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Research Article

Fatty Acids Analysis, Antioxidant and Biological Activity of Fixed Oil of *Annona muricata* L. Seeds

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The total oil yield and the fatty acid composition were determined in the *Annona muricata* L. fixed oil using organic solvent extraction and GC-FID. The seeds were found to contain about ~21.5% of crude fixed oil on a dry weight basis. The crude oil containing fatty acid was converted into methyl esters and analysed by GC-FID. Fourteen fatty acids were identified using GC-FID. The major monounsaturated and saturated fatty acids were oleic acid (39.2%) and palmitic acid (19.1–19.2%), respectively, whereas the α-linolenic acid (1.2%) and linoleic acid (34.9%) were polyunsaturated fatty acid. The other saturated acids were stearic acid (3.3%), arachidic acid (0.4%), myristic acid (0.1%), heptadecanoic acid (0.1%), behenic acid (0.1%), and lignoceric acid (0.1%). Some of the fatty acids have not been reported earlier from the oil of *Annona muricata* L. Fixed oil exhibited significant free radical scavenging activity which was measured using DPPH and is also known to inhibit the gastrointestinal motility significantly.

1. Introduction

Soursop (*Annona muricata* L.; family: Annonaceae; common name graviola) is a large spiny, green tropical fruit with a sweet and white pulp. It is widely distributed in India and Central America and is used in beverages, ice cream, and food [1]. As its major nutrient composition, the fruit contains carbohydrate, in addition to abundant vitamin C and several B complex vitamins along with calcium, phosphorus, and iron [2]. In Folk medicine, the fruit and leaves are used as remedies to relieve stomach distress, ulcer, pain, fever, and respiratory problems, such as cough and asthma [3, 4]. In South America, it is taken internally as astringent for diarrhea and dysentery. The plant is also reported to have good antioxidant activity [5]. Furthermore, the extract of leaves, twigs, and root are found to have antiproliferative, antispasmodic, and hypotensive activities and are rich in *Annonaceous* acetogenins [6–8]. Literature survey indicates that the extract of soursop inhibited the growth of herpes virus in the laboratory animals [9] and compounds extracted were effective against breast cancer cells. The pharmacological effects of *Annona muricata* L. leaves extract were studied on WRL-68, MDA-MB-435S, and HaCaT cell lines. The butanolic leaves extract of *Annona muricata* L. produces significant cytotoxic activity on cancerous cells [10, 11]. Local community in Africa (Nigeria, Kenya, and South Africa), Southern America (Brazil, Argentina, and Columbia), and India have used the fruit of graviola for different ailments [12]. The fruit of *Annona squamosa* is different from *Annona muricata*, which is wildly grown in India. The antimalarial, antifungal, antibacterial, and larvicidal activity of extract from seed has been reported [13–16]. Although the seeds contain 15.0 to 23.9% fixed oil, they may generate environmental pollution if these are not properly disposed. A few fatty acids (palmitic, stearic, palmitoleic, oleic, and linoleic acid) were separated and identified using TLC and GC [17, 18]. However, reports on antioxidant and other activities of *Annona muricata* L. seeds have not been evaluated so far. The seeds of the *Annona muricata* L. are toxic [17] and resistant to weathering conditions; it is thought to be worthwhile to study the fixed oil of *Annona muricata* seed and explore its therapeutic potential. In the present paper we are reporting the capillary GC analysis of fatty acid composition of fixed oil of *Annona muricata* L. and its biological activity.

2. Experimental

2.1. Materials. The seeds were powdered using mixer grinder after separating it from the pericarp of the mature fruit which was dried at room temperature. In the experiments the chemical and the reagents (analytical grade) were procured from Sigma-Aldrich, USA. Animals (Albino mice) that weighed around 20–25 g were purchased from local market. Animals were acclimatized at room temperature of 24 ± 2°C and relative humidity of 55–60%, with free access to food and water for one week. Animal ethics committee guidelines were followed for the experiments on animals.

2.2. Extraction of Fixed Oil. Round bottomed flask containing the 50 g of powdered seed was taken and extracted with 200 mL of *n*-hexane, which was then sonicated for 15 minutes. After sonication the contents were filtered and the residue was extracted again using *n*-hexane (2 × 150 mL). The solvent was evaporated using rotary evaporator. Oil was collected in amber colored vials, which were then sealed with Teflon caps and stored in refrigerator until analysis at −20°C. Similarly oil was extracted using diethyl ether from the seed powder. Oil samples extracted using either *n*-hexane or diethyl ether were analysed for their different fatty acid constituent and their effect on different biological activities.

2.3. Determination of Physical Properties. Physical properties (specific gravity and refractive index) were determined according to standard procedure [19]. Iodine, peroxide, saponification value, and free fatty acid content were estimated using reported methods [19]. UV spectra of oil samples were recorded in *n*-hexane using Shimadzu double beam UV-Visible-1800 spectrophotometer (Shimadzu Co., Japan), while infrared spectra of oil (as thin film) were recorded on Shimadzu Infinity IR spectrophotometer (Shimadzu Co., Japan).

2.4. Determination of α-Tocopherol in Oil. HPLC consists of Shimadzu Prominence HPLC (Shimadzu Co., Japan) equipped with autosampler; PDA detector and low pressure quaternary gradient pump were used. Signals were recorded at 290 nm and analysed using LC-solution (version 1.25), Windows 7 based software. The separation of different isomers was achieved using BDS-Hypersil column (150 mm × 4.6 mm, 5 μm) and a mixture of methanol and acetonitrile (50 : 50, v/v) as mobile phase was pumped at a constant flow rate of 1 mL/min. Different samples of α-tocopherol were prepared in mobile phase and 5 μL sample was injected. The calibration curves were linear from 10 to 60 μg/mL. Different samples (qualitative and quantitative) of oil were prepared and analysed using present chromatographic conditions. The α-tocopherol content was calculated in the oil from the calibration curve.

2.5. Determination of Fatty Acid Methyl Esters (FAME) by GC-FID. Transmethylated fatty acid methyl esters were prepared using sodium methoxide. Oil samples were mixed with methyl acetate in *n*-hexane and treated with sodium methoxide. The reaction was stopped after heating for short period by adding solution of oxalic acid in diethyl ether. From the reaction mixture sodium oxalate was precipitated by centrifuging the mixture at 4000 rpm for 10 min; then the aliquots of supernatant were injected for analysis. FAME was determined using Shimadzu GC Instrument (Shimadzu Corp., Japan), equipped with DB-23 capillary column with 0.25 μm of film thickness, length of 60 m, and i.d. of 0.025 mm. The operating condition for GC was as follows: carrier gas helium flowing at 35 mL/min of linear velocity; the temperature of the oven was maintained at 70°C for 2 min and then increased to 200°C from 70°C, by steadily increasing the temperature at a rate of 4°C/min. The temperature was then held at 200°C for 15 min. Samples were directly injected to the GC. Injection volume was 1 μL (with split ratio of 1 : 25). The injector and flame ionised detector were maintained at 240°C. Signals were recorded and analysed using Windows 7 based GC-solution software (version 1.25).

Standard FAME mixture (Sigma-Aldrich Inc., St. Louis, California, USA) containing 37 pure methyl ester of C_4–C_{24} saturated and unsaturated fatty acids was used for the identification of different fatty acid methyl ester derivatized from the experimental oil. The samples were quantitatively determined and results are averaged on three different chromatograms of three independent reactions. Relative percent fatty acid composition is reported in the results.

2.6. Antioxidant Activity [20–22]. Oil samples were evaluated using DPPH radical for free radical scavenging activity using the method reported earlier [20–22]. Solutions were freshly prepared. DPPH (0.006 g%), different concentration of oil (20 μg/mL–1280 μg/mL), and α-tocopherol (12.5–1000 μg/mL) were prepared in *n*-hexane. One mL of DPPH was mixed with either oil (1 mL) or α-tocopherol (1 mL). These solutions were vortexed at 25°C for 30 s and kept aside for 30 min in dark. Using *n*-hexane as blank at 517 nm the instrument spectrophotometer was set at zero. The free radical scavenging activity of residual DPPH against the blank was determined at 517 nm using the following formula:

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

All the experiments were conducted in triplicate. The values of the calculated IC_{50} are expressed as mean ± SD.

2.7. Effect of Oil on Gastrointestinal Motility [23]. For the motility studies the mice were fasted overnight with only access to water. The study was performed using the procedure [23] in which the mice are divided into different groups. Oil samples (100, 200, and 400 mg/kg p.o.) containing Tween 80 (0.1%, 0.1 mL) were administered to different animals. Animals of standard groups received loperamide (8 mg/kg, p.o.), while the control group received Tween 80 (0.1%, 0.1 mL). Charcoal meal (0.3 mL, 10% suspension of charcoal in 1% sodium CMC) was given to animals, 15 minutes after the treatment. Twenty minutes later, animals were sacrificed

and small intestine was removed. The distance travelled by the charcoal plug in the intestine (T_c) and the total length of intestine (T_t) were measured for each animal. Percentage of charcoal advance in the intestine was calculated using the following formula:

$$\% \text{ inhibition} = \left[1 - \frac{T_t}{T_c}\right] \times 100, \quad (2)$$

where T_c is the percentage of gastrointestinal transit in the vehicle treated group and T_t is the percent of gastrointestinal transit in sample treated group.

2.8. Antiproliferative Activity [24]. The following cell lines were studied to ensure the coverage of a wide range of cancer types: human ductal breast epithelial tumor T47D, human breast adenocarcinoma MCF-7, human epithelial carcinoma HeLa, human epithelial colorectal adenocarcinoma Caco-2, human colorectal adenocarcinoma cell line HRT, and human kidney carcinoma cell line A498. Cell lines were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) containing 10% heat inactivated fetal bovine serum (HI-FBS) (Invitrogen, USA), 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. Cells were passaged weekly, and the culture medium was changed twice a week. The optimal plating densities were determined according to the cells growth profiles. To assess the antiproliferative activity, MTT assay was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich, USA) assay, as previously described [24]. The concentration that inhibits the proliferation of oil-treated cells by 50% relative to the control (untreated cells) was used to evaluate the cytotoxic activity of the oil. To ensure exponential growth throughout the experimental period, as well as a linear relationship between absorbance at 570 nm and cell number when analysed by the MTT assay, 2×10^4 cells per well were seeded of each cell line. Treated cells were incubated in a 37°C incubator with 5% CO_2 for 48 h. As a positive control, vincristine sulphate (Sigma) was used at concentrations of 50 and 100 nM. In addition, a negative control, control wells without oil treatment, was employed and prepared under the same experimental conditions. All treatments were carried out in triplicate (in same 96-well plate) and repeated two times for each cell line.

2.9. Statistical Analysis. Results are expressed as mean ± standard deviation (SD). GraphPad Prism 5 (San Diego, CA, USA) for Windows was used for statistical analysis of experimental data. The t-test and one-way ANOVA were used to test for statistical significance with a p value < 0.05 considered as significant.

3. Results and Discussion

The average oil content in *Annona muricata* L. seed was 21.52 ± 0.05% (w/w). Iodine values and UV spectrum (λ_{max} 220 nm) of oil indicate the existence of unsaturated compounds in oil. The measured extinction of oil (in hexane) at 220 nm ($A^{1\%}_{1\,cm}$) was ranged from 75.21 to 75.35. Characteristic signal at 725 (CH_2 rocking); 951 (-CH_2 wagging), 1097 (O-CH_2-C), and 1292 (C-CO-O); 1711, 1736 (C=O esters); 2851 and 2918 cm^{-1} (CH_2 *sym*. and *asym*. str.) was observed in IR spectrum (Figure 1). Acid, saponification, iodine, and peroxide values were 3.13, 205.5, 102.5, and 8.5, respectively. The percentage of α-tocopherol was 12.5%. Other physical parameters were recorded in Table 1.

Table 1: Physical and chemical properties of fixed oil of *Annona muricata* L. seeds.

Characteristics	Oil (extracted using *n*-hexane)
Specific gravity (20°C)	0.9281 ± 0.0005
Refractive index (25°C)	1.4514 ± 0.0004
$A^{1\%}_{1\,cm}$ at 220 nm	75.21 ± 0.15
Acid value	3.13 ± 0.10
Saponification value	205.5 ± 2.5
Iodine value (Wijs method)	102.5 ± 1.4
Peroxide value* (mEq/kg)	8.5 ± 1.1
α-Tocopherol content (mg/kg)	12.5 ± 0.9

*α-Tocopherol (2.01 ± 0.19).

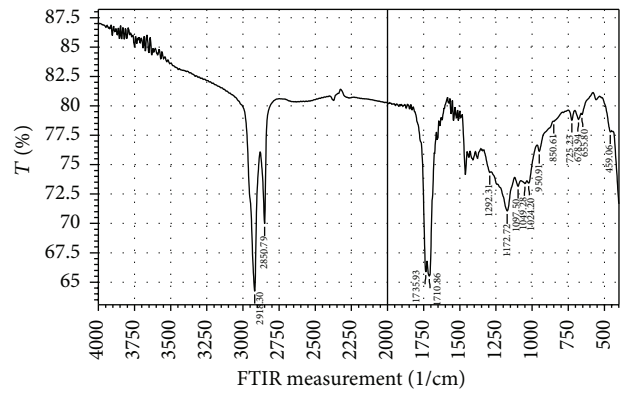

Figure 1: IR spectrum of fixed oil of *Annona muricata* L. seed (as thin film, number of scans: 30, resolution: 4 cm^{-1}, apodization; Happ-Genzel).

In this study, we report the fatty acid composition of *Annona muricata* seed analysed by capillary GC-FID. The SFA content detected was ~23.16% with palmitic acid and stearic acid as major fatty acid (19.1% and 3.3%, resp.). The MUFA content was ~40.77% with oleic acid (39.20%) and palmitoleic acid (1.36%) was the main MUFA content. The major PUFA content was linolenic acid (~34.9%) and ALA (~1.16%). These results are partially similar to the previously reported findings on the fatty acid content of *Annona muricata* [18]. Results are comparable with the reported studies of da Silva and Jorge [25] and Kimbonguila et al. [26] who have reported the presence of 25.0% SFA (containing 19.4% palmitic acid), 44.8% MUFA, and 41.41% and 30.60% of oleic and linoleic acid, respectively. The present study reports for

Table 2: Fatty acid composition of fixed oil of *Annona muricata* L. seeds.

Fatty acid	Oil extracted using hexane (%)*	Oil extracted using diethylether (%)*
Myristic (C14:0)	0.06 ± 0.01	0.05 ± 0.01
Palmitic (C16:0)	19.12 ± 0.02	19.22 ± 0.02
Heptadecanoic (C17:0)	0.07 ± 0.01	0.07 ± 0.01
Stearic (C18:0)	3.30 ± 0.01	3.22 ± 0.01
Arachidic (C20:0)	0.42 ± 0.01	0.41 ± 0.01
Behenic (C22:0)	0.10 ± 0.00	0.09 ± 0.00
Lignoceric (C24:0)	0.10 ± 0.00	0.09 ± 0.00
∑SFA[a]	**23.17**	**23.15**
Cis-10-Pentadecenoic (C15:1)	Trace	Trace
Palmitoleic (C16:1)	1.36 ± 0.02	1.35 ± 0.02
Cis-10-Heptadecenoic (C17:1)	0.05 ± 0.01	0.05 ± 0.01
Oleic (C18:1)	39.18 ± 0.03	39.22 ± 0.02
Cis-11-Eicosenoic (C20:1)	0.16 ± 0.01	0.17 ± 0.01
∑MUFA[b]	**40.75**	**40.79**
Linoleic (C18:2)	34.91 ± 0.03	34.85 ± 0.03
α-Linolenic (18:3)	1.16 ± 0.01	1.17 ± 0.01
∑PUFA[c]	**36.07**	**36.02**

*Values are expressed as mean ± SD ($n = 3$).
[a]SFA: saturated fatty acids; [b]MUFA: monounsaturated fatty acids; [c]PUFA: polyunsaturated fatty acids.

Table 3: Antioxidant activity of the fixed oil.

Sample	Free radical scavenging activity	
	Max. activity	IC_{50} (μg/mL)
Fixed oil (extracted using hexane)	77.5 ± 3.1[a]	202.5 ± 3.1
Fixed oil (extracted using diethylether)	78.0 ± 3.3[b]	190.1 ± 2.9
α-Tocopherol	95.1 ± 2.2[c]	87.5 ± 1.3

[a,b]Fixed oil (1280 μg/mL); [c]α-tocopherol (500μg/mL).

Table 4: Effect of orally administered fixed oil of *Annona muricata* L. seeds on gastrointestinal transit in mice.

Treatment	Dose (mg/kg)	Charcoal meal advance (%)[a]	% Inhibition[b]
Control	Vehicle	78.5 ± 2.5	—
Fixed oil	100.0	69.4 ± 3.7	11.6
	200.0	59.0 ± 3.8	24.8
	400.0	36.0 ± 3.1	54.1
Loperamide	8.0	33.8 ± 1.8	56.9

[a]Values are presented as mean ± SD; [b]statistically different from control ($p < 0.05$).

the first time the presence of fatty acids like myristic, heptadecanoic, behenic, lignoceric, cis-10-pentadecenoic, cis-10-heptadecenoic, and cis-11-eicosenoic acid in the oil (Table 2). The extraction solvent used did not produce any impact on the fatty acid composition and extraction efficiency. These fatty acids are within the limits in our studies which were not in accordance with the result or the findings of Rana [27], on *Annona squamosa*, where these fatty acids were absent.

DPPH scavenging activity (IC_{50}) was calculated graphically (Table 3 and Figure 2). The extracted oil (at concentration of 1280 μg/mL) showed 77.5% and 78.0% activity comparable to that of α-tocopherol. The IC_{50} values ranged from 190.1 ± 2.9 to 202.5 ± 3.1 μg/mL against DPPH. The antioxidant activity may be attributed to the presence of α-tocopherol (~12.5 mg/kg), phenolic compounds, and unsaturated fatty acids. The lipid content may change due to oxidation of the oil which in turn affects the antioxidant potential of the oil; hence it is important to determine peroxide value of extracted oil. The peroxide value of oil was lower than the other edible oils [25]. The lower value indicates the better stability of the oil. The oil was also studied for its effect on gastrointestinal motility and was found to inhibit the motility significantly (Table 4). Fixed oil retarded significantly the intestinal transit of charcoal meal by 54.1%

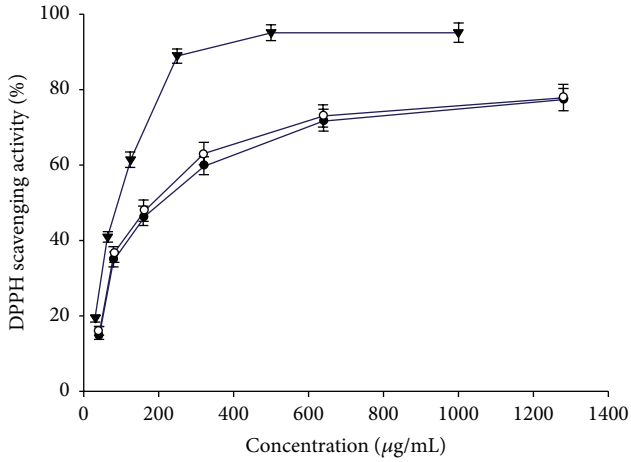

Figure 2: DPPH radical scavenging activity of fixed oil of *Annona muricata* L. seeds extracted using hexane (●), ether (○), and Vit. E (▼).

at a dose of 400 mg/kg b.wt. p.o. Significant inhibition in GIT motility might be due to the direct effect of the oil on intestine ($p < 0.05$).

According to our results, the examined oil did not exhibit significant antiproliferative effects on the various cancerous cell lines. Although the content of these seeds is a rich source of oleic acid and linoleic and palmitic acid that produce significant antioxidant and reduce the g.i.t. motility, these constituents failed to provoke cytotoxic effects on the cell line under investigation. Further work is to be carried out to explore more the other chemical constituent and pharmacological activity of seeds.

4. Conclusion

Fatty acid composition of *Annona muricata* L. fixed oil was studied using GC-FID and biological studies. The results indicate that various fatty acids like oleic acid, linolenic acid, palmitic acid, and ALA present in oil are important source of an ingredient in oleochemical, soap, and cosmetic industries. As the fixed oil is free from any toxic effects, it can be exploited for industrial, cosmetic, and medicinal purposes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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