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RESEARCH ARTICLE

Qualitative Phytochemical Analysis in Determination of Antioxidant Activity of Methanolic Extract of *Oenothera biennis* by GCMS – A Preliminary Research Study

Afroz Patan*, M. Saranya, S. Vignesh, A. Bharathi, G. Vikram, P. Yuvaraj, M. Vijeyaanandhi
Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, Vels Institute of Sciences, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai – 603117, Tamilnadu, India.
*Corresponding Author E-mail: **afroz.sps@velsuniv.ac.in**

ABSTRACT:

Introduction and Aim: *Oenothera biennis* an important medicinal plant which belongs to Onagraceae family. It is used for various medicinal purposes in ayurvedic medicine and herbal remedy. The aim of the present study was to evaluate the antioxidant activity of phenol and flavonoid extract of plant of *O. biennis* and GC-MS analysis for active compound identification. **Materials and Methods:** Radical scavenging assay and reduction assay methods were used for antioxidant activity. The antioxidant capacity of methanolic plant extractHP-5 column was used for GCMS analysis. **Results:** The IC_{50} of DPPH radical scavenging activity of methanol leaves extract of *O. biennis* was 31.43µg/mL concentration, IC_{50}value of superoxide radical scavenging activity was 37.71µg/mL concentration. The RC_{50} of phosphomolybdenum reduction of methanolic plant extract of *O. biennis* was 49.90µg/mL concentration and the $RC_{50}$of Fe^{3+} reduction was 37.25µg/mL concentrations. Antioxidant compounds such as Phenol, 2, 6-bis(1,1-dimethyl)-4-[(4-hydroxy-3,5-dimethylphenyl)methyl]-, Cromaril and Oleic acid were eluted by GCMS analysis. **Conclusion:** The data showed that the methanolic plant extract of *O. biennis* has significant antioxidant activity. The flavone compounds identified in GCMS could be responsible for antioxidant activity. Further research work needed to isolate active compounds to kill diseases.

KEYWORDS: *Oenothera biennis*, Antioxidant activity, DPPH, GC-MS.

INTRODUCTION:

In life of humans, herbal plants plays a major role in different sources such as in pharmacology, cosmetics, perfumery, nutraceuticals, beverages and dying industries than the synthetic drug[1]. In this research article, we are discussing about Ornamental plant *Oenothera biennis* or Evening primrose is a species of Oenothera belonging to family Onagraceae. It is the second largest genus with 145 species of flowering plants. The plant is a biennial weed of Onagraceae native to North America and found in parts of Asia and Europe. The yellow fragrant flower blooms allevening[2].

Other species found in India were *Oenothera macrocarpa*, *O. rosea*, *O. caespitose*, *O. clelandii*, *O. drummondii*, *O. oclorata*. Evening primrose oil is a natural product extracted by cold-pressed from *Oenothera biennis* L seeds[3]. The plant is a rich source of omega-6 series fatty acids[4]. Evening Primrose (*Oenothera biennis*) is a wild medicinal herd of Central American origin that is now globally widespread. Its traditional uses include treatment of rheumatoid arthritis and premenopausal pain[5]. *Oenothera biennis* is the most numerous species in the genus Oenothera, illustrated to biological activity on Chemical compound of various parts of plant mainly leaves, stems and seeds[6]. The seed of this genus species have great economic properties in industrial application i.e. in medicine and in nutraceutics but it remains unexplored in development of nano-emulsion formulation[7]. It is a seed drug plant rich source in gamma-linolenic acid established and mainly important in food and component of pharmaceutical products[8].

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Taxonomical classification:
Domain: Eukaryotic
Kingdom: Plantae
Phylum: Spermatophytae
Subphylum: Angiospermae
Class: Dicotyledonae
Order: Myrtales
Family: Onagraceae
Genes: Oenothera
Species: *Oenothera biennis*

Ecology:
Colour: Yellow
Size: 5-20cm long; 1-2.5cm broad
Life span: 2 years
Leaves: Lanceolate, tight rosette in first year, spiral on stem in second year.
Flowers: Hermaphrodite, nectar guide, pollinators.
Fruits: Capsule, containing 1-2mm long seeds.

Agricultural practice:
The plant requires sunny and arid place with loamy soil. The seeds are sown in April (spring seeds) or July-August (autumn seeds). The time of harvesting is 75 to 85 days after flowering. There is no much need of water during the vegetation period. It is a biennial herb cultivated in temperate regions of the world and in Indian gardens[9].

A study has shown that the irrigation with salt water could increase the oil yield and quality in Evening primrose seeds. Thus, the plant could be a valuable alternative oil crop in arid regions.

Phytochemical Constituents:
The plant contains linoleic acid, γ-linolenic acid, flavonoids, carbohydrates, sterols, tannins and xanthone derivatives[10]. In genus Oenothera mainly occurring constituent in plant material is Oenothein B, a dimeric macrocyclic ellagitannin[11]. Some of the compounds isolated in roots and seeds of this species used in activity are–Esters: methyl ester of procatechuic acid, methyl ester and ethyl ester of gallic acid[12]. Triterpenoids: oleanolic acid, Oenotheralanosterol A and B[13,14]. Tannins: ellagitannins OeB. Fatty acids: gamma-linolenic acid and linolenic acid[15].

Traditional Medicinal Uses:
The whole plant especially the leaves were boiled to tea and used as a stimulant to treat laziness and against over fatness. The roots are used externally to treat piles and boils. It is also chewed and rubbed onto the muscle to improve strength. The bark and leaves are used as astringent and sedative. A syrup made from flowers is effective in treatment for whooping cough. In food it is used as a dietary aid.

Other Uses:
The plant is used to treat gastro-intestinal disorders, eczema, whooping cough, asthma, blood disorders, laziness, obesity, piles and boils[9]. The oil is used in the preparation of soaps and cosmetics. The plant is widely used as a dietary supplement, rheumatic, arthritic pain, atopic dermatitis, psoriasis, premenstrual syndrome, menopausal syndrome and diabetic ne neuropathy having beneficial effects[16].

MATERIALS AND METHODS:

Preparation of Extract:
Oenothera biennis plants were collected and were washed, shade dried for 15 days and make into coarse powder by mechanical blender. About 20g of powdered material was soaked in 100mL of methanol and kept it for 72 hrs. Then the supernatant was filtered and condensed by rotor evaporator, which yields greenish gummy extract.

Qualitative phytochemical analysis:
The methanolic plant extract of *O. biennis* was subjected to preliminary phytochemical analysis using specific reagents[18] as followed in standard methods[19].

Determination of total phenols:
Folin-Ciocalteau reagent method was used to determine the total phenolic compounds with slight modifications[20]. One hundred µL of methanolic plant extract (1mg/mL) of *O. biennis* was mixed with 900µL of distilled water and 1mL of Folin Ciocalteu reagent (1:10 diluted with distilled water). After 5 min, 1mL of Na_2CO_3 (20%) solution was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured by UV-VIS spectrophotometer at 765nm. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

Determination of total flavonoids:
The total flavonoid content of methanolic plant extract of *O. biennis* was determined using aluminium chloride method with slight modification[21]. Five hundred µL of leaf extract (1mg/mL) was mixed with 0.5mL of

methanol. To the mixture 5% (w/v) sodium nitrite solution and 0.5mL 10% (w/v) aluminium chloride solution were added. Then 50µL of 1 M NaOH solution was added and the absorbance was measured at 510nm using spectrophotometer. The result was expressed as (µg/mg of extract) quercetin equivalent.

Antioxidant Activity:

DPPH radical scavenging activity:

The antioxidant activity of methanolic plant extract of *O.biennis* was measured by stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) and free radical scavenging activity[22]. One mL of 0.1mM DPPH solution in methanol was mixed with 1mL of various concentrations (20-120µg/mL) of plant extracts. The mixture was then allowed to stand for 30 min incubation in dark. One mL methanol mixed with 1mL DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis spectrophotometer at 517nm. Ascorbic acidwas used as the reference standard. The percentage of inhibition was calculated using the following formula:

$$\% \text{ of DPPH radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Superoxide radical scavenging activity:

Superoxide radical scavenging activity was carried out by riboflavin-UV light-NBT system[23]. The reaction mixture contains different concentrations (20-120 µg/mL) of methanolic plant extract of *O.biennis*, 1.5mM of riboflavin, 12mM of EDTA and 50mM of NBT, added in that sequence. All the reagents must be prepared in 50mM of phosphate buffer (pH 7.6). The reaction was started by illuminating the reaction mixture under UV light for 15 min. Immediately, the absorbance was measured at 590nm. Ascorbic acid was used as positive control. The percentage of inhibition was calculated using the formula.

$$\% \text{ of superoxide radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Phosphomolybdenum reduction assay:

The reduction capacity of methanolic plant extract of *O. biennis* was assessed by phosphomolybdenum reduction method[25]. The plant extract with concentrations ranging from 20- 120µg/mL was combined with reagent solution containing ammonium molybdate (4mM), sodium phosphate (28mM) and sulphuric acid (600mM). The reaction mixture was incubated in water bath at 90ºC for 90min. The absorbance of the coloured complex was measured at 695nm. Ascorbic acid was used as standard reference. The percentage of reduction was calculated using the following formula

$$\% \text{ Of phosphomolybdenum Reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

Ferric (Fe^{3+}) reducing power assay:

The reducing power of the plant of methanolic extract of *O. biennis* was determined by slightly modified method of Yen and Chen[26]. One mL of plant extract of different concentrations (50 - 300µg/mL) was mixed with 1mL phosphate buffer (0.2 M, pH 6.6) and 1mL of 1 % (w/v) potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were then incubated at 50°C for 20 min. One mL of 10% (w/v) trichloroacetic acid was added to each mixture. Then to the 1 mL mixture of 0.1% (w/v) $FeCl_3$ was added and the absorbance was measured at 700nm using Spectrophotometer. Ascorbic acid was used as the standard reference. The formula

$$\% \text{ of ferric } (Fe^{3+}) \text{ reduction} = \frac{\text{Sample-Control}}{\text{Sample}} \times 100$$

RESULT AND DISCUSSION:

DPPH radical scavenging assay:

Scavenging of DPPH free radical is the popular antioxidant assay that will measure the capacity of antioxidants to scavenge DPPH radicals, which was measured at 517nm[27]. The ability of methanol leaves extract of *O.biennis* to scavenge free radicals was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The maximum DPPH radical scavenging activity was 72.72% at 120µg/mL concentration (Table 1, Fig 1). The methanolic plant extract of *O. biennis* showed free radicals scavenging activity by reducing the stable purple coloured DPPH (1,1-diphenyl-2-picrylhydrazyl) radical to the yellow coloured 1,1-diphenyl-2- picrylhydrazyl and the reducing capacity increased with increasing concentration of the extract. The IC_{50} was found to be 31.43µg/mL concentration and was compared with standard.

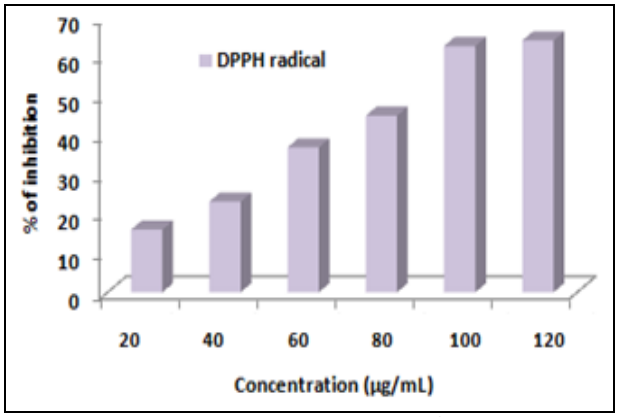


Fig. 1: DPPH radical scavenging activity of methanolic plant extract of *O.biennis*

Table 1: DPPH radical scavenging activity of methanolic plant extract of *O. biennis*

S. No.	Concentration (µg/mL)	% of inhibition DPPH radical
1	20	56.81
2	40	63.63
3	60	65.90
4	80	69.69
5	100	71.21
6	120	72.72

Superoxide ($O_2^{\cdot-}$) radical scavenging activity:

Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. Superoxide anions derived from dissolved oxygen by the riboflavin-light-NBT system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which is measured at 590nm. Antioxidants are able to inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture[28]. The maximum superoxide radical scavenging activity of methanolic plant extract of *O.biennis* was 75.75% at 120 µg/mL concentrations (Table 2, Fig 2) and the IC_{50} was 37.71µg/mL concentration.

Table 2: Superoxide ($O_2^{\cdot-}$) radical scavenging assay of methanolic plant extract of *O.biennis*

S. No.	Concentration (µg/mL)	% of inhibition Superoxide radical
1	20	40.90
2	40	53.03
3	60	57.57
4	80	59.09
5	100	62.12
6	120	75.75

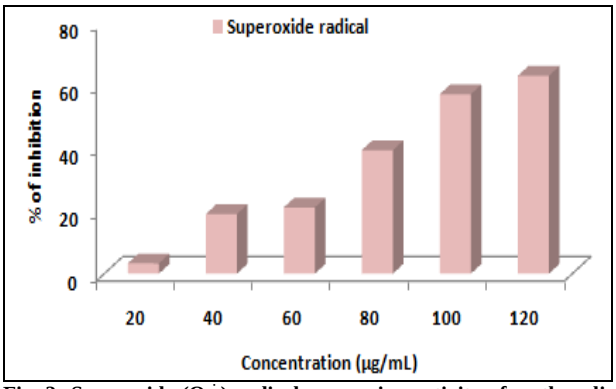


Fig. 2: Superoxide ($O_2^{\cdot-}$) radical scavenging activity of methanolic plant extract of *O.biennis*

Phosphomolybdenum reduction activity:

The reduction of radicals of methanolic plant extract of *O.biennis* was measured by phosphomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo(V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695nm[23]. The maximum phosphomolybdenum reduction was 65.02% at 120µg/mL concentration (Table 3, Fig 3) and the RC_{50} was 49.90µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50} = 6.34µg/mL concentration). PM assay is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand. It involves in thermally generating auto-oxidation during prolonged incubation period at higher Temperature.

Table 3: Phosphomolybdenum reduction activity of methanolic plant extract of *O.biennis.*

S. No.	Concentration (µg/mL)	% Of reduction Phosphomolybdnum reduction
1	20	24.26
2	40	25.58
3	60	60.12
4	80	62.24
5	100	64.24
6	120	65.02

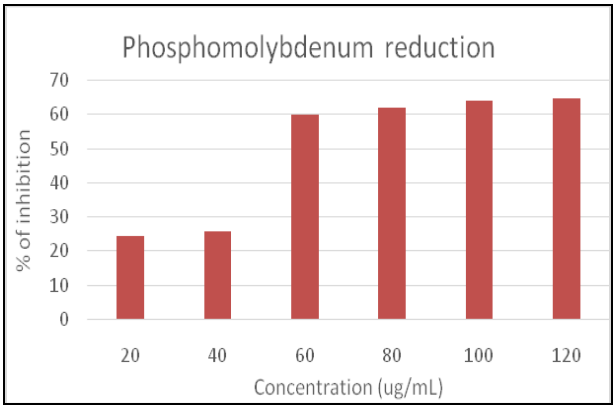


Fig. 3: Phosphomolybdenum reduction activity of methanolic plant extract of *O.biennis*

Ferric (Fe^{3+}) reducing power activity:

The reducing power ability of methanolic plant extract of *O. biennis* was carried out by the reduction of Fe^{3+} to Fe^{2+} by the electron donating ability of extract and the subsequent formation of ferro-ferric complex. The reduction ability increases with increase in concentration of the extract[24]. The maximum Fe^{3+} reduction was 85.05% at 120µg/mL concentrations (Table 4, Fig 4) and the RC_{50} was 37.25µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50} = 7.72 µg/mL concentration). Also in this assay, higher absorbance of the reaction mixture indicates higher reduction potential. The reducing capacity of aqueous extract poses as a significant indicator of its potential antioxidant activity. The reducing capacity of the extract was performed using Fe^{3+} to Fe^{2+} reduction assay as the yellow colour changes to green or blue colour depending on the concentration of antioxidants[30]. The antioxidants

such as phenolic acids and flavonoids were present, considerable amount in plant of methanolic extract of *O.biennis* and showed the reducing capacity in a concentration dependant manner.

Table 4: Fe3+ reducing power activity of methanolic plant extract of *O.biennis*

S. No.	Concentration (µg/mL)	% Of reduction Fe3+ reduction
1	20	15.18
2	40	53.68
3	60	66.27
4	80	79.81
5	100	84.04
6	120	85.05

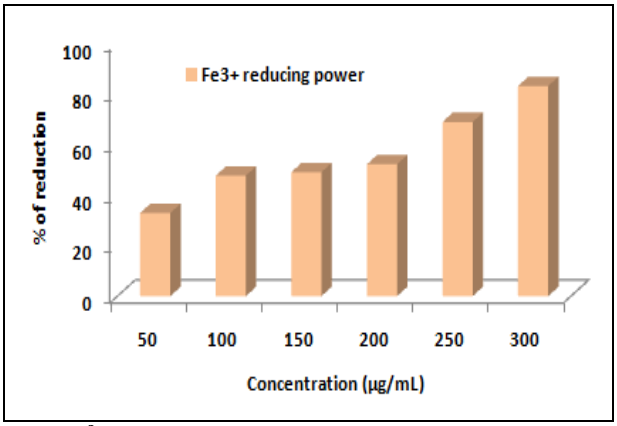


Fig 4: Fe^{3+} reducing power activity of methanolic plant extract of *O. biennis*

GC-MS analysis:

Gas chromatography Mass Spectrometry (GCMS):

The sample was injected into a HP-5 column (30m X 0.25mm i.d with 0.25µm film thickness), Agilent Technologies 6890 N JEOL GC Mate II GC-MS model for GC-MS analysis. Following chromatographic conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250°C; interface temperature of 250°C; mass range of 50-600 mass units[31].

Identification of components:

The database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation of the mass spectrum of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

GC-MS analysis of the whole plant of methanolic aqueous extract of *O.biennis* was shown in Table 5. Antioxidant compounds such as Phenol,2,6-bis(1,1-dimethyl)-4-[(4-hydroxy-3,5-dimethylphenyl)methyl]- , Cromaril and Oleic acid were eluted and recorded.

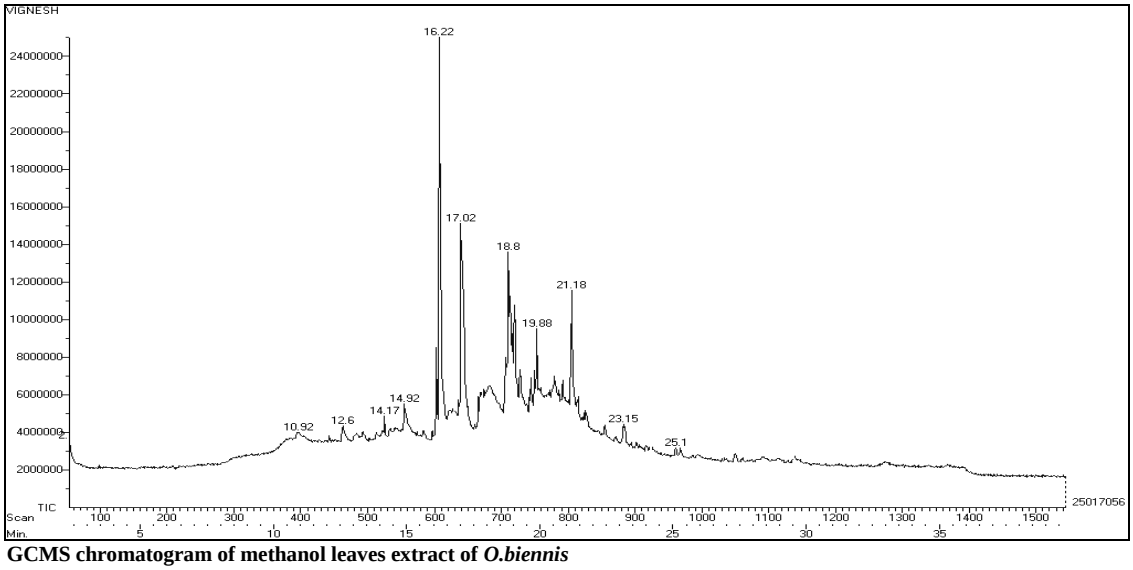
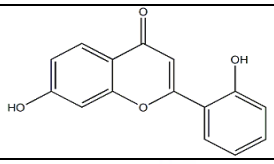
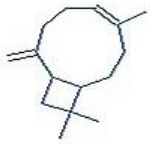
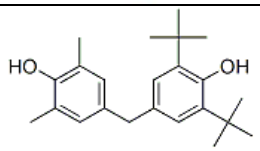
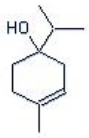
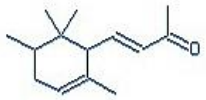
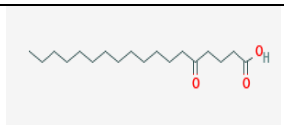
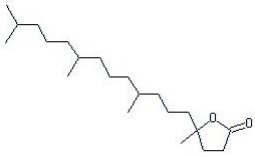
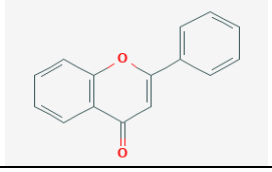


Fig. 5: GCMS chromatogram of methanol leaves extract of *O.biennis*

Table 5: GCMS analysis of the leaves of methanol extract of *O.biennis*

S. No.	Compound name	RT	Compound structure	Mol. Weight g/mol	Mol. Formula	Biological Activities
1.	10-Octadecenoic acid methyl ester	18.8		296.5	$C_{19}H_{36}O_2$	Antibacterial, antifungal, antioxidant, decrease blood cholesterol
2.	4H-1-Benzopyran-4-one,7-hydroxy-2-(4-hydroxyphenyl)	16.22		284.26	$C_{16}H_{12}O_5$	Antiviral
3.	Caryophyllene	12.6		204.36	$C_{15}H_{24}$	Anti-inflammatory and analgesic, Alcohol craving reduction. Anti-cancer, Anti-anxiety and anti-depressant.
4.	Oleic acid	17.02		282.47	$C_{18}H_{34}O_2$	Reduces Blood Pressure, Lower Cholesterol level Antidiabetic.
5.	2,6-bis(1,1-dimethyl)-4-[(4-hydroxy-3,5-dimethylphenyl)methyl]- Phenol,	25.1		340.50	$C_{23}H_{32}O_2$	Anti infective, Hair colourants
6.	4-methy-1-(1-methylethyl)-3-Cyclohexen-1-ol	10.92		154.24	$C_{10}H_{18}O$	antimicrobial and antioxidant
7.	3-Buten-2-one, 4-(2,5,6,6-tetramethyl-2-cyclohexane-1-yl)	14.17		206.32	$C_{14}H_{22}O$	Flavouring agent, Dermatology
8.	a-Ketostearic acid	19.88		298.46	$C_{18}H_{34}O_3$	-
9.	4,8,12,16-Tetramethylheptadecan-4-olide	21.18		324.5	$C_{21}H_{40}O_2$	Anti viral
10.	Isopropyl stearate	23.15		326.5	$C_{21}H_{42}O_2$	Anti-bacterial Cosmetics
11.	Cromaril	14.92		222.24	$C_{15}H_{10}O_2$	Immunology, Inflammation

CONCLUSION:

Antioxidants are substances that significantly delay or prevent the oxidation of an oxidisable substrate when present in low concentrations. Plants are potential sources of valuable antioxidants.[32] The results of the present study indicate that the methanolic plant extract of *O.biennis* have significant antioxidant activities to reduce harmful effect of radicals. The results of the present study provide promising hope to use *O. biennis* as an antioxidant agent.

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