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ORIENTAL JOURNAL OF CHEMISTRY
An International Open Free Access, Peer Reviewed Research Journal

www.orientjchem.org

ISSN: 0970-020 X
CODEN: OJCHEG
2015, Vol. 31, No. (4):
Pg. 1929-1935

GC-FID Analysis of Fatty Acids and Biological Activity of *Zanthoxylum rhetsa* (Roxb.) DC Seed Oil

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http://dx.doi.org/10.13005/ojc/310409

(Received: July 30, 2015; Accepted: November 04, 2015)

ABSTRACT

The Fatty acid content and composition of fixed oil from *Zanthoxylum rhetsa* seeds was determined. The seeds were found to contain about ~19.5% of crude fixed oil on a dry weight basis. Fatty acids were converted into methyl esters and analyzed by GC-FID. Ten fatty acids were identified using GC-FID. The major monounsaturated and saturated fatty acids were oleic acid (41.6 - 43.5%) and palmitic acid (26.8-30.2%) respectively, whereas the α-linolenic acid (12.1 - 12.5%) and linoleic acid (10.0%) were polyunsaturated fatty acid. Stearic acid (5.2 - 6.0%), myristic acid (0.1%), traces of pentadecanoic, heptadecanoic and arachidic acid were also identified. These fatty acids have not been reported earlier from the oil of *Z. rhetsa*. Fixed oil exhibited significant free radical scavenging activity which was measured using DPPH, and is also known to inhibit the gastrointestinal motility significantly.

Key words: *Zanthoxylum rhetsa* (Roxb.) DC, Biological evaluation, Antioxidant, Gastrointestinal motility, GC-FID, Fatty acid methyl ester (FAME).

INTRODUCTION

The main components of the seed oil are the triglycerols, fatty acid that are present in the plant oil are the saturated or unsaturated like olefinic acid with 16 to 18 carbon that have a carboxyl group at one end. The double bonds are interrupted by methylene group in some derivatives like diene and triene, members that ingrate this structure include palmitic, stearic, palmitoleic, oleic, linoleic, and linolenic acid. All these are identified as components of the seed oil[1-3]. Alpha-linolenic acid (ALA) found in plants is an essential fatty acid that belongs to group of fatty acid called omega-3 fatty acid. Seed like soyabean, rapeseed, walnuts, flaxseed, perilla, chia and hemp have high source of ALA[4,5]. Longer chain fatty acid like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are synthesized by ALA in the body and plays a vital role in functioning of the body. It is known to reduce the risk of harmful disorders like cardiac arrhythmia, high

cholesterolemia, hypertension, thrombosis, allergy and cancer[6,7]. The risk of coronary disease is potentially reduced by daily intake of omega-3 fatty acids. Inadequate daily intake of ALA is associated with the disorders like hypertension, diabetes mellitus, coronary heart disease, schizophrenia, Alzheimer's disease, atherosclerosis and cancer, emphasizing on the importance of adequate daily intake of ALA in our diet. Food and agriculture organization of United Nations and World Health Organization have issued statement on the importance of adequate intake of ALA [8-11]. The demand for ALA has increased to 2 million kg annually, making such huge amount available is beyond the capacity. One way to overcome such requirement is to look for a natural source.

Zanthoxylum rhetsa (Roxb.) DC (Hindi Name-Triphal) is a small deciduous tree that belongs to the family Rutaceae, commonly grown in coastal Karnataka, southern part of Maharashtra and other part of India. Tree bears green color fruits which turns dark brown to black upon drying, exposing the seed. It's mainly used as condiment in fish curries and in preparation of some vegetables, the outer cover of the fruit (pericarp) is used in cooking by grinding it against stone releasing all the flavours. The major constituents present in the essential oil of seed coat were terpinen-4-ol (32.1%), α-terpineol (8.2%), sabinene (8.1%), β-phellandrene (7.4%) and 2-undecanone (7.1%)[12]. Sabinene (66.3%), α-pinene (6.6%), β-pinene (6.3%) and terpinen-4-ol (3–5%) were the major components of the essential oil of seed [13]. It has been reported that essential oil has comparatively better anthelmintic activity against *Tanenia solium, Ascaridia galli* and *Pheretima postuma* when compared with synthetic compound like piperazine phosphate[14]. Local community used the essential oil in the treatment of cholera[15]. Essential oil is used as antiseptic, disinfectant and for the treatment of asthma, toothache and rheumatism[16].

To the best of my knowledge the seed (kernel) oil has never been studied, although the seeds contain fixed oil but are discarded. This paper reports for the first time the GC-FID analysis of fatty acids and biological activity of fixed oil of *Zanthoxylum rhetsa* seeds.

EXPERIMENTAL

Materials and methods

DPPH, (±) α-tocopherol and loperamide hydrochloride were purchased from Sigma-Aldrich USA. Analytical grades solvents, reagents and chemical were used for the experiments. Plants materials (Trifal seeds with pericarp) were collected from the trees grown in Karwar City, Karnataka, India (Western Coast) in April, 2013. Voucher specimens (FPMS-2013-001) were deposited at Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, Amman-19328, Jordan. Plant material consisting of mature fruits was dried at room temperature. After separation from the pericarp, seeds were grounded using a mixer and powdered material was used for extraction of oil.

Albino mice (20–22.g) were purchased from local market. All animals were acclimatized (temperature 25±2 °C; humidity 60%) for 10 days, had free access to water and food. All experiments on animals were conducted as per the animal ethics guideline.

Extraction of Fixed oil

Fifty gram of seed flour was placed in a 500 ml round bottomed flask and 200 ml n-hexane was added to it. It was sonicated for 15 minutes. Then the content were filtered and again extracted with 2 x 150 ml of n-hexane. N-hexane was combined and the oil was recovered removing the solvent with a rotary evaporator. Oil was transferred into amber colored glass vials, flushed with stream of nitrogen, closed with Teflon scaled caps and stored in a -20 °C until analyses. In a separate experiment 50g seed flour was also extracted with diethyl ether and processed as mentioned earlier to receive the oil. Both oils were analysed for constituents and different activities.

Determination of physical properties

Specific gravity at 20 ºC and refractive index (RI) at 25 ºC with an Abbe's refractometer for oils were determined according to standard procedure (AOCS) [17]. The free fatty acid content, saponification value, iodine value, peroxides value and $A^{1\%}_{1cm}$ at 220 and 254nm were determined using standard procedure.

Preparation of Fatty acid methyl esters (FAME)

The fatty acid methyl esters were prepared by transmethylation using sodium methoxide in the presence of methyl acetate in n-hexane. After brief heating the reaction was stopped by adding a saturated solution of oxalic acid in diethyl ether with brief agitation. The mixture was centrifuged at about 4000 rpm for 10 minutes to precipitate sodium oxalate and aliquots of the supernatant were directly injected for GC analysis using split-split injection mode.

Determination of FAME by GC-FID

GC analysis was performed by a Gas Chromatograph (Model Shimadzu 2010, Shimadzu Co. Japan) equipped with a Teknocroma TRB-WAX-Omega capillary column (length-60 m, i.d.-0.025mm, film thickness, 0.25µm, Spain). The injector was maintained at 240°C. Operating conditions were as follows: carrier gas-helium, linear velocity 35 ml/min, injection volume-1 µl and split ratio-1:25. Oven temperature was maintained at 70 °C for 2 min., and then temperature was increased from 70 to 200 °C at a rate of 4 ºC/min, followed by 15 min hold at 200 °C. Fame were identified by comparing their GC retention time with those of 37 pure component FAME mix (Sigma-Aldrich Inc., St. Louis, California, USA), methyl ester of C_4-C_{24} saturated and unsaturated fatty acids. The samples were quantitatively determined through the normalization method without using correction factors: the relative peak area for individual constituents was averaged on three different chromatograms of three independent reactions. The fatty acid composition in the oil was expressed as the percentage of the total fatty acids.

Antioxidant activity

Radical scavenging activity of the oil samples from *Zanthoxylum rhetsa* seeds were performed with DPPH radical according to a method developed in house using reported method[18]. DPPH solution was freshly prepared at a concentration of 0.006 g % in n-hexane, solvent that allowed the complete solubility of lipid fraction and having high stability of the DPPH radical. Different solutions of oil (2000 µg/ml -125µg/ml) were prepared in n-hexane. Hexane solution of DPPH radical (1ml) and each oil (1ml) in *n*-hexane were mixed and then vortexed for 30s at 25±2 ºC. These samples were stored in dark for 30 mins. The spectrophotometer was set to zero using n-hexane as blank at 517nm. Against the *n*-hexane as blank the DPPH radical scavenging activity was determined as residual DPPH percentage of absorbance between DPPH solution (without oil) and sample in 517 nm in quartz cell with a spectrophotometer (Shimadzu UV-Vis spectrophotometer). The calculated IC_{50} is mentioned as mean ± SD (n=3).

$$\text{DPPH Scavenging activity} = \left[1 - \frac{\text{abs. of control}}{\text{abs of sample}}\right] \times 100$$

Effect of oil on gastrointestinal motility

Mice were kept overnight fasted and had free access to water. The effect of oil on intestinal motility in mice was evaluated using the reported procedure[19]. Mice were divided into the different groups. Oil samples (50, 100, 200, 300 mg/kg p.o.) were mixed with 0.1 % Tween 80 (0.1ml) and administered orally. Loperamide (8 mg/kg, p.o.) was given to animals in the standard group. The animals of control group received 0.1 % Tween 80 (0.1ml). Fifteen minutes after drug administration, the animals were treated orally with 0.3 ml of charcoal meal (10% charcoal in 5 % gum acacia). After 20 minutes, all animals were sacrificed and the entire length of small intestine was removed carefully. The distance travelled by the charcoal plug in the intestine (T1) and the total length of intestine (T2) was measured for each animal. Percentage of charcoal advance in the intestine was calculated using formula:

$$\% \text{ inhibition} = [1-(T2/T1)] \times 100$$

Where T1 is the percentage of gastrointestinal transit in the vehicle treated group and T2 is the percent of gastrointestinal transit in sample treated group. The statistical significance of differences between the groups was determined by one-way ANOVA, using the software GraphPad Prism 5 (San Diego, CA, USA) and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The total oil content in the seed was 19.50±0.05 %. The Iodine values indicate the presence of unsaturated fatty acids. The absorption maxima at 220 nm also support the presence of

Table 1: Some physical and chemical properties of *Zanthoxylum rhetsa* seed oil

Characteristics	Oil extracted using hexane	Oil extracted using ether
Specific Gravity (20 °C)	0.925 ± 0.001	0.923 ± 0.001
Refractive Index (25 °C)	1.4710 ± 0.0004	1.4708±0.0002
$A^{1\%}_{1cm}$ at 220 nm	13.51 ± 0.15	13.52 ± 0.13
$A^{1\%}_{1cm}$ at 254 nm	3.89 ± 0.04	3.91 ± 0.03
Acid Value	6.9 ± 0.10	6.4 ± 0.12
Saponification Value	199.5 ± 2.5	199.3 ± 2.2
Iodine Value (Wijs Method)	95.8 ± 1.2	98.9 ± 0.8
Peroxide Value* (mEq/kg)	12.1 ± 1.5	10.2 ± 0.0
α-tocopherol content (mg/kg)	20.5 ± 1.2	21.5 ± 1.1

***** Activity of α-tocopherol (2.0 ± 0.2)

Table 2: Fatty acid composition (%) of *Zanthoxylum rhetsa* seed oil samples

R_t, min	Fatty acid	Oil extracted using hexane	Oil extracted using ether
25.5	Myristic (C14:0)	0.14 ± 0.01*	0.09 ± 0.00*
30.4	Palmitic (C16:0)	30.20 ± 0.02	26.78 ± 0.02
32.6	Heptadecanoic (C17:0)	tr.	0.20 ± 0.01
34.9	Stearic (C18:0)	5.17 ± 0.02	5.96 ± 0.01
39.1	Arachidic (C20:0)	0.05 ± 0.00	0.48 ± 0.01
	ΣSFA[a]	35.56	33.51
28.0	Cis-10-pentadecenoic (C15:1)	tr.	0.06 ± 0.00
30.9	Palmitoleic (C16:1)	0.78 ± 0.02	0.50 ± 0.01
35.4	Oleic (C18:1)	41.63 ± 0.03	43.52 ± 0.02
	ΣMUFA[b]	42.41	44.08
36.3	Linoleic (C18:2)	9.97 ± 0.01	9.92 ± 0.01
37.7	α-Linolenic (18:3)	12.06 ± 0.02	12.49 ± 0.02
	ΣPUFA[c]	22.03	22.41

*Each value in the table represents the Mean ± SD of three replicates;

[a]SFA = Saturated fatty acids; [b]MUFA= monounsaturated fatty acids; [c]PUFA= Polyunsaturated fatty acids

Table 3: Antioxidant activity of the oil

Sample	DPPH radical activity	
	Maximum activity	IC_{50} (µg/ml)
Oil extracted using hexane	78.5±2.1[a]	265.5 ± 1.8
Oil extracted using ether	75.5±2.0[b]	264.5 ± 1.5
α-tocopherol	95.1±3.0[c]	85.1 ± 1.2

[a,b] oil concentration (1200 µg/ml), [c] α-tocopherol (500 µg/ml)

Table 4: Effect of oil from *Zanthoxylum rhetsa* seed on gastrointestinal transit in mice

Treatment	Dose (mg/kg p.o.)	Charcoal meal advance (%) [a]	Percent Inhibition [b]
Control	Vehicle	80.6 ± 4.5	-
Seed Oil	50.0	72.1 ± 2.6	10.6
	100.0	68.1 ± 3.8	15.5
	200.0	59.2 ± 3.5	26.6
	300.0	55.2 ± 3.6	31.5
Loperamide	8.0	36.4 ± 3.0	54.8

[a] Values expressed as Mean ± SD, [b] statistically difference from control ($p \leq 0.05$)

unsaturated fatty acids. The Extinction of oil (in hexane) at 220 nm ($A^{1\%}_{1cm}$) was 13.5, while at 254 nm it was 3.90. Other physical parameters were recorded in Table. 1. The fatty acid composition of *Zanthoxylum rhetsa* seed oil was analysed by GC, is presented in the Fig.1 a-b and Table 2. The major fatty acid identified in the oil extracted using hexane were oleic acid (41.6%), α-linolenic acid (12.1 %), palmitic acid (30.2%), stearic acid (5.2%) , linoleic acid (10.0%), with traces of pentadecanoic, heptadecanoic acid. There was no significant changes in the content or the composition of oil extracted using ether was similar to that of the composition of seed oil extracted using hexane, which is in accordance to earlier findings on cantaloupe seed oil[20] composition over extraction time 1st fraction, 2nd fraction and 3rd fraction. It may be confirmed that the fatty acid composition is similar

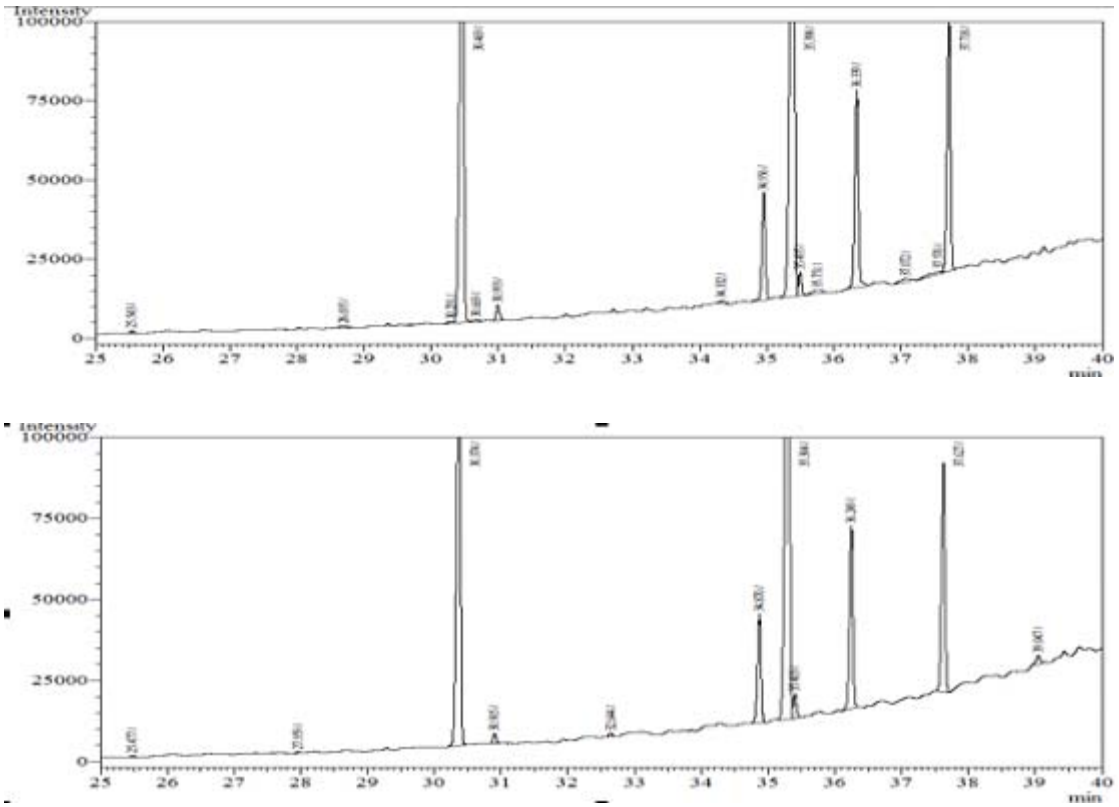

Fig. 1: GC-FID chromatogram of fatty acid methyl ester (FAME) of *Zanthoxylum rhetsa* seed oil extracted using (a) n-hexane and (b) diethyl ether

to some edible oil, mart palm oil and palm oil in respect to oleic acid and palmitic acid content.

The fatty acid composition of *Zanthoxylum* seed oil extracted using hexane contains saturated fatty acid (SFA) 35%, monosaturated fatty acid (MUFA) 42% and 22% of polyunsaturated fatty acid (PUFA). Seed oil extracted using ether showed presence of 33% of SFA, 44% MUFA and 22% PUFA. The content of MUFA was higher, than SFA and PUFA. Oleic acid was the predominant fatty acid in both the oil content extracted from hexane and ether. The fatty acid content of the *Zanhoxylum rhetsa* seed oil was similar to that of the work or the findings on the cucurbitaceae species with the major fatty acid content like Oleic acid, linoleic acid and stearic acid[21-24]. As evident from result this seed oil may be considered as an edible source of ALA and linoleic acid which can possibly be used as food supplements to reduce the fatal risks like cardiovascular diseases[25]. ALA along with linoleic acid is known to have anti–thrombotic effect in rats[26], this oil may have other biological properties. Dietary related diseases like cancer heart disease and stroke is mainly due to the food intake. Adequate intake of suitable fatty acid in diet can reduce this risk. Oleochemical industries use lauric oils as one of the main ingredients and *Zanthoxylum rhetsa* seeds can be suggested as vital source in this regard[28, 29]. The present study showed the presence of ALA in *Zanthoxulum rhetsa* seed oil which was similar to the oil reported for the same genus[26, 27] and cucurbitaceae species [22-25].

To test the free radical scavenging activity of various samples DPPH is used as it is a free radical compound with characteristic absorption of 517 nm[19]. When antioxidants react with DPPH, it either donates an electron or hydrogen to DPPH as result neutralizing it free radical activity changing its color from purple to yellow and decreasing its wavelength absorbance at 517 nm. The IC_{50} values for DPPH scavenging activity were calculated from the graphs (Concentration of sample required to scavenge 50% free radical). In the antioxidant activity test the extracted oil (at concentration 1200µg/ml) showed 78.5 % and 75.5% activity comparable to that of α - tocopherol. This seed oil is a potential antioxidant comparable to α-tocopherol. This may be partly due to the presence of constituents like tocopherol, phenolic compounds and fatty acids[30-32]. A significant higher antioxidant activity may be attributed to various reason or factors like the level of α - tocopherol, relatively high content of PUFA. Oxidation of oil leads to the changes in the lipids which may affect the antioxidants activity, so it's important to determine the level of oxidation of the oil obtained. The peroxide values suggests that the oxidation of the oil was relatively low compared to other studies[32] as it was extracted using either cold ether or hexane. Indicating the oil has better stability due to lower peroxide activity lower oxidation rate. *Zanthoxylum* is used by the local tribe to treat gastrointestinal disorders; this prompted us to study the effect of the seed oil on the gastrointestinal motility in albino rats. The oil showed a significant inhibition in gastrointestinal activity due to its direct effect on intestine. The significant percent inhibition ($p \leq 0.05$) was observed at a dose of 300 mg / kg b.w. which retarded the intestinal transit by 31.5% (compared to that of loperamide).

CONCLUSION

The *Zanthoxylum rhetsa* seed oil was studied for the first time for their fatty acid composition, antioxidant activity and its effect on gastrointestinal motility. On the basis of the result obtained in regard to the content of ALA, linoleic acid, and oleic acid content it may be suggested as vital source as food supplement or as an ingredients in cosmetic and in oleo-chemical industries. Similar to the suggestions made for other seed oils. Further research may be required for making it more define and for possible use as food supplement to overcome the demand for products from natural source. This may help to exploit the plant for other commercial and medicinal purposes.

ACKNOWLEDGEMENTS

Author wish to thank, Dean, Faculty of Pharmacy and Medical Sciences Dean, Higher Education and Scientific Research, Al Ahliyya Amman University, Amman, Jordan for providing necessary facilities.

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