Immunomodulatory activity and chemical characterization of fixed oils obtained from different parts of *Oxytropis glabra* DC

Rabia Mehtab[1], Muhammad Ibrahim[2,3], Asia Naz[1]*, Ambreen Faiyaz[4], Aisha Faheem[5], Munawwer Rasheed[6] and Zafar Saeed Saify[7]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan
[2]Department of Chemistry, Faculty of Science, University of Karachi, Karachi, Pakistan
[3]Pakistan Standards and Quality Control Authority, Pakistan Secretariat, Karachi, Pakistan
[4]Department of Biochemistry, King Saud bin Abdul Aziz University, Jeddah, Saudi Arabia
[5]Dr. Panjwani Centre for Molecular Medicine and Drug Research Centre, International Centre for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan
[6]Centre of Excellence in Marine Biology, University of Karachi, Karachi, Pakistan
[7]HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

Abstract: *Oxytropis glabra* DC. is a plant with enormous therapeutic vitality. In the present study a comparison of lipophilic profiling of different parts of *O. glabra* has been carried out by using gas chromatography-mass spectrometry. A total of 32 compounds have been identified from this plant, amongst which 31 have been identified for the first time. These compounds have been further confirmed from their Van den Dool and Kratz (*I*) Indices. Out of these 32 compounds, 18 have been identified from flower (80.94%), 15 from fruit (85.36%), 11 from leaves (66.35%) and 11 from root (45.96%). The major class of metabolite identified from different parts is fatty acid. Hydrocarbons have also been detected in flower and fruit but not in root and leaves. The extracts were screened for their immunomodulatory activity on whole blood cells. The root oil was found to be moderately active (IC_{50} 32.3 μg/ml). At present only limited data is available on the phytochemical composition of *O. glabra*.

Keywords: *Oxytropis glabra* DC, Leguminosae, fixed oil, GC-MS analysis, anti-inflammatory.

INTRODUCTION

Genus *Oxytropis* is native to Siberia and belongs to the family *Leguminosae* (subfamily *Papillionoideae*) which is considered as the largest and versatile family of plant kingdom. More than 300 species of *Oxytropis* are distributed all over the world (Li *et al*., 2012). Members of this genus are reported from Turkey, North America, China, Afghanistan, Iran, Iraq, Pakistan, Italy, France, Austria, Sweden, Yugoslavia, Spain, Albania, Poland, *Czech* Republic, *Slovakia*, Romania, Bulgaria, England, Greece, Germany, Norway, Africa and Finland (Erkul & Aytac, 2013). Species of *Oxytropis* reported from Pakistan includes; *O. chitralensi, O. tatarica, O. lapponica, O. glabra, O. immerse, O. mollis, O. platonychia and O. gloriosa.* Most of these are reported from Chitral, Kashmir, Skardu and surroundings. *O. glabra* is a perennial herb, having a flowering season from July to August (ALI, 1964).

Herbal medicinal system is the integral part of traditional Chinese medicine (Wohlmuth, 2008). One of the Chinese herbal formulation contains *O. glabra* and is reported for treating arthralgia and rheumatic arthritis (Weiwei, 2015; Xianfu, 2016).

A topical preparation made from different herbs along with *O. glabra* is reported for curing periodontitis (Yandi, 2015). Topical use of formulation containing *O. glabra* as a main antiviral agent for HPV (human papilloma virus) has been reported in 2015 (Hong, Yao, Xia, & Weixin, 2015). Along with these traditional medicinal applications, extracts from *O. glabra* in combination with other selected herbs are cosmetically applied for skin whitening purpose, which is thought to produce anti-tyrosinase activity and inhibit melanogenesis in pigment cells (Tabuchi, 1995).

Recent studies on biochemical activities of *O. glabra* generally focus on its antioxidant, anti-tumor, analgesic and bacteriostatic properties (Li *et al*., 2012; Wang, Li, Ma, Zhang, & Ma, 2012). Chemical investigation has revealed various medicinally important compounds from *O. glabra*. Flavonoids including quercetin, kaempferol, 3', 7-dihydroxy-2',4'-dimethoxy-isoflavane, kaempferol-7-*O*-α-L-rhamnopyranoside have been reported (Zhang & Zhu, 1992). A very important indolizidine alkaloid, swainsonine have also been isolated from *O. glabra.* (Lu, Wang, & Zhao, 2012). A study on aerial parts of *O. glabra* led to the discovery of some important triterpenoidsaponins namely oxytrogenol, soyasapogenol E and soyasapogenol B (Rong-Qi & Zhong-Jian, 1990). Barring a few exceptions (Palmitic acid, daucosterol and tetratriacontane), *O. glabra* has not been analyzed for lipophilic components. (Yu, 1991).

**Corresponding author:* e-mail: asia.naz@uok.edu.pk

Subjected to the degree of saturation and carbon chain length, there are about 3 dozen of fatty acids found abundantly in nature (Boullata, 2006). Human body is not capable to synthesize the so called essential fatty acids and these should only be taken in the diet (De Pablo & De Cienfuegos, 2000). Essential fatty acids are required in a number of processes starting from immunity building to normal cognitive function (Aluko, 2012; De Pablo & De Cienfuegos, 2000). These fatty acids are believed to be involved in preventive effects from Alzheimer's disease by inhibiting formation of amyloid in brain (Aluko, 2012). Moreover, they also play an important role in modulation of immune system by reducing proliferation of lymphocytes, decreasing cytokine production, activating phagocytosis and modifying the activity of defensive cells of human body (De Pablo & De Cienfuegos, 2000). Specifically, omega-3 and 6 fatty acids prevent normal cells from getting damaged due to effects of oxidative stress, by halting activation of NF-κB (Natural killer cell kappa-B) (Aluko, 2012). This mechanism is also accompanied by up regulating transcription of antioxidant enzyme and down regulation of transcription of those enzymes which take part in the formation of reactive oxygen species (ROS)(Kremmyda, Tvrzicka, Stankova, & Ales, 2011).

Production of ROS is a basic part of immune response which in turn activates inflammatory process. ROS are responsible for various pathological states including rheumatic arthritis, systemic lupus erythromatosus (SLE), cancer and atherosclerosis. Immunomodulators are required to control a number of diseases either by activating the immune system such as in AIDS or by inhibiting it as in the case of arthritis or grafting conditions (Mesaik *et al*., 2013).

In the present study fixed oil from different parts of *O. glabra* were screened for their immunomodulatory activity. The investigation also encompasses comparative profiling of fatty acids among different parts of the plant using GC and GC-MS.

MATERIALS AND METHODS

Plant material

Whole plant of *O. glabra* was collected during August to September (2014) from Skardu Valley, Pakistan. The herbal sample was identified by Dr. Muneeba Khan and a specimen (No. 92172) was submitted in the Centre for Plant Conservation, University of Karachi. Leaves, flowers, fruits and roots were separated manually and shade dried. Each part was then ground in an electric grinder and stored in zip-lock bags.

Chemicals

n-Hexane, methanol (purity >99.8%), dimethyl sulfoxide (DMSO) and hydrochloric acid (37%) of ACS grade were purchased from Merck Millipore, Germany, while anhydrous sodium sulphate, Hanks balance salt solution (HBSS), luminol (3-amino-phthalhydrazide) and zymosan A (*Saccharomyces cerevisiae* origin) were obtained from Sigma-Aldrich, Germany.

Extraction of fixed oil

Extraction of fixed oil with little modification has been carried out as per the reported protocol(Walia, Rawat, Bhushan, Padwad, & Singh, 2014). Briefly, 7g of each part of plant was extracted in soxhlet apparatus using *n*-hexane for 5 hours. The extracts were concentrated under reduced pressure to get a viscous oily concentrate.

Esterification of fatty acids

The extracts were derivatized to esters(Weston, Derner, Murrieta, Rule, & Hess, 2006). 25mg of each extract of flower, fruit, leaves, and fruit were weighed in a screw capped vial containing Teflon line. To each vial 5 ml methanolic hydrochloric acid (1M) was added and subjected to heating at 80°C with continuous stirring on water bath for 1h. The esterified derivatives were extracted by the addition of 1:1 mixture of distilled water: *n*-hexane and centrifuged for 10 minutes at 3000 rpm. The upper *n*-hexane portion was collected and dried over anhydrous sodium sulfate. This esterified extract was filtered and preserved for GC-MS analysis.

GC-FID analysis

The esterified extracts were subjected to GC-FID using Shimadzu GC-17A which was fitted with a ZB-5 capillary column (60m length and 0.53mm ID). Nitrogen as carrier gas was set at a flow rate of 10 ml/minute. Initially the oven temperature was held at 70°C and scheduled to 250°C with a rise of 5°C/min. Temperatures of injector and detector were set at 240°C and 260°C, respectively.

GC-MS analysis

GC-MS analysis was carried out on Agilent 7890A gas chromatograph system attached with Agilent 7000 GC-QQQ mass spectrometer on EI positive mode at an electron energy of 70 eV preset at 250°C. HP-5MS capillary column (30m, 0.25mm ID and 0.25µm film thickness) was used for analyses, the column temperature was programmed as follows: 50°C for 10 minutes and then rise at a rate of 6°C /min to 180°C and hold for 20 min, again rise from 180°C to 290°C at 15°C /min and finally hold for 23 min at 290°C. The carrier gas used was helium at a flow rate of 1.2ml/min and injection port temperature was kept at 250°C.

Identification of compounds

Lipophilic components including fatty acids methyl esters (FAMEs) were identified through comparing their spectra from NIST library, 2005. These compounds were further confirmed by matching their retention indices with the reported data. The retention indices of compounds were calculated by Van den Dool and Kratz (*I)* method (Van den Dool & Kratz, 1963). The identified compounds are listed in table 1.

Table 1: Fixed oil composition after esterification from roots, flowers, leaves and fruits of *Oxytropis glabra* DC.

Compounds	$I_{Cal.}$	I_{lit}	(I_{lit}) References
1,1,2,3-tetramethylcyclohexane (1)	921	936	(Surmaghi & Bahreini, 2012)
1-ethyl-4-methylbenzene (2)	954	961	(Leffingwell, Alford, Leffingwell, Penn, & Mane, 2013)
p-cymene (3)	1016	1026	(Leffingwell, Alford, Leffingwell, *et al*., 2013)
1,8-cineole (4)	1036	1037	(Boix, Victório, Lage, & Kuster, 2010)
Tridecane (5)	1296	1300	(Kotowska, Żalikowski, & Isidorov, 2012)
Tetradecane (6)	1396	1400	(Kotowska *et al*., 2012)
Dodecanoic acid methyl ester (7)	1519	1527	(Lazari, Skaltsa, & Constantinidis, 2000)
Nonanedioic acid diemthyl ester (8)	1546	1548	(El Alfy, El Tantawy, Motaal, & Gamal, 2015)
Diethyl toluamide (9)	1579	1585	(Leffingwell, Alford, Leffingwell, *et al*., 2013)
Hexadecane (10)	1596	1600	(Zhao *et al*., 2006)
Tetradecanoic acid methyl ester (11)	1718	1723	(El Alfy *et al*., 2015)
6-phenyldodecane (12)	1723	1727	(Kotowska *et al*., 2012)
Pentadecanoic acid methyl ester (13)	1819	1820	(El Alfy *et al*., 2015)
6,10,14-trimethyl-2-pentadecanone (14)	1835	1847	(Kowalski, 2005)
Hexadecanoic acid methyl ester (15)	1918	1926	(Vedernikov & Roshchin, 2010)
Hexadecanoic acid ethyl ester (16)	1987	1995	(Leffingwell, Alford, & Leffingwell, 2013)
9,12-Octadecadienoic methyl ester (17)	2086	2076	(Martins *et al*., 2014)
9-Octadecenoic acid methyl ester (18)	2094	2084	(Martins *et al*., 2014)
Octadecanoic acid methyl ester (19)	2131	2130	(Lazari *et al*., 2000)
9,12-Octadecadienoic ethyl ester (20)	2174	2159	(Pino, Marbot, & Vazquez, 2004)
9-Octadecenoic acid ethyl ester (21)	2179	2152	(Martins *et al*., 2014)
Eicosanoic acid methyl ester (22)	2327	2329	(Leffingwell, Alford, & Leffingwell, 2013)
Tetracosane (23)	2397	2400	(Lazari *et al*., 2000)
Heneicosanoic acid methyl ester (24)	2428	2429	(Leffingwell, Alford, & Leffingwell, 2013)
Docosanoic acid methyl ester (25)	2528	2531	(Kowalski, 2005)
Tricosanoic acid methyl ester (26)	2628	2631	(Leffingwell, Alford, & Leffingwell, 2013)
Heptacosane (27)	2697	2699	(Kotowska *et al*., 2012)
Tetracosanoic acid methyl ester (28)	2728	2731	(Zhao *et al*., 2006)
Nonacosane (29)	2900	2900	(Zhao *et al*., 2006)
Hexacosanoic acid methyl ester (30)	2931	2941	(Zhao *et al*., 2006)
Octacosanoic acid methyl ester (31)	3137	3144	(Vedernikov & Roshchin, 2010)
β-Sitosterol (32)	3337	3329	(Bataglion *et al*., 2015)

I cal. =Linear retention indices of samples; *I* lit. =Linear retention indices cited in literature. No. in bold shows the elution order of compounds.

Chemiluminescence immunoassay

Chemiluminescence immunoassay was done in accordance with the method described (Haklar, Sayin-Özveri, Yüksel, Aktan, & Yalçin, 2001; Helfand, Werkmeister, & Roder, 1982; Sultana, Arayne, Naz, & Mesaik, 2013). As per the protocol 10ml blood was drawn from healthy individual, into heparinized tubes. The dilution of extracts (10µg, 50µg and 250µg) were prepared in Hanks balanced salt solution (HBSS++). The blood was also diluted (1:50), and suspended in HBSS++. The 25µL of whole blood and extracts were added in a 96 well plate which was placed in luminoskan chamber at 37˚C for 15-20 min. Final volume was adjusted with HBSS++ after incubation, following the addition of 25µL of zymosan (SOZ) and luminol to each well. The sample was scanned for 50 min in a repeated scan mode using Luminometer (Labsystems Luminoskan Helsinki, Finland). The chemiluminescence was expressed in RLU (relative light unit). Ibuprofen was used as a standard drug while negative control was run without the addition of extract/standard and zymosan. All tests were performed in triplicates. The results of immunoassay of extracts are presented in table 3.

RESULTS

Oxytropis glabra when subjected to soxhlet extraction using *n*-hexane yielded yellow thick oil from its roots and fruits with nutty odor, while its flower and leaves provided green thick oil with aromatic, tamarind like odor.

A total of 18 constituents were identified in fixed oil from flowers of *Oxytropis glabra* DC. constituting 80.94%. Flower oil was dominated by the presence of fatty acid esters (68.81%) followed by hydrocarbons (10.41%).

Among (7, 11, 15, 17, 18, 19, 22, 24, 25, 26, 28, 30, and 31) fatty acids, the dominant constituents were found to be docosanoic acid methyl ester (13.80%), hexadecanoic acid methyl ester (11.12%), followed by 9-octadecenoic acid methyl ester and tetracosanoic acid methyl ester that appeared in equal percentages (8.32%). Major constituents among (12, 27 and 29) hydrocarbon class were nonacosane (6.37%) and heptacosane (3.57%). *β*-sitosterol was found to be 1.13%. Out of 18 constituents identified from flower, only hexadecanoic acid methyl ester (15) has been reported earlier from *O. glabra* whereas 17 compounds (7, 11, 12, 14, 17, 18, 19, 22, 24, 25, 26, 27, 28, 29, 30, 31 and 32) are reported in this study for the first time from the plant. 5 compounds (14, 18, 27, 29 and 32) are reported from genus *Oxytropis* and 1 compound, 6-phenyldodecane (12) is reported for the first time from *Leguminosae* family.

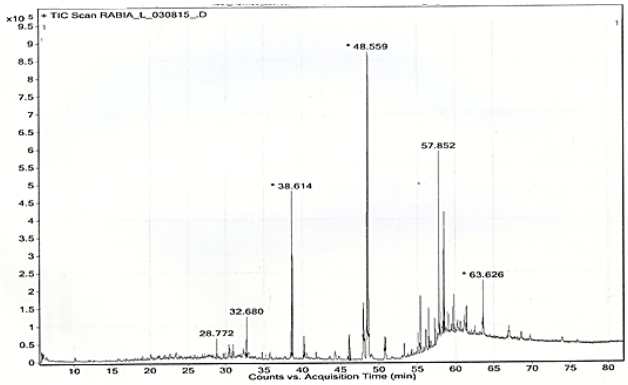

Fig. 1: GCMS chromatogram of leaf oil from *Oxytropis glabra* DC

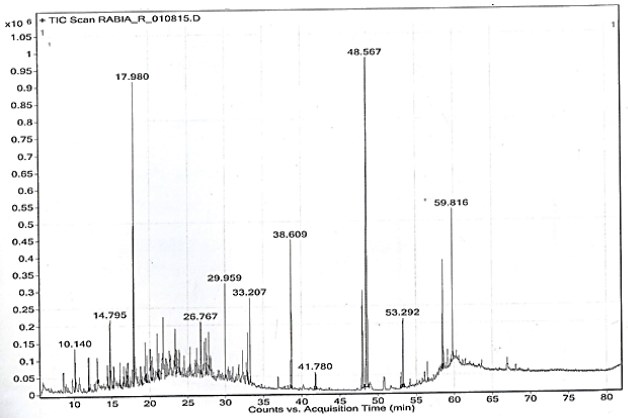

Fig. 2: GCMS chromatogram of root oil from *Oxytropis glabra* DC

Fruit oil from *O. glabra* constituted 8, 11, 13, 15, 17, 18, 19 and 22 which are fatty acid methyl ester (71.27%). 9-Octadecenoic acid methyl ester (18) was present in excessive amount (31.8%) in fruit followed by hexadecanoic acid methyl ester (15) and 9,12-Octadecadienoic methyl ester (17) (19.72% and 14.0% respectively). Hydrocarbons (7.79 %) obtained from fruit oil are 1, 2, 5, 6, 10 and 23. The most abundant hydrocarbon was 1-ethyl-4-methyl benzene (2) (3.3%). Out of 15 identified compounds, 14 are reported for the first time from *O. glabra*, out of which 2 compounds (18 and 23) are identified first time from the genus *Oxytropis* and 2 constituents 1,1,2,3-tetramethylcyclohexane (1) and 1-ethyl-4-methylbenzene (2) are reported for the first time from the family.

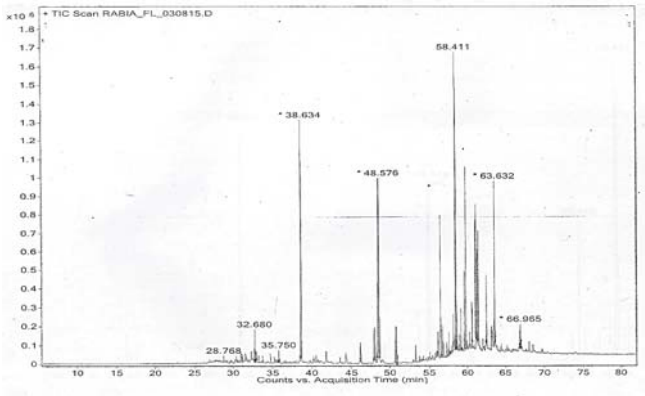

Fig. 3: GCMS chromatogram of flower oil from *Oxytropis glabra* DC

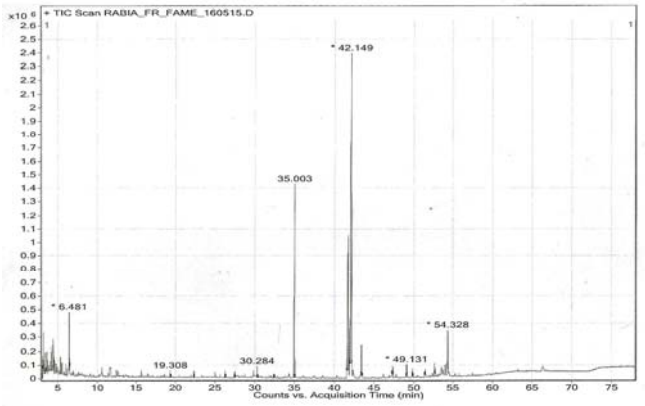

Fig. 4: GCMS chromatogram of fruit oil from *Oxytropis glabra* DC

Furthermore, 11 fatty acid methyl esters (7, 11, 15, 17, 18, 19, 22, 25, 28, 30 and 31) were identified from leaves. Among these 9-octadecenoic acid methyl ester (18) was present in the highest ratio (23.93%) followed by hexadecanoic acid methyl ester (15) (13.88%) and docosanoic acid methyl ester (25) (6.84%). Out of 11 identified compounds, 10 are reported for the first time from *O. glabra* and 9 from genus *Oxytropis.*

A total of 45.96% of the oil obtained from root were accounted for the presence of 11 compounds (4, 9, 15, 16, 17, 18, 20, 21, 22, 25 and 28). The most abundant class was fatty acids esters (39.91%) in which, 9-Octadecenoic acid methyl ester (18) was the most dominating (14.6%). Hexadecanoic acid methyl ester (15) and tetracosanoic acid methyl ester (28) both were found to be 6.57% followed by 9,12-Octadecadienoic methyl ester (17)

Table 2: Percentage Composition of different classes of compounds in fixed oil of *Oxytropis glabra* DC.

Compounds	Source (%)			
	Flower	Fruit	Leaves	Root
1,1,2,3-tetramethylcyclohexane	--	1.92	--	--
1-ethyl-4-methylbenzene	--	3.28	--	--
p-cymene	--	6.3	--	--
1,8-cineole	--	--	--	1.15
Tridecane	--	0.82	--	--
Tetradecane	--	0.54	--	--
Dodecanoic acid methyl ester	0.23	--	1.71	--
Nonanedioic acid diemthyl ester	--	0.68	--	--
Diethyl toluamide	--	--	--	4.9
Hexadecane	--	0.68	--	--
Tetradecanoic acid methyl ester	1.48	1.1	3.42	--
6-phenyldodecane	0.47	--	--	--
Pentadecanoic acid methyl ester	--	0.27	--	--
6,10,14-trimethyl-2-pentadecanone	0.59	--	--	--
Hexadecanoic acid methyl ester	11.12	19.72	13.88	6.57
Hexadecanoic acid ethyl ester	--	--	--	0.73
9,12-Octadecadienoic methyl ester	1.55	14.0	3.21	4.32
9-Octadecenoic acid methyl ester	8.32	31.8	23.93	14.6
Octadecanoic acid methyl ester	1.80	3.15	1.18	--
9,12-Octadecadienoic ethyl ester	--	--	--	0.73
9-Octadecenoic acid ethyl ester	--	--	--	3.04
Eicosanoic acid methyl ester	6.54	0.55	3.42	0.85
Tetracosane	--	0.55	--	--
Heneicosanoic acid methyl ester	1.07	--	--	--
Docosanoic acid methyl ester	13.80	--	6.84	2.50
Tricosanoic acid methyl ester	1.90	--	--	--
Heptacosane	3.57	--	--	--
Tetracosanoic acid methyl ester	8.33	--	2.99	6.57
Nonacosane	6.37	--	--	--
Hexacosanoic acid methyl ester	5.06	--	1.92	--
Octacosanoic acid methyl ester	7.61	--	3.85	--
β-Sitosterol	1.13	--	--	--
Fatty acid esters	68.81	71.27	66.35	39.91
Hydrocarbons	10.41	7.79	--	--
Miscellaneous	1.72	6.30	--	6.05
Unidentified	19.06	14.64	33.65	54.04
Total identified	80.94	85.36	66.35	45.96

(4.32%) and 9-Octadecenoic acid ethyl ester (21) (3.04%). Diethyl toluamide (9) was also found (4.9%) in roots. Out of 11 identified compounds, 10 are reported for the first time from *O. glabra*, 8 compounds from genus *Oxytropis* and 1 compound, (9) from *Leguminosae* family. Fixed oils were subjected for their immunomodulatory potential. Oils from different parts of the plant were compared and results are presented in table 3. Fixed oils from root of *O. glabra* showed moderately positive results as an immunomodulator (IC_{50}= 32.3 μg/ml) when compared with standard drug Ibuprofen (IC_{50}= 11.2 ± 1.9 μg/ml), whereas, other parts did not offer any significant inhibitory activity.

DISCUSSION

In literature, various reports have discussed the antioxidant potential of hexadecanoic acid methyl ester (Belakhdar, Benjouad, & Abdennebi, 2015; Patra, Das, & Baek, 2015). An edible seaweed, *Laminaria japonica* L. containing major portion of hexadecanoic acid methyl ester (16.57%) in its essential oil showed strong inhibition of lipid per oxidation yielding a good antioxidant activity. It is reported that a good portion of the same seaweed contains 9,12-octadecadienoic acid (12.09%) which could be used as an antioxidant, anti-inflammatory, acne reductive and moisture retaining properties in various

cosmeceutical formulations (Patra *et al*., 2015). A report discussed the biological effects of fatty acids pointed out the antioxidant and anticancer effects of 9-octadecenoic acid methyl ester (Belakhdar *et al*., 2015). Beside antioxidant capacities of fatty acids, antibacterial action is usually attributed as being a property of long-chain unsaturated fatty acids more specifically 9-octadecenoic acid and 9,12-octadecadienoic acid (Choi *et al*., 2013). In comparison to polyunsaturated fatty acid, a study on peanut oil showed eicosanoic acid (arachidic acid) and n-docosanoic acid (behenic acid) as a source of saturated fatty acid. Peanut oil exhibited a strong antioxidant potential when tested for its radical scavenging effects and this is reported to have a sound ratio between polyunsaturated and saturated fatty acids as recommended by WHO/FAO organization (Nile & ParK, 2013). 9-octadecenoic acid which is reported as a major type of fatty acid in *C. tenuifolia* seed oil. The oil showed excellent *in vivo* anti-oxidative activity as well as good antibacterial effect (Wei *et al*., 2016).

Table 3: Immunomodulatory activity of fixed oil from *Oxytropis glabra* DC

n-Hexane Extract	IC_{50} (μg/mL)
Flower	> 250
Fruit	> 250
Leaves	> 250
Root	32.3
Ibuprofen	11.8

In the present study, different parts of *O. glabra* DC are screened for lipid profiling using GC-MS and subjected to immunomodulatory activity. Fixed oil composition showed that it contains fatty acids as its major components. Fatty acids are well known for immunomodulation, synergistic for anticancer agents, neuroprotective and as potential anti-Alzheimer's group (Aluko, 2012; De Pablo & De Cienfuegos, 2000). In this study, 31 compounds have been reported for the first time from *O. glabra.* The study revealed that each part of *O. glabra* is equally enriched with good fatty acids such as 9-octadecenoic acid methyl ester, 9,12-octadecadienoic methyl ester and hexadecanoic acid methyl ester showing its possible utility for medicinal purposes. It is important in particular that the fruit of *O. glabra* is worthy enough to isolate 9-octadecenoic acid methyl ester as it carries a good proportion which is about 31.8% of the total oil. Besides fatty acids, soxhlet extraction also afforded some other compounds which were not reported earlier from this specie including *p*-cymene, 1,8-cineole, β-sitosterol and diethyl toluamide. The root oil has showed moderate inhibitory effect on the formation of reactive oxygen species (ROS) using whole blood cells. This activity is possibly attributed due to the presence of some miscellaneous compounds specifically 1,8-cineole. Eucalyptus oil which contain 1,8-cineole as a major component is popular for its antimicrobial, anti-inflammatory, antioxidant and even spasmolytic effects (Sadlon & Lamson, 2010). A previous study on anti-inflammatory effect of monoterpenes investigated the effects of 1,8-cineole on gross damage of colonic tissues by the induction of trinitrobenzenesulfonic acid. This study not only proved that 1,8-cineole significantly reduce tissue injury but also reduce myeloperoxidase activity, therefore suggesting its anti-inflammatory effects (de Cássia da Silveira e Sá, Andrade, & de Sousa, 2013).

CONCLUSION

In conclusion, 32 compounds from different parts of *O. glabra* have been identified. Out of which, 31 compounds have not been reported before. The major class of compounds among all identified constituents is fatty acid which is well-known for its antioxidant potential. Upon immunomodulatory screening root oil was found to be active. Further investigation on root oil against lymphocyte proliferation and antineoplastic potential is required. This overall assessment of *O. glabra* however furnishes its usefulness as a herb of immense utility.

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