu 1601) against a reagent blank.

Lipid peroxidation was also performed using cumene hydroperoxides oxidants. Lipid peroxidation was induced by incubating PMS/microsomes for one hour at 37°C in presence of 5 mM tertiary butyl hydroperoxide. After the addition of TCA and TBA, the absorbance was measured at 535 nm.

DNA sugar damage thiobarbituric acid reactive species (TBARS) assay

The sugar damage was assayed by the method of Gutteridge and

Wilkinson (1983). The hydroxyl radical was generated by using ferric nitrate (Fe^3), ascorbic acid (reducing agent), and hydrogen peroxide.

The reaction mixture in a total volume of 2 ml contained 1 ml of calf thymus DNA (1 mg/ml), 0.57 ml of Tris HCl buffer (0.001 M, pH 7.5), 0.2 ml of Fe_2NO_3 (2 mM), 0.2 ml of Ascorbic acid (100 mM) and 0.03 ml of H_2O_2 (30 mM). Various concentrations of plant extract (1000, 500 and 250 µg/ml) were added, wherever needed. The reaction mixture was incubated at 37°C in a shaking water bath for 24 h. After incubation was over, the deoxyribose degradation was assessed by theTBARS. To reaction mixture 1 ml of 1.67% of thiobarbituric acid (TBA) was added. Tubes were kept in a boiling water bath for 10 min. Tubes were cooled and centrifuged at 10,000 g for 5 min. The supernatant TBARS showing a characteristic absorption at 535 nm was read on spectrophotometer.

Radical scavenging activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH assay was performed according to the method of Yamaguchi et al. (1998). An aliquot (200 µl) of the various concentrations of the extract (1000, 500 and 250 µg/ml) was mixed with 800 µl of Tris-HCl buffer (100 mM, pH 7.4). To this was added 1 ml of 500 µM DPPH in ethanol, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 20 min in the dark, and the absorbance was taken at 517 nm %DPPH scavenging activity was calculated as:

$$\frac{\text{Control absorbance} - \text{Extract absorbance}}{\text{Control absorbance}} \times 100$$

Ferric thiocyanate (FTC) assay

This assay was carried out as described in the method of Kikuzaki and Nakattani (1993). A mixture of 4.0 mg of test sample (extract of *O. vulgare*) in 4 ml of absolute ethanol, 4.1 ml of 2.5% linoleic acid in absolute ethanol, 8.0 ml of 0.02 M phosphate buffer (pH 7.0) and 3.9 ml of water contained in a screw-cap vial was placed in an oven at 40°C in the dark. The final concentration of sample was 0.02% w/v. To 0.1 ml of this mixture, 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% ammonium thiocyanate was added. Three minutes after the addition of 0.1ml of 2.0×10^{-2}M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance was measured at 500 nm at every 24 h interval until one day after absorbance of the control reached its maximum value.

Thiobarbituric acid (TBA) assay

This test was conducted according to the method of Kikuzaki and Nakattani (1993). The same samples prepared for FTC method were used. To 2.0 ml of the sample solution, was added 1.0 ml of 20% aqueous TCA and 2.0 ml of aqueous thiobarbituric acid solution. The final sample concentration was 0.02% w/v. The mixture was placed in boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on absorbance on the final day. In both methods, antioxidant activity is described by percent inhibition.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Determination of total phenolics

The amount of total phenolics in extracts was determined according to Singletons procedure (Singleton and Rossi, 1965). To the sample solution in (0.5 ml duplicates) were added 2.5 ml of folin-ciocalteus reagent (0.124 g/ml) and 2 ml of sodium carbonate (7.5%). The reaction mixtures were allowed to stand for 120 min. Absorption at 675 nm was measured by UV-VS spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/gm of dry material.

PMS oxidation in the presence of various extracts of *O. vulgare*

Figure 1 represents the effects of ethanolic and aqueous extracts of the *O. vulgare* on the PMS oxidation induced by Fe^{3+}/A.A in the presence of hydrogen peroxide.

In the presence of ethanolic extract

At a concentration of 250 µg/ml, the value of MDA decreased from 17 nmoles to 9.8 nmoles in Fe^{3+}/A.A/H_2O_2 oxidant system. At a higher concentration of 500 and 1000 µg/ml of the same extract, the value of peroxidation decreased to 7.0 and 4.3 nmoles respectively.

In the presence of aqueous extract

At a concentration of 250, 500 and 1000 µg/ml the extract decreased the value of MDA formation from 17 to 13.7 nmoles, 11.4 and 9.7 nmoles respectively (Figure 1).

Microsomal lipid peroxidation in the presence of various extracts of *O. vulgare*

Figure 2 represents the effect of alcoholic and aqueous extracts of *O. vulgare* on Fe^{3+}/A.A mediated oxidant damages in the presence of hydrogen peroxide.

In presence of ethanolic extract

The values observed are 4, 3.2 and 2.0 nmoles at extract concentration of 250, 500 and 1000 µg/ml respectively (Figure 2).

In the presence of aqueous extract

At a concentration of 250, 500 and 1000 µg/ml, the aqueous extract decreased the value from 9.7 to 6.4 nmoles, 5.3 and 4.2 nmoles respectively (Figure 2).

Effect of *O. vulgare* extracts on deoxyribose damages

Figure 3 represents the effects of the ethanolic and aqueous extracts of *O. vulgare* extracts on deoxyribose sugar damages calculated through estimation of TBARS.

Effect of absolute ethanolic extract

With the pretreatment of extracts at a concentration of 250, 500 and 1000 µg/ml the TBARS formation decreased from 15 to 6.8 nmoles, 4.7 and 3.0 nmoles respectively (Figure 3).

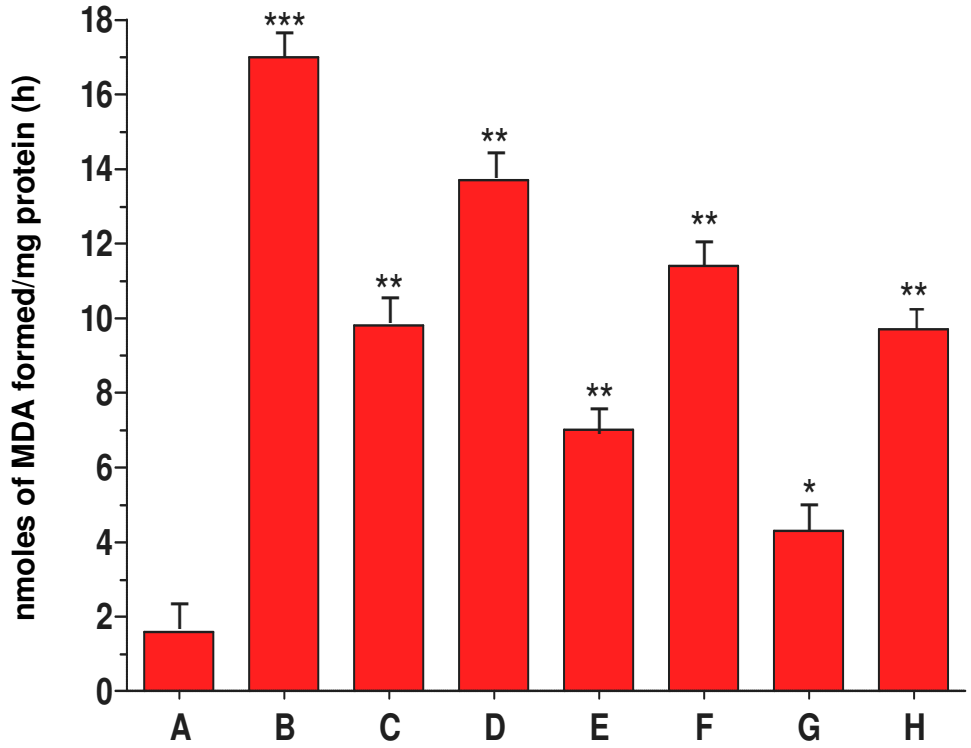

Figure 1. Effect of ethanolic and aqueous extracts of *O.vulgare* on ferric nitrate/ascorbic acid mediated induction of PMS membrane lipid peroxidation in the presence of hydrogen peroxide. A represents PMS (4 mg/ml) alone (control), B represents PMS, ferric nitrate (20 mM) and ascorbic acid (500mM) and hydrogen peroxide (30 mM), C represents B+250 µg/ml of *O.vulgare* (absolute ethanolic extract), D represents B+250 µg/ml of *O vulgare* (aqueous extract), E represents B+500 µg/ml of *O vulgare* (ethanolic extract), F represents B+500 µg/ml of *O vulgare* (aqueous extract), G represents B+1000 µg/ml of *O. vulgare* (ethanolic extract) and H represents 1000 µg/ml of *O. vulgare* (aqueous extract).Each value represents mean ± S.E of six experiments,*** P< 0.001 with respect to their control, ** P< 0.01 with respect to their control,*P<0.05 with respect to their control.

Effect of aqueous extract

The value of TBARS at 250, 500 and 1000 µg/ml decreased to 8.7, 6.0 and 4.9 nmoles respectively in the presence of aqueous extracts of *O. vulgare* (Figure 3).

DPPH radical scavenging activity

Another way of measuring antioxidant activity through free radical abrogating potential of our testing plants is by determining the free radical inhibitory ability of the extracts of *O. vulgare*. The results are depicted in Table 1. The 1, 1 Di phenyl 2, picryl hydrazyl radical scavenging activity shown by the extracts is concentration dependent. With the increase in concentration, the radical scavenging activity increases in both absolute ethanolic and aqueous extracts of *O. vulgare*. The maximum percentage inhibition is shown by absolute ethanolic extract of *O. vulgare* at a concentration of 1000 µg/ml. Vitamin C was taken as a reference compound which shows percent inhibition of 92% at a concentration of 1000 µg/ml.

Antioxidant activity assay (ferric thiocyanate assay)

The FTC method measures the amount of peroxide produced during the initial stages of oxidation which are the primary products of oxidation. As shown in Figure 4 all tested extracts exhibited varying antioxidant activity when compared with Vitamin E (α-tocopherol) in the FTC method by using *O. vulgare* (absolute ethanolic) and aqueous extracts, the absorbance values ranged from 0.005-0.007 which shows a percent inhibition of 88.09-83.3 respectively (Table 2). The percent inhibition observed for Vit E was 92.3%.

The TBA assay measures the total peroxide content at a later stage of lipid oxidation, involving the quantitation of the secondary products formed from oxidation. The results of the TBA assay agreed well with that of the FTC assay. As shown in Table 2 absolute ethanolic extract of *O. vulgare* showed a high percentage inhibition of (84.6%) as in FTC assay while the aqueous extract showed percentage inhibition of 82.05%.

Antioxidant potential due to phenolics

The total concentration of phenolic compounds in the extracts was determined using a series of gallic acid standard solutions (1 mg/ml) as described by Singleton and Rossi (1965) but with some modifications. Results indicate the higher amounts of gallic acid equivalents in ethonolic extract of *O. vulgare*. Aqueous extracts also possess good phenolic content (Table 3).

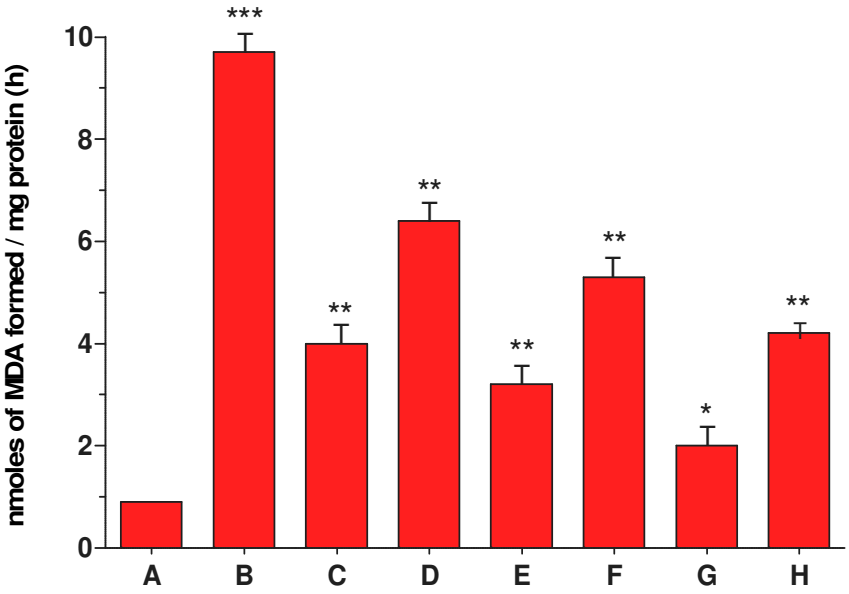

Figure 2. Effect of ethanolic and aqueous extracts of *O. vulgare* on microsomal lipid peroxidation induced by Ferric nitrate/Ascorbic acid in presence of various peroxides. A represents microsomes alone (control), B represents microsomes, ferric nitrate (20 mM) and ascorbic acid (500 mM) and hydrogen peroxide (30 mM), C represents B+250 µg/ml of *O. vulgare*(absolute ethanolic extract), D represents B+250 µg/ml of *O. vulgare* (aqueous extract), E represents B+500µg/ml of *O. vulgare* (ethanolic extract), F represents B+500µg/ml of *O. vulgare* (aqueous extract), G represents B + 1000µg/ml of *O. vulgare* (ethanolic extract) and H represents 1000µg/ml of *O. vulgare* (aqueous extract). Each value represents mean ± S.E of six experiments, *** P< 0.001 with respect to their control, ** P< 0.01 with respect to their control,*P<0.05 with respect to their control.

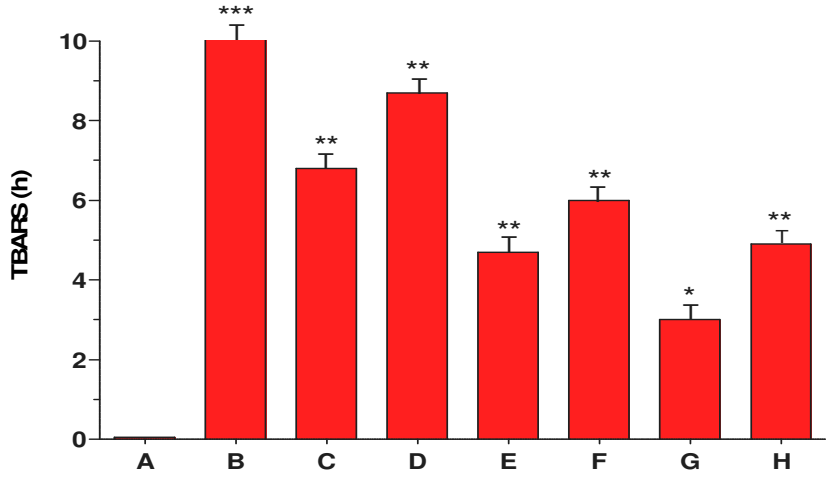

Figure 3. Effect of absolute ethanolic and aqueous extracts of *O. vulgare* on DNA damage induced by Ferric nitrate/Ascorbic acid alone and in presence of various peroxides. A represents DNA alone (control), B represents DNA +Ferric nitrate/Ascorbic acid and Hydrogen peroxide (30 mM), C represents B+25 0 µg/ml of *O. vulgare* (absolute ethanolic extract), D represents B + 250 µg/ml of *O. vulgare* (aqueous extract), E represents B + 500µg/ml of *O. vulgare* (ethanolic extract), F represents 500 µg/ml of *O. vulgare* (aqueous extract), G represents B+ 1000 µg/ml of *O. vulgare* (ethanolic extract) and H represents B + 1000 µg/ml of *O. vulgare* (aqueous extract). Each value represents mean ± S.E of six experiments, *** P< 0.001 with respect to their control,** P<0.01 with respect to their control, *P< 0.05 with respect to their control.

Table 1. DPPH radical scavenging activity of absolute ethanolic and aqueous extracts of *O. vulgare*.

Sample	Percent inhibition
DPPH alone	
DPPH+ ethanolic *O. vulgare*	
1000 µg/ml	79.6
500 µg/ml	69.5
250 µg/ml	54.0
DPPH+ aqueous *O. vulgare*	
1000 µg/ml	77.8
500 µg/ml	70.2
250 µg/ml	62.0
DPPH+Vit.C	
1000 µg/ml	92
500 µg/ml	89.8
250 µg/ml	85.6

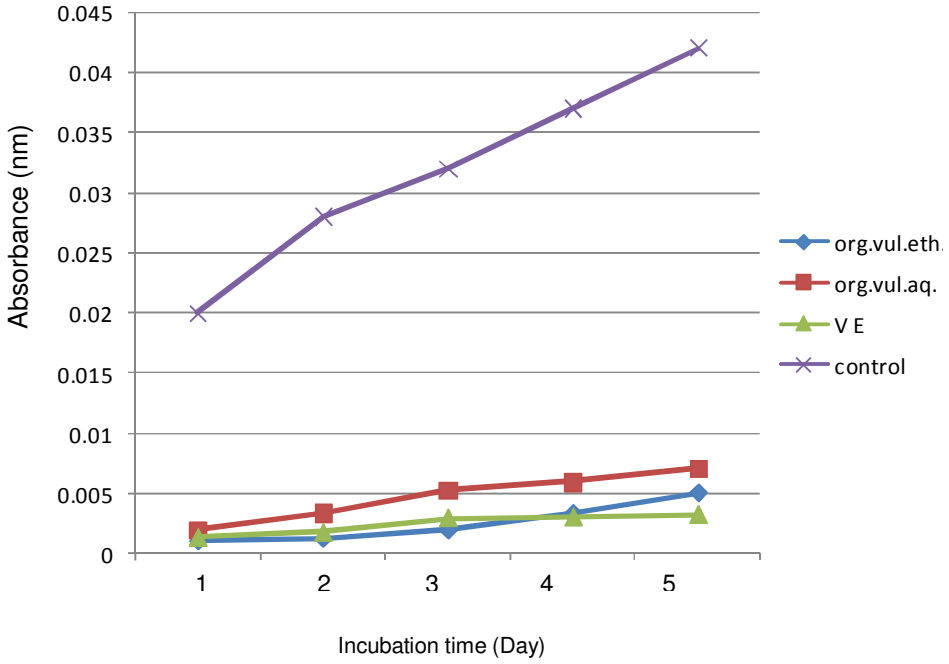

Figure 4. Antioxidant activity of ethanolic and aqueous extracts of *Origanum vulgare* as measured by FTC method.

DISCUSSION

Oxidative agents are highly reactive intermediates (ROS, RNS) which interact with several extracellular and intracellular molecules and with each other, thus generating a complex network of responses culminating in an outcome that may be detrimental or beneficial for the host (Virgillio, 2004).

Our data on experiments with PMS and microsomes clearly reflects that the extracts isolated from *O. vulgare* display effective free radical scavenging property as signified by the suppression of the lipid peroxidation initiated by Fe^{3+}/A.A and hydrogen peroxide. Concentration dependent percentage inhibition of lipid peroxidation was observed with both ethanolic and aqueous extracts. The herb of *O. vulgare* has been shown to possess volatile oil, tannins, thymol, carvacrol, free alcohols, esters, and bicyclic sesquiterpenes. The antioxidant property of

Table 2. Comparison of absorbance values and percent inhibition of linoleic acid peroxidation as measured by the FTC and TBA antioxidant assays.

Sample	Absorbance* (FTC)	Percent inhibition	Absorbance* (TBA)	Percent inhibition
O. vulgare (ethanolic)	0.005	88.09	0.006	84.6
O. vulgare (aqueous)	0.007	83.3	0.007	82.05
Vitamin E	0.0032	92.3	0.0037	90.5
Control	0.042	0	0.039	0

*Absorbance reading on the 5th day (one day after control reached maximum).

Table 3. Total phenolic content µg equivalents of gallic acid/mg of plant extract.

Plant	Total phenol (µg GAE/mg extract)
O. vulgare (ethanolic)	105.4±0.2
O. vulgare (aqueous)	51.5±0.10

the extract could be attributed to these compounds present in this plant. Tannins and carvacrol has been reported with antioxidant activity (Mukerjee et al., 1940). The antioxidant properties of substances possessing reactive phenolic groups can be explained by chain breaking activity of polyphenolic, which act as H-atom donors to peroxy radical, involving termination of radical chain formation (Flatmark and Romslo, 1975).

Various studies suggest that cyclic reduction and oxidation of iron with the perpetual production of free radicals are involved in mediating the DNA damage. The OH attacks the C4 sugar moiety of the DNA, abstracts H-atom from it and form C4 centered radical. The addition of an oxygen molecule generates the peroxyl radical. The successive rearrangement leads to sugar cleavage by the β-elimination. The extracts of the plants have the ability to inhibit free radical production or through scavenging properties can be helpful in diminishing Fe^{3+}/A.A hydroperoxide DNA damages.

The percentage DPPH scavenging activities of the extracts were concentration dependent. Significant DPPH radical scavenging activity was evident at all the tested concentrations of the plant extracts. The DPPH free radical scavenging by the plant extracts is due to their hydrogen donating ability due to their rich contents of polyphenols.

In the FTC and TBA antioxidant assays, the low absorbance values corresponding to a good percent of inhibition for alcoholic and aqueous extracts of *O. vulgare* indicate their good antioxidant potentials. The antioxidant activity of these plants could not be easily attributed to a single compound or a single class of compounds. However, flavonoids and its glycosoids are known to be good antioxidants and the presence of these compounds in our test extracts would be certainly contributing to this antioxidant activity.

Although average content of total phenolics has been determined in both test extracts and it cannot be excluded that the scavenging and antioxidant activity could be attributed to their presence but there is a possibility of a synergistic effect with other metabolites. Our data suggest enough evidence to support the enormous antioxidant potential of the extracts of our testing plants.

ACKNOWLEDGEMENT

Authors are immensely thankful to Indian Institute of Integrative Medicine Jammu, Department of Biochemistry Jamai Hamdard New Delhi, Department of Biotechnology, Department of Botany University of Kashmir and Department of Floriculture SKAUST- Kashmir for providing all the facilities and constant encouragement during this period of work.

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