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Proximate Chemical Composition and Antimicrobial Activities of Fixed oils from *Diospyros lotus* L.

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Abstract

Diospyros lotus roots are traditionally used in various diseases including its use in microbial infections. We designed a study to identify chemical constituents of oil from *D. lotus* and its antimicrobial activities. Gas chromatography coupled to mass spectrometer (GC-MS) was used for chemical analyses. Results showed that oil contained saturated as well as unsaturated compounds. Oil was investigated for its antimicrobial properties. Oil showed moderate antibacterial activity against two gram positive and two gram negative bacterial strains however oil showed low activity against four fungal strains. Interestingly no cytotoxicity was observed in Brine shrimp model; these encouraging results indicate further yet extensive studies to explore therapeutic potential in microbial infections.

Keywords: *Diospyros lotus*; Antibacterial; Antifungal

Introduction

Diospyros lotus belongs to family Ebenaceae, which consists of about 500 species. Genus *Diospyros* is widely distributed in tropical and subtropical region throughout world. Characteristic feature of *Diospyros* species is that all members of the genus are trees [1]. *D. lotus* tree grows up to 9 m in height and around 6 m in width. This plant is rarely available in Britain and it is native to Himalayan region. In folk medicine, *Diospyros* species are known for their multiple medicinal uses. In many traditional and folk medicinal systems all over the world, *Diospyros* plants are well known for their medicinal and therapeutic value. All parts of these plants have been used for medicinal purposes e.g. the leaves are used for lumbago; the fruits are carminative, astringent and cure biliousness the seeds are sedative and the bark is bitter, astringent and febrifuge [2]. Leaf extract of Japanese persimmon *D. kaki* in combination with jasmine is used in Japan for making anti-tobacco smoking candies. Triterpenoids belonging to lupane, oleanane and ursane series have been isolated and showed anti-inflammatory activity [3]. *Diospyros* species are used in folk medicinal systems for various medicinal uses such as their use used as antifungal for internal hemorrhage, for bedwetting in children, Woman's drug for insomnia hiccough, as an antihypertensive, dysponea, vermicide and vermifuge, sedative, antifebrile, to promote secretions, astringent, bactericidal [4,5]. There are only a few reports on the studies of the roots of this plant [6,7] hence in the current study we have made an effort to analyze the chemical constituents of this part and evaluate its antimicrobial and cytotoxicity activity.

Material and Methods

Plant material

D. lotus roots were collected from Razagram, Toormang distic Dir Khyber Pakhtunkhwa (KPK) province of Pakistan in the month of august, 2009. The plant was identified by Prof. Dr. Abdur Rahsid, Department of Botany, University of Peshawar, Peshawar, KPK, Pakistan and a Voucher specimen No Rauf (6645) [8] was deposited at the herbarium of the said department.

Extraction of oil

Shade dried and crushed roots of *D. lotus* (14 kg) were subjected to cold extraction with MeOH (3×10L) at room temperature. Extract was then concentrated under reduced pressure at temperature below 55°C. Final residue obtained was 400 g. This methanolic extract was suspended in water and successively partitioned with hexane, chloroform, EtOAc. Hexane fraction (8 g) was subjected to Column chromatography on silica gel (Merck, 5×60 cm). Column was eluted with hexane/EtOAc (100:0→0:10) as solvent system. A total of 30 fractions, RF-1 to RF-30 were obtained based on TLC profiles. Fraction RF-10 to RF-13 contained yellow colored oil. Extracted oil was subjected GC-MS analysis and injected 1 µl to GCMS using auto injector system.

Chemicals and reagents for GC-MS

Boron triflouride solution in methanol (10%) was purchased from Fluka Chemie (Buchs, Switzerland). Sodium hydroxide solution (methanolic; 0.5N) and sodium chloride (analytical grade) were obtained from Merck (Darmstadt, Germany) while methanol (HPLC grade), *n*-hexane (HPLC grade) were from Fischer Scientific (Leicestershire, UK). Helium gas (99.9999%) from Pak gas (United Arab Emirates) was procured. Tridecanoic acid methyl ester and Fatty acid methyl esters (FAMEs) having 37 components (Table 1) standard mixture were obtained from AccuStandard (Newhaven, Connecticut USA).

Preparation of standard for GC-MS

Internal standard was prepared by dissolving 13.7 mg of tridecanoic acid methyl ester in 1 mL hexane. External standard was prepared by diluting 10 mg of 37 component FAMEs mix standard to 10 mL with dichloromethane. From this solution further working standard solutions were prepared.

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ID#	Name	R. Time	Area	Conc. (%)	Std. Dev.
1	C6:0; Hexanoic acid, methyl ester	3.028	2637	40.00	0.001
2	C8:0; Caprylic acid, methyl ester	4.911	43548	40.00	0.002
3	C10:0; Capric acid, methyl ester	6.743	59804	40.00	0.002
4	C11:0; Undecanoic acid, methyl ester	7.608	32300	20.00	0.003
5	C12:0; Lauric acid, methyl ester	8.493	67697	40.00	0.002
6	C13:0; Tridecanoic acid, methyl ester	9.554	35734	20.00	0.003
7	C14:0; Myristic acid, methyl ester	10.897	72212	40.00	0.003
8	C14:1c; Myristoleic acid, methyl ester	11.466	8237	20.00	0.003
9	C15:0; Pentadecanoic acid, methyl ester	12.548	38432	20.00	0.003
10	C15:1; Pentdecanoic acid, methyl ester	13.234	7517	20.00	0.002
11	C16:0; Palmitic acid, methyl ester	14.533	118819	60.00	0.002
12	C16:1c; Palmitoleic acid, methyl ester	15.066	7253	20.00	0.002
13	C17:0; Margaric acid, methyl ester	16.829	34317	20.00	0.003
14	C17:1; Heptadecenoic acid, methyl ester	17.417	7643	20.00	0.003
15	C18:0; Stearic acid, methyl ester	19.500	67063	40.00	0.001
16	C18:1c; Oleic acid, methyl ester	20.038	17841	40.00	0.003
17	C18:1n9T; Elaidic acid, methyl ester	20.115	7232	20.00	0.003
18	C18:2T; Linoleic acid, methyl ester	21.608	8777	20.00	0.004
19	C18:2C; Octadecadionoic acid, methyl ester	21.856	9261	20.00	0.001
20	C18:3n6; G-linoleic acid , methyl ester	22.762	5708	20.00	0.002
21	C18:3n3; Linolenic acid , methyl ester	24.160	6455	20.00	0.003
22	C20:0; Arachidic acid, methyl ester	27.058	66297	40.00	0.004
23	C20:1; Eicosenoic acid , methyl ester	27.659	8757	20.00	0.003
24	C20:2; Eicosadienoic acid , methyl ester	29.395	6480	20.00	0.002
25	C20:3n6; 8,11,14-Eicosatrienoic acid , methyl ester	30.312	6128	20.00	0.004
26	C21:0; Heneicosanoic acid , methyl ester	30.745	30613	20.00	0.004
27	C20:4n6; Arachidonic acid , methyl ester	31.073	5846	20.00	0.003
28	C20:3n3; Eicosatrienoic acid , methyl ester	31.717	9586	20.00	0.001
29	C20:5N3; (EPA) Eicosapentaenoic acid , methyl ester	33.359	6262	20.00	0.001
30	C22:0; Behenic acid methyl ester	34.188	62867	40.00	0.005
31	C22:1; Eruccic acid methyl ester	34.753	6847	20.00	0.001
32	C22:2; Locosadienoic acid , methyl ester	36.323	9365	20.00	0.002
33	C23:0; Tricosanoic acid methyl ester	37.440	29002	20.00	0.002
34	C24:0; Tetracosanoic acid methyl ester	40.521	60828	40.00	0.003
35	C22:6n3; (DHA) Docosahexaenoic acid , methyl ester	40.859	5333	20.00	0.002
36	C24:1; Tetracosenoic acid methyl ester	41.098	9144	20.00	0.002

Table 1: Quantification results of 37 components standard.

Chromatographic separation of FAMEs

A gas chromatograph from Shimadzu hyphenated to a mass spectrometer QP 2010 plus (Tokyo, Japan) equipped with an auto-sampler (AOC-20S) and auto-injector (AOC-20i) was used. Helium was used as carrier gas. All chromatographic separations were performed on a capillary column (TRB-FFAP; Technokroma) having specifications: length; 30 m, id.; 0.35 mm, thickness; 0.250 µm, treated with polyethylene glycol. Other GC-MS conditions are: ion source temperature (EI): 250°C, interface temperature: 240°C, pressure: 100 KPa, solvent cut time; 1.6 min. 1 µL of sample and standard were injected into the GC column. Injector was operated in a split mode with a split ratio 1:50. Injection temperature was 240°C. The column temperature program started at 50°C for 1 min and changed to 150°C at the rate of 15°C/min. The temperature was raised to 175°C at the rate of 2.5°C/min and held for 5 minutes. Then the temperature was increased to 220°C at the rate of 2.5°C/min and kept constant for 5 minutes. Total elution time was 45 minutes. MS scanning was performed from m/z 85 to m/z 380. GC-MS solutions software provided by the supplier was used to control the system and to acquire the data. Identification of the compounds was carried out by comparing the mass spectra obtained with those of standard mass spectra from the NIST library (NIST 05).

Antimicrobial activities

In this study six fungal and five bacterial strains were used as reported earlier [9,10]. Bacterial strains used were *Escherrchia coli, Bacilus subtilis, Staphylococcus aureus, Klebsiella pneumonia* and *Straptococcus epidermis.* Fungal strains chosen for this study were *Trichophyton longifusus*, *Candida albicans*, *Aspergillus flavus*, *Microspoum canis*, *Fusarium salani* and *Candida glaberata*. All these strains were maintained on agar slant at 4°C. Slant was allowed to activate at 37°C for 24 hours on Müller-Hinton agar (for bacteria) and Sabouraud glucose agar (fungi) before any screening. Cultures were taken in triplicates at incubation temperature of 37°C for 24 to 72 hours. Broth culture (0.6 mL) of the test organism was placed in a sterile Petri-dish to which 20 ml of the sterile molten MHA was added. Holes were bored in to the medium using 0.2 ml of the oil. Streptomycin was the standard antimicrobial agent at a concentration of 2 mg /ml. Inoculation was done for 1 h to make possible the diffusion of the antimicrobial agent into the medium. Incubation was done at 37°C for 24 h and the diameters of the zone of inhibition of microbial growth were measured in the plate in millimeters. Extent of antimicrobial activity was obtained by measuring the diameter of zone of inhibition around the hole. Bioassay was repeated three times and then the mean diameter was determined. In this study streptomycin, miconazole and

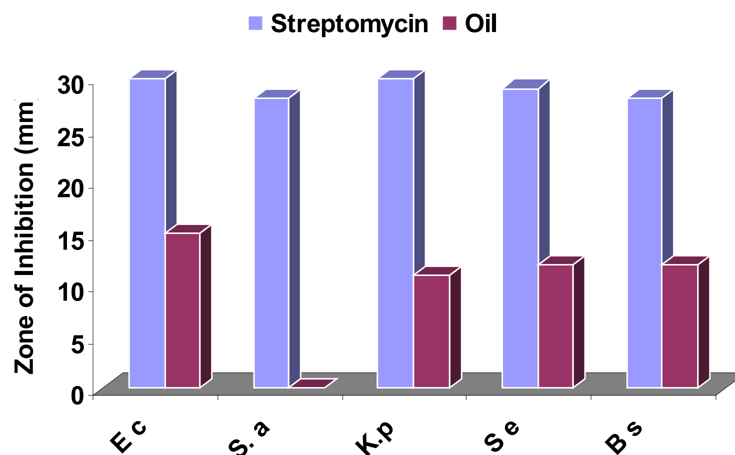
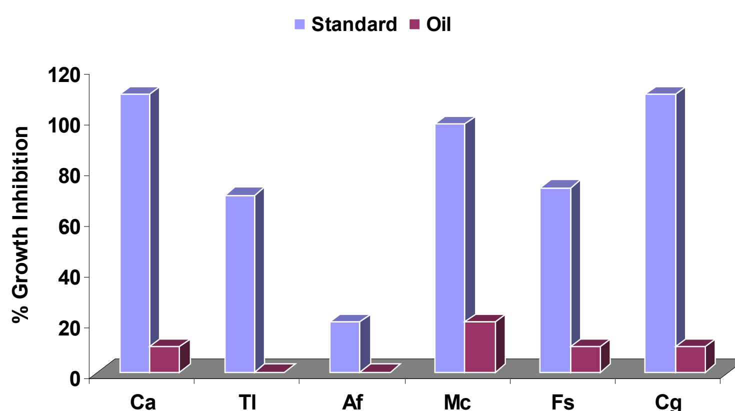


Figure 1: Antibacterial activity of oil isolated from the roots of *D. lotus.*

Peak #	Name	Relative area	Conc.	R. Time	m/z	Std. Dev
1	C12:0; Lauric acid, methyl ester	2878	2.21	8.265	87.00	0.0001
2	C14:0;Myristic acid, methyl ester	1916	1,47	10.989	87.00	0.0001
3	C16:0;Palmitic acid, methyl ester	75624	58.07	14.657	87.00	0.0000
4	C18:0; Stearic acid, methyl ester	12414	9.53	19.670	87.00	0.0002
5	C18:1c; Oleic acid, methyl ester	13481	10.35	20.216	97.00	0.0002
6	C18:1n 9T; Elaidic acid, methyl ester	845	0.65	20.457	97.00	0.0002
7	C18:2c; Linoleic acid, methyl ester	23066	17.71	21.822	97.00	0.0003

Table 2: Quantification results of fixed oil isolated from *D. lotus roots.*



Quantification results of fixed oil isolated from *D. lotus roots*

Figure 2: Antifungal activity of oil isolated from the roots of *D. lotus.*

amphotericin B were used as standard antibiotics to compare extract and fraction with it.

Cytotoxicity activity

A shallow rectangular plastic dish (22×32 cm), filled with artificial sea water was taken. The sea water was prepared with commercial salt mixture mixed with double distilled water. Brine shrimp (*Artemia salina* leach) eggs were hatched the dish. Dish was made unequally partitioned by using an artificial perforated device. About 50 mg of the eggs were sprinkled in to large compartment which becomes darken. Minor compartment was exposed to the ordinary light. After two days, nauplii were collected and removed by a pipette from lighted side. A sample of the compounds to be tested was prepared by dissolving 20 mg of each compound in DMF (2 ml). Three different stock solution i.e., 550, 50, and 5 mg/mL were transferred to 9 vials (three for every dilution were used for each test sample and LD_{50} is the average of the three values) with one vial containing DMF was reserved as a control. Solvent was allowed to evaporate keeping overnight. Two days, later when the shrimp larvae were ready, 1 mL of sea water and 10 shrimp were added to each vial (30 shrimps/ dilution) with a volume adjusted with sea water to 5 mL per vial. After 24 h, the numbers of survivors were counted using standard procedure [11-13]. The data was analyzed by the use of finny computer program to determine LD_{50} values (Figure 1).

Results and Discussion

GC-MS analysis

Table 2 presents the results obtained from GC-MS analysis indicating relative concentration of individual FAMEs based on the external standard method. Analysis was repeated three times and values of area and concentration are given in Table 2. Quantification of FAMEs was performed using three points calibration curve with R2 value less than 0.99 (R^2>0.99) in each case. Table 2 shows the Quantification results obtained from fatty acid standard mixture of 37 components while Table 1 is the GC-MS chromatogram of *D. lotus* roots oil with properly labeled signals of analytes detected (Figure 2).

Both the saturated and unsaturated FAMEs were detected in sample under investigations. Palmitic acid, methyl ester was found in highest concentration (58.07%) among the identified analyzes of interest which shows anti-inflammatory and antibacterial activities. Second FAME with higher concentration was Linoleic acid methyl ester (17.71%). Among the other FAMES with concentrations more than 1% were: Oleic acid, methyl ester (10.35%), Stearic acid, methyl ester (9.53%), Lauric acid, methyl ester (2.21%) and Myristic acid, methyl ester (1.47%) were found. Concentrations of Llaidic acid methyl ester were less than 1% (Table1). Fixed oil revealed moderate antibacterial activity against all test bacterial strain except *Staphylococcus aureus* and *E. coli* was found to be the most susceptible bacterial strain. However streptomycin (standard) exhibited comparatively better activity than oil. In case of antifungal activity, only low activities were observed against. *Candida albicans*, *Aspergillus flavus*, *Microspoum canis*, *Fusarium salani* and *Candida glaberata*. Interestingly oil showed no considerable toxicity in brine-shrimp lethality assay which indicates safety of oils for pharmacological use. This study highlighted potential of *D. lotus* to be investigated further for its antibacterial effects at cellular and molecular levels.

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