A COMPARATIVE STUDY OF TOTAL PHENOLIC, TOTAL FLAVANOID AND ANTIOXIDANT ASSAYS OF AQUEOUS AND DMSO EXTRACT FROM LEAVES OF ORIGANUM VULGARE

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ABSTRACT

In this study, total phenolic content, total flavonoid content, radical scavenging activity and antioxidant power of aqueous and DMSO extracts of oregano (*Origanum vulgare L.*) leaves by spectrophotometric method. The total phenolic content ranged from 28.48 to 98.86 mg/g of dry/fresh weight, expressed as Gallic acid equivalents. The flavonoid concentrations varied from 14.45 to 19.53 mg/g of dry/fresh weight, expressed as Quercitin. Radical scavenging activity of extracts against DPPH radicals inhibition ranged from 1.90% to 92.02%. and IC50 values were expressed in μg/ml. The antioxidant power assay activity ranged from 18.63 to 673.67 μg/ml of extract, expressed in ascorbic acid equivalents. Aqueous extract of fresh leaves showed highest phenolic content, flavonoid content, DPPH radical inhibition and strong antioxidant activity. Also positive correlation was attained between radical scavenging and antioxidant power as well as radical scavenging and total phenolic content. So, aqueous extract of fresh oregano leaves can be considered as having high natural antioxidant activity amongst plant sources.

KEY WORDS: *Origanum vulgare* L., Antioxidant power, DPPH, phenols, flavonoids.

INTRODUCTION

Medicinal plants are the good sources for the discovery of pharmaceutical compounds, potential drugs and diverse substances possessing antioxidant properties having ability to protect the human body against cellular damage due to oxidation.[1],[2] Many medicinal
plants, especially members of the Lamiaceae family (mint family) such as origanum contain
good amount of phytochemicals with various bioactivities including antioxidants such a
polyphenols, ascorbic acid and carotenoids, anti-inflammatory and anticancer.\[2\] The
Antioxidant activity of phenolic compounds is due to their redox properties and chemical
structure which play an important role in the absorption and neutralization of the reactive
oxxygen radicals, chelating metals, quenching singlet and triplet oxygen or by delocalization
or decomposition of peroxides.\[1\].\[3\] Phenolic compounds in plants provide an array of
rosmarinic acid, in inhibiting the formation and also the natural sources of antioxidants to use
in foods and decomposition of hydroperoxides in tocopherol-stripped nutraceuticals.\[4\] Also,
Phenolic antioxidants which are products of secondary metabolism are good sources of
natural antioxidants in human diets.\[3\]

Here we concentrate on one of the such medicinal plant that is \textit{Origanum Vulgare L.}
commonly known as Oregano. Oregano belongs to genus Origanum of lamiaceae family
which is also known as mint family.\[5\] It is widely used in form of spices, fragrance,
medicinal as well as ornamental plants. Origanums are native to Mediterranean and Eurasia
and are grown as Herbaceous perennials or sub-shrubs in mountainous areas with rocky,
calcareous soil.\[6\]

The total antioxidant capacity of plant material depends upon the content and composition of
phenolics and synergism or antagonism between the active compounds.\[7\] The total phenolic
content from plant extracts can be used as a suitable indirect index to estimate the antioxidant
capacity and effectiveness of different extract preparation methods.\[8\],[9] The present study has
been designed to evaluate better antioxidant potential of dried and fresh leaves extracts of
oregano leaves in Aqueous and DMSO solvents by TPC, TFC, DPPH and FRP.

**MATERIALS AND METHODS**

**Materials**

All the chemicals were purchased from Sigma Aldrich Chemicals, India and Himedia
chemicals India Pvt. Ltd. Oregano leaves were grown in moist and humid conditions of
garden environment collected from Ahmedabad district 23.0061° N, 72.5647° E during
month of August and September. Fresh leaves were weighed and used for different
experimental protocols on the other hand for dried leaves, fresh oregano leaves were
processed for drying.

**Methods**
Extraction procedure
The extraction procedure was carried out with slight modifications[2], [3], [10] and the dry residues obtained were reconstituted in Aqueous (Millipore; double distilled water; neutral pH was maintained throughout the procedure) and DMSO solvents to make stock solution of 10mg/ml. The extracts were stored in air tight dark containers and stored at 4°C and analysed within 2 weeks. Working solutions (10µg/ml–2000µg/ml) were prepared from stock solutions to carry out different antioxidant properties.

Dried Leaves Extract
The leaves were shade dried for 8-10 days and then powdered by using domestic blender For every 1 gram of powdered dry leaves, 25 ml of solvent was used and subjected to reflux at 45°C for 6-7 hours. The extract was then filtered through Whatmann No. 1 filter paper and evaporated to obtain completely dry residues.

Fresh Leaves extract
The fresh leaves were used directly after cutting them into 1mm x 1mm pieces. For every 1 gm 25 ml of solvent was used and subjected to reflux at 45°C for 6-7 hours. The extract was then filtered through Whatmann No. 1 filter paper and evaporated to obtain completely dry residues.

Total Phenolic Assay
The Total Phenolic Content (TPC) of dried and fresh leaves of Origanum vulgare sp. was determined spectrophotometrically (Systronics Visiscan 167) by using Folin Ciocalteau reagent and Gallic acid as standard with some modifications.[11] The absorbance was noted at 725 nm against blank. After deriving the regression formula, total phenolic content values were expressed as mg of Gallic acid equivalents (GAE) per gram of dry and fresh extract.

Total Flavonoid Assay
The Total Flavonoid content (TFC) of dried and fresh leaves of Origanum vulgare sp. was determined spectrophotometrically by using Quercitin as standard.[12] Absorbance was measured at 430 against blank. After deriving the regression formula, Total flavonoid content values were expressed as mg of quercitin equivalents per gram of dry and fresh extract.

Antioxidant Assays
1, 1-diphenyl-2-picrylhydrazyl (DPPH) Free Radical Scavenging Activity Assay

In DPPH assay, lower the absorbance of the sample mixture indicates high DPPH free radical scavenging activity.\cite{13} The absorbance was measured at 515 nm. The scavenging activity of extracts with DPPH radicals was calculated by $I\% = [(A_o - A_s)/A_o] \times 100$ where $I\%$ is the percentage inhibition of DPPH, $A_o$ is the absorption of control and $A_s$ is the absorption of the sample extract. The antioxidant activity of each extract was expressed as $IC_{50}$. $IC_{50}$ is the concentration of extract in which 50 % inhibition is achieved and it was acquired by regression analysis.

Ferric Reducing Antioxidant Power Assay (FRP)

The FRP method is a simple, rapid, inexpensive and reproducible method, which can be applied to the assay of antioxidants in plasma or botanicals.\cite{14} This assay was performed using TPTZ, acetate buffer and FeCl$_3$\cite{15} and absorbance was measured at 593 nm. Results were expressed in µg AAE per ml of extract.

Statistical analysis

The results were documented after repeating the experiments in triplicates for five times (n=5). The experimental results were expressed as mean ± Standard Deviation (SD). The statistical analysis of the data were carried out by one way ANNOVA and the results were considered significant when $p<0.05$ and highly significant when $p<0.01$.

3. RESULTS AND DISCUSSION

Total Phenolic Content

Quantitative analysis was carried out to determine the Total Phenolic Content present in aqueous and DMSO extracts of dried and fresh Origanum vulgare sp. (Table: 1).The highest amount of total phenolic content between DMSO and aqueous extracts was obtained in aqueous extract of fresh leaves (98.86 mgGAE/g) and lowest quantity was obtained in aqueous extract of dried leaves(23.48 mgGAE/g). This method measures the total concentration of phenolic hydroxyl groups in the plant extract and these Polyphenols react with specific redox reagents like Folin-Ciocalteu reagent to form a blue complex that are quantified. The linear regression formula of Gallic acid standard curve was $y=0.010x$ ($r^2=0.993$). Results were significant in dried oregano leaves extracts aqueous ($p<0.01$) and DMSO ($p<0.02$) and highly significant in fresh oregano leaves extract aqueous ($p<0.004$) and DMSO($p<0.0052$).
Table: 1 Total phenolic content of dry and fresh extracts of aqueous and DMSO solvents

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Dry leaves extract</th>
<th>Fresh leaves extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>23.48 ± 1.21</td>
<td>98.86 ± 6.42</td>
</tr>
<tr>
<td>DMSO</td>
<td>70.98 ± 7.18</td>
<td>89.69 ± 6.50</td>
</tr>
</tbody>
</table>

Results are expressed in mean ± SD mg of Gallic acid equivalent per gram of dry/fresh weight (mg GAE/g) of the extract (n=5).

Total Phenolic assay is also used as an indirect index to estimate the antioxidant capacity and to investigate the effectiveness of different extraction and preparation methods.\textsuperscript{[16]} The total phenolic content of the plant extracts depends on the type and polarity of the extract.\textsuperscript{[12]} Aqueous (Millipore double distilled water) and DMSO are highly polar solvents and high solubility of phenols in polar solvents provides high concentration of these compounds in the extracts.

According to a study conducted, total phenolic content of fresh oregano leaves and dry oregano leaves of methanol extract obtained was 857 mg GAE/100g and 6120 mg GAE/100g respectively.\textsuperscript{[17]} On the contrary, in the present study total phenolic content of fresh leaves and dry leaves in aqueous extract were found to be 9886 mg GAE/100g and 2348 mg GAE/100g respectively and of fresh and dry leaves from DMSO solvent was 7098 mg GAE/100g and 8960 mg GAE/100g respectively. The total phenolic content of acetone extract of fresh and dried leaves were found to be 46.15(± 0.04) g/100g d.m. and 2.90(± 0.10) g/100 g d.m. respectively\textsuperscript{[18]} which as compared to our present study was very low. Methanol, acetone, DMSO and water all being polar solvents, the values obtained in present study for both fresh and dried oregano leaves were distinctly high.

**Total Flavonoid Content**

The concentrations of Total Flavonoid Content in aqueous and DMSO extracts of Origanum vulgare sp. were determined spectrophotometrically(Table:2). The quantitative analysis revealed that highest concentration of flavonoids was present in aqueous extract of fresh leaves (19.53 mg QE/g) and moderately less concentration was obtained in DMSO extracts of fresh leaves (14.45 mg QE/g). The linear regression formula of Quercitin standard curve was $y=0.04x$ ($r^2=0.989$). Results were significant in case of Aqueous extract of dry leaves (p<0.02) and fresh leaves (p<0.18) while in DMSO extract of dry leaves (p<0.04) results were significant and non significant in case of fresh leaves (p<0.08).
**Table: 2 Total flavonoid content of dry and fresh extracts of aqueous and DMSO solvents**

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Dry leaves extract</th>
<th>Fresh leaves extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>17.01 ± 10.25</td>
<td>19.53 ± 5.42</td>
</tr>
<tr>
<td>DMSO</td>
<td>19.46 ± 9.22</td>
<td>14.45 ± 7.42</td>
</tr>
</tbody>
</table>

Results are expressed in mean ± SD mg of Quercitin equivalent per gram of dry/fresh weight (mg QE/g) of the extract (n=5).

Flavonoids are class of secondary plant metabolites with significant antioxidant and chelating properties. So, the concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation and the antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups.

According to one study, total flavonoid content of fresh and dry oregano leaves in methanol extract attained was 189mg QE/100g and 1790 mg QE/100g respectively while in the present study total flavonoid content of aqueous extract of fresh leaves and dry leaves obtained was 1953 mg QE/100g and 1701 mg QE/100g respectively and for DMSO extract for dried and fresh leaves was 1445 mg QE/100g and 1946 mg QE/100g respectively. As methanol, DMSO and water are polar solvents, in the present study the total flavonoid concentration of fresh leaves in aqueous and DMSO solvents was very high as compared to the study referred while for dried leaves the values of methanol extract and aqueous extracts were found to be almost similar while for the flavonoid content in dried leaves of DMSO extract was higher.

**Antioxidant Assays**

1. 1-diphenyl-2-picrylhydrazyl (DPPH) Free Radical Scavenging Activity Assay

The DPPH radical has been used widely to test the antioxidant activities of plant extracts. This method is purely based on the reduction of DPPH in methanol by hydrogen donating antioxidant which changes the colour from purple to yellow. In table: 3 and Fig:1 inhibition % of DPPH radicals by extracts of dried and fresh oregano leaves in water and DMSO solvents is shown. It is apparently observed that aqueous extract of fresh oregano leaves have highest percentage of scavenging DPPH radicals (92.02 %) and least was observed in fresh leaves; DMSO(55.08%). As the concentration of extracts increases the scavenging activity of extracts with DPPH radical also increased. All the data obtained is significant within all the extracts (p<0.05).
Antioxidant activity -IC$_{50}$ (half maximal inhibitory concentration) of different extracts was calculated. The lower the IC$_{50}$ value higher the antioxidant activity. IC$_{50}$ value of Aqueous extract of fresh leaves was found to be the lowest (80.482 ± 1.72 µg/ml) confirming its highest antioxidant activity followed by IC$_{50}$ values of DMSO extract of Dried oregano leaves (144.442 ± 0.75 µg/ml)>Aqueous extract of Dried oregano Leaves (805.193 ± 7.98 µg/ml)> DMSO extract of Fresh oregano leaves (5136.886 ±0.16 µg/ml). Ascorbic acid was taken as standard its IC$_{50}$ value was (2.344 ± 1.19 µg/ml) establishing the highest antioxidant activity amongst all extracts.

Table: 3 Free radical scavenging activity (inhibition percentage %) of dry and fresh extracts of aqueous and DMSO solvents

<table>
<thead>
<tr>
<th>Concentrations µg/ml</th>
<th>% Inhibition of DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry leaves Aqueous</td>
</tr>
<tr>
<td>10</td>
<td>1.90 ± 2.22%</td>
</tr>
<tr>
<td>60</td>
<td>5.31 ± 2.61%</td>
</tr>
<tr>
<td>100</td>
<td>5.88 ± 4.04%</td>
</tr>
<tr>
<td>500</td>
<td>33.51 ± 3.70%</td>
</tr>
<tr>
<td>1000</td>
<td>51.07 ± 4.87%</td>
</tr>
<tr>
<td>1500</td>
<td>66.83 ± 4.25%</td>
</tr>
<tr>
<td>2000</td>
<td>75.54 ± 3.41%</td>
</tr>
</tbody>
</table>

Results are expressed as 1% mean ± SD for Aqueous and DMSO solvent extracts (n=5)

According to a report, the percentage of inhibition by acetone extract of fresh and dried leaves was 81.51 % and 38.33% respectively,[18] while in the present study inhibition percentage of DPPH radicals by aqueous extract of fresh and dried leaves was found to be 92.02% and 75.54 % respectively and of DMSO extract was 55.08% and 75.31% of fresh and dried leaves correspondingly. In both cases, the inhibition percentage obtained in present data was comparatively very high. In one of the studies, in dry extract with methanol water and acetic acid, IC$_{50}$ value was 0.601±0.034 (mg/ml)[4] while in present study DMSO extract of dried oregano leaves was 0.144 ± 0.75 mg/ml and that of aqueous extract of dried leaves were 0.805 ± 7.98 mg/ml. The IC$_{50}$ value of DMSO extract was much lower than that of study reported and for aqueous extract it was found to be almost equivalent.
Ferric Reducing Antioxidant Power Assay (FRP)

Ferric Reducing Antioxidant Power assay is based on the reduction, at low pH, of a colorless ferric complex (Fe3+ tripyridyltriazine) to a blue-colored ferrous complex (Fe2+ tripyridyltriazine) by the action of electron-donating antioxidants and changes in absorbance are noted spectrophotometrically.\textsuperscript{20} In table 4 and fig:2; µg of Ascorbic acid equivalent per ml of dried and fresh leaves oregano extracts in water and DMSO extracts is documented. It is clearly seen that highest antioxidant power is of fresh oregano leaves in aqueous solvent (673.67 µg AAE/ml). The antioxidant power of Fresh oregano leaves in DMSO solvent (671 µg AAE/ml) and dried Oregano Leaves in DMSO solvent (578.63 µg AAE/ml) was found to be almost similar while Aqueous extract of dried oregano leaves (250 µg AAE/ml) had moderately less antioxidant power as compared to all the other extracts. As the concentration increased the antioxidant activity of extracts also increased. The standard curve of ascorbic acid was $y=0.008x+0.029(r^2=0.991)$. All the data obtained were found to be significant ($p<0.05$).

Table: 4 Ferric reducing antioxidant power (FRP) of dry and fresh extracts of aqueous and DMSO solvents

<table>
<thead>
<tr>
<th>Concentrations µg/ml</th>
<th>Dry leaves Aqueous</th>
<th>Dry leaves DMSO</th>
<th>Fresh Leaves Aqueous</th>
<th>Fresh leaves DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>18.63 ± 2.63</td>
<td>19.00 ± 0.75</td>
<td>29.88 ± 1.13</td>
<td>22.25 ± 4.50</td>
</tr>
<tr>
<td>60</td>
<td>25.50 ± 0.25</td>
<td>30.75 ± 0.75</td>
<td>62.25 ± 2.75</td>
<td>29.23 ± 0.04</td>
</tr>
</tbody>
</table>
Results are expressed in mean ± SD µg of ascorbic acid equivalent per ml of extract (µg AAE/ml of extract; n=5)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>1500</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>32.88 ± 0.38</td>
<td>79.75 ± 6.75</td>
<td>94.25 ± 6.25</td>
<td>55.50 ± 1.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94.00 ± 0.75</td>
<td>207.88 ± 22.38</td>
<td>165.00 ± 5.50</td>
<td>213.88 ± 5.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>133.13 ± 1.88</td>
<td>384.75 ± 54.50</td>
<td>262.00 ± 22.5</td>
<td>366.25 ± 3.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>206.38 ± 5.88</td>
<td>480.25 ± 64.50</td>
<td>471.5 ± 1.75</td>
<td>465.38 ± 4.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250.00 ± 2.00</td>
<td>578.63 ± 41.38</td>
<td>673.67 ± 0.58</td>
<td>671.00 ± 3.00</td>
<td></td>
</tr>
</tbody>
</table>

Fig: 2 Showing antioxidant activity of different solvent extracts of leaves of *Origanum vulgare* sp.

Water, DMSO and methanol are all polar solvents. In studies performed, the highest antioxidant activity in methanolic extract of fresh leaves (781 mg GAE/100 g) and dried leaves (8200 mg GAE/100 g) was documented\(^{[17]}\) while in the present study the antioxidant activity even at the highest concentration dry Aqueous (250 µg AAE/ml), dry DMSO (578.63 µg AAE/ml), fresh Aqueous (673.67 µg AAE/ml) and fresh DMSO (671.00 µg AAE/ml) was found to be extremely less. Strong antioxidant values are related to its phenolic compounds and decrease in antioxidant activity can be attributed to degradation of phytochemicals, phenolic compounds and enzyme activities.

**Co-rrelation**

Positive correlation was obtained between values of radical scavenging activity DPPH and antioxidant power assay FRP, antioxidant power assay and total phenolic content and between values of Radical scavenging activity and total phenolic content. Negative correlation was attained between values of total phenolic content and total flavonoid content as well as antioxidant power assay and total flavonoid content.
<table>
<thead>
<tr>
<th></th>
<th>DPPH v/s FRP</th>
<th>DPPH v/s TPC</th>
<th>TPC v/s TFC</th>
<th>FRP v/s TPC</th>
<th>FRP v/s TFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous Dry</td>
<td>0.989654</td>
<td>0.91837</td>
<td>-0.86019</td>
<td>0.86019</td>
<td>-0.79972</td>
</tr>
<tr>
<td>Aqueous Fresh</td>
<td>0.63974</td>
<td>0.94145</td>
<td>-0.6867</td>
<td>0.6867</td>
<td>-0.34545</td>
</tr>
<tr>
<td>DMSO Dry</td>
<td>0.898476</td>
<td>0.92591</td>
<td>-0.77013</td>
<td>0.77013</td>
<td>-0.78313</td>
</tr>
<tr>
<td>DMSO Fresh</td>
<td>0.963901</td>
<td>0.79565</td>
<td>-0.72065</td>
<td>0.72065</td>
<td>-0.77403</td>
</tr>
</tbody>
</table>

**CONCLUSION**

The results in the present analysis report the quantitative analysis of total phenolics and total flavonoids, radical scavenging activity and antioxidant activity present in various solvent extracts of dried and fresh leaves of *Origanum Vulgare sp.* However, further investigations are required to isolate and characterize the active constituents from this plant to evaluate their therapeutic role. Total phenolics and antioxidant assays showed linear correlation, the aqueous solvent of fresh leaves had the highest phenolic content and aqueous solvent of dried leaves had low phenolic content. Flavonoid content in all the extracts was found to be almost similar, numbers of variations were observed in radical scavenging and antioxidant activity of the extracts. While fresh extract of Aqueous solvent showed high percentage of inhibition and antioxidant power, fresh extract of DMSO solvent showed low percentage of inhibition and high antioxidant activity. DMSO and water both are highly polar solvents and DMSO has ability to replace some water molecules associated with cellular components, so it can be stated that due to replacement of water molecules by DMSO the extracts have low phenolic content was well as low antioxidant activity.

There is significant linear co-relation between phenolics and antioxidant activity by both radical scavenging activity and antioxidant assay as phenolics have redox properties which make tem act as reducing agents, singlet oxygen quenchers and hydrogen donors by neutralizing free radicals with radical scavenging activity. Studies have reported that Oregano is an efficient blocker of pro-oxidant events and hence can defend the body from deleterious action of free iron. The extracts of dried leaves did not show high phenolics, flavonoids and antioxidant activity one of the possible reasons could be loss of antioxidant properties and degradation in phytochemicals due to temperature effect.

Since the extracts (aqueous) of fresh oregano made are comparable to cooking conditions and minimal extracts showed high phenol, flavonoid and antioxidant properties, it can be used in day to day life as a nutritive ingredient.
REFERENCES


