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Research Article

Phytochemical analysis and biological activities of *Diospyros lotus* L. fruit extracts

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Abstract

Diospyros lotus L. (Locally known as Amlok) is a common tree growing in the forests of Northern part of Pakistan. This fruit is small in size having blackish color and astringent taste. Qualitatively and quantitatively analysis of fruit was carried out for secondary metabolites like flavonoids, quercitin, alkaloids, saponins, tannins and total phenols by using standard methods. It was observed that *D lotus* contained higher amount of tannins as compared to flavonoids, phenols, alkaloids and other metabolites. The DPPH scavenging and Reducing power assays revealed that *D. lotus* fruit contained significant amount of antioxidants that might be helpful for reducing the heart diseases and carcinogenic infections in human population. Furthermore, antibacterial activities of fruits extracts against both Gram Positive and Gram Negative bacteria revealed that fruits of *D. lotus* have potential to provide defensive mechanism against broad spectrum infections causes by pathogenic microorganisms;

Keywords: antioxidant activity, antimicrobial activity, *Diospyros lotus*, secondary metabolites

1. Introduction

Diospyros lotus. L belongs to family Ebenaceae and it is originated from Balkans, Caucasia to China and Japan. It is commonly known as "*Amlok"* or "*Kala Amlok*" in Pakistan. Ripened fruits of *D. lotus* are blackish blue globes having 1.5-2 cm diameter. Maturity of fruit is easily determined by the color development that varies from green to yellow[1]. The consumption of un-ripened fruits is not suitable due to having sharp smell. However, after ripening of fruits different changes take place in phyto-chemistry that enhance taste and the quality of fruit[2]. Color become brown after ripening and contains 2 or 6 seeds per fruit[3].

According to information collected from available literature *D. Lotus* possess anti-diabetic, anti-septic and anti-tumor activities due to availability of various phyto-nutrients. Different research worker reported that *D. Lotus* contained significant amounts of flavonoids, phenols, tannins, spanins, triterpenoids and alkaloids.

Furthermore triterpenoids found in this fruit have anti-oxidant, anti-allergic and anti-cancer activities. Tannins present in persimmon (Amlok) are considered more efficient than tocopherol[4]. It was observed that tannins improved life style of hypertensive and strokes level was reduced by tannins in experimental animals. These persimmon tannins are 20 times more effective than other antioxidants like, vitamin E as reported[5]. It is reported that *D. lotus* contained sedatives, astringents, laxatives, nutritive, febrifuges, antitussives, antiseptics, antidiabetics and antitumors activities[6]. The fruits of *D. lotus* commonly use by local inhabitants for diarrhea, dry coughs and hypertension[7].

Therefore keeping in view the importance of this fruit present research work was carried out with following aims and objectives.

1) Qualitative and quantitative analysis of secondary metabolites for *D. lotus* fruit
2) Determination of antimicrobial activities of various fruit extracts.
3) Assessment of antioxidant activities of *D. Lotus* fruit extracts

2. Material and Methods

2.1 Sample collection and preparation

Fruit samples of *Diosypros lotus* were collected from different locations of Kotli Sattian areas for analysis. Samples were identified by expert taxonomist registered (voucher specimen) for future reference at ASL herbarium, Quaid-i-Azam University Islamabad Pakistan. Fruit sample were shadow dried followed by oven drying and subjected to fine grinding (40 mashes). Resultant fine powder was again dried at 37 °C in incubator to remove the moisture. Samples were preserved in clean bags with proper labeling of names and locations of samples and then stored at 4 °C for further analysis.

2.2 Extraction

Fifty grams of powder form of fruit samples were soaked separately for 48 hours in 200 ml distilled water, 50% (v/v) methanols, acetone and petroleum ether for aqueous, alcoholic, acetone and petroleum ether extraction, respectively. The soaked material was agitated at regular time intervals and after 48 hours the soaked material was filtered using muslin cloth followed by filtration using Whattman filter paper No 1. The final filtrates were collected and dried under room temperature. The extracts were stored at 4 °C until further uses.

2.3 Proximate Analysis

Analysis of *D. lotus* fruit for total proteins, crude fat, fiber, moisture, ash and carbohydrate contents were determined[8].

2.4 Assessment of secondary Metabolites

Qualitative and Quantitative analysis of secondary metabolites from fruit samples of *D. louts* were carried out for alkaloids, phenols, flavonoids, tannins and saponins by using methods reported[9].

2.5 Assessment of Quercetin

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Qualitative estimation of quercetin from the fruit of *D. lotus* was carried out by using method reported[10]. Briefly 100 mg of fruit sample was added in 20 mL volumetric flask containing 80 % ethyl alcohol and subjected to shaking followed by sonication for 10 minutes to extract quercetin. The solution was filtered with Whatman filter paper No. 42 and filtrate was collected in 15 ml falcon tubes and was stored at -20 °C until further analysis.

2.6 DPPH radical scavenging activity assay

Radical scavenging activity of fruit extract against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined using standard method[11]. The DPPH radical scavenging activities of various extracts of *D. lotus* were assessed. Total 100 µl of sample extract was added with 2.9 ml of DPPH reagent (0.1 mM in methanol) and mixture was vortexed vigorously. The absorbance of the mixture at ambient temperature was recorded for 60 minutes after every 10 minutes intervals. Gallic acid was used as a reference antioxidant compound. The absorbance of the remaining DPPH radicals was read at 519 nm using UV/Visible spectrophotometer (Bionate 5). Triplicate analysis was carried out to verify the results. The scavenging of DPPH radical activity was calculated according to the following equations

$$(\%) = [\{A_{control} - A_{sample}\}/\{A_{control}\}]x100$$

Whereas, $\mathbf{A}_{\mathbf{control}}$ is the absorbance of DPHH radical in methanol, $\mathbf{A}_{\mathbf{sample}}$ is absorbance of DPHH radical + sample extract/standard.

2.7 Reducing capacity

Concentrations of 0.01, 0.05 and 0.075 mg/ml of fruit extracts of *D. lotus* were mixed with 2.5 ml of 0.02M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide [K_3 Fe(CN)$_6$]. The mixture was then incubated at 50 ^oC followed by addition of 2.5 mL of aliquots of 10% trichloroacetic acid and finally absorbance was measured at 54 nm by using UV/Visible spectrophotometer (Bionate 5).

2.8 Preparation of extract for antimicrobial activity

The ground sample (80 mashes) was extracted with solvents like, n-hexane, chloroform, acetone ethanol, methanol and aqueous media. Extraction of sample with ethanol (1:10) was performed by shaking for 24 hours followed by centrifugation at 10,000 rpm for 15 minutes. Supernatants were shifted into pre-weighed falcon tubes and residue was further re-extracted with other solvents. The same process was repeated with all solvents and the extracts were dried in an incubator. The dried extracts were dissolved in dimethylsulfoxide (DMSO) for antimicrobial assay.

2.9 Microorganism Tested

The bacterial strains were cultured and maintained on Lauria-Broth media as reported[12]. The fruits extracts were tested against Staphylococcus *aureus* (ATCC 25923), *Streptococcus pyogenes* (19615), *Escherchia coli*, (ATCC 8739), *Klebsiella Pneumoniae* (ATCC 10031) and *Pseudomonas aeruginosa* (ATCC 9027) and standard antibiotic gentamicin as reported[9]. Antimicrobial activities of bacterial strains were tested by using well diffusion method. Preparation of all microbes inoculums was performed in Lauria-Broth in different test tubes which were put in shaking incubator at 37 °C for 24 hours containing 10^8 cfu/ml.

2.10 Statistical analysis

Data obtained was subjected to ANOVA for calculation of mean and standard values.

3. Results

3.1 Proximate Analysis of *D. Lotus* Fruits

Data regarding analysis of *D. Lotus* fruit for proximate contents are given in Table 1. Information regarding proximate parameters of fruits, vegetables and other foods are necessary for the quality, development and application of food for public health. Furthermore a reliable amount of water, ash, fiber total proteins, crude fat, fiber and carbohydrates plays important role for health of human and animals[13].

3.2 Estimation of phytochemicals from fruit samples

Analysis of *D.lotus* fruit extracts was carried out for various phytochemicals and results are presented in Tables 2-3 and Fig 1, According to results higher concentration of tannins (37.05 ± 0.61 to 41.49 ±0.64 %) followed by flavonoids (30.52 ±2.51 to 34.42 ± 2.89 %), phenols (16.05 ± 0.82 to 17.4 ±1.2 %), alkaloids (2.45 ±0.22 to 2.53 ±0.25 %) and saponins (0.90 ± 0.05 to 0.98 ±0.08 %). All phytoconstituents found in *D. lotus* fruit play an important function in the human body and necessary for better quality of fruits.

Tannins perform antimicrobial activity as they do not allow the microbes to connect with cell wall. Tannins help in protein transportation and also attach to polysaccharides in cell membrane. Furthermore, flavonoids are considered as the important source of antioxidants found in fruits and vegetables. The flavonoids also provide defensive mechanism for animals and plants against microbial infections[12]. Phenols play an important role to enhance growth of tissues and cells of human and animals. Phenols perform different functions in living organisms such as antioxidant and anti microbial activities and play protective role against infections. Alkaloids are important secondary metabolites and play vital role in defense mechanism of plants and also in human after consumption. Saponins help to reduce heart diseases[14,15,16].

Amount of quercetin (0.50 ± 0.01 to 0.55 ± 0.02 mg/ml) was found in fruit of *D. Lotus* (Table 3. Fig 2), quercetin is an important flavonoid and considered as essential components for many biochemical reaction Presences of above mentioned secondary metabolites in *D. Lotus* fruits indicates its importance in fresh or dry condition.

Table 1. Proximate analysis (%) of *D. Lotus* fruits

Sr No.	Moisture	Crude Protein	Crude Fat	Ash	Carbohydrate
1	71.5±0.5	2.5±0.6	4.6 ±0.5	1.5±0.5	19.9±0.3
2	69.3±0.2	1.8±0.2	3.7 ±0.6	1.2±0.8	24.5±0.7
3	72.6±0.4	2.6±0.3	4.3±0.8	1.6±0.4	18.9 ±0.6

Mean ± SD, triplicate analysis (n=3)

Table.2. Quantitative estimation (%) of Phytochemicals from *D. lotus* fruit extracts.

Sr. No	Flavonoid	Phenol	Saponin	Tannin	Alkaloid
1	34.42±2.89	16.91±0.84	0.98±0.08	41.49±0.64	2.53±0.25
2	30.52±2.51	17.41±1.2	0.90±0.05	37.05±0.61	2.45±0.22
3	36.91±2.96	16.05±0.82	0.95±0.06	40.12±0.63	2.50±0.24

Values are expressed in terms of Mean ± SD after triplicate analysis.

Table.3. Estimation of quercetin from *D. lotus* fruit samples

Sr. No	Quercetin (mg/ml)
1	0.052±0.03
2	0.050±0.01
3	0.055±0.02

Mean values ± SD, after triplicate analysis

3.4 Determination of Antioxidant Activities

3.4.1 DPPH Radical Scavenging Assay

1,1,diphenyl-2-picrylhydrazyl (DPPH) is considered as the best reported antioxidant assay[17]. The antioxidant activity of *D. lotus* fruit extracts (22.57 ± 0.59 %) as compared to Gallic acid (39.06 % ± 0.73) (Table 3, Fig. 2) . Phenols and flavonoids as antioxidant donate free radicals and get paired. Naturally occurring antioxidants are employed in food and pharmaceutical industries to manufacture relevant ingredients required for human health.

3.4.2 Reducing power assay

Reducing power (0.53 ± 0.2 µg/g GAE /g assay was evaluated capability of Fe^{3+} to Fe^{2+} (Figure 5). Therefore it could be predicted that antioxidant activities of *D lotus fruit* is due to higher phenolic contents. Several antioxidants including Alkaloids, flavonoids and phenolics, found in *D. lotus* and other fruit as reported by various authors.

IC50 (The half maximal inhibitory concentration, (50) is the concentration concerned drug or plant extract that inhibit the activity of a biological system, therefore IC50 values of D lotus (1.89 ± 0.5 µg /g as compared Gallic acid (1.04 ±0.3 µg/g) (Table 5).

Table 4 . Determination of DPPH free radical scavenging potential (%) of *D. lotus* fruit at 517 nm

Fruit extracts conc. (µg/ml)	*D. lotus*	Gallic acid
20	6.28±0.01	11.13±0.19
40	11.75±0.21	16.42±0.23
60	13.70±0.37	30.38±0.53

Results are obtained as triplicate analysis mean ± SD

Table. 5. IC_{50} and anti radical power assay of different fruits and Gallic acid

Samples	IC_{50} (µg)	ARP
Gallic acid	1.04±0.3	0.96±0.4
D. lotus	1.89±0.5	0.53±0.2

Mean values ± SD after triplicate analysis (n=3)

Fig 1. Graphical presentation of quercetin concentration from fruit samples

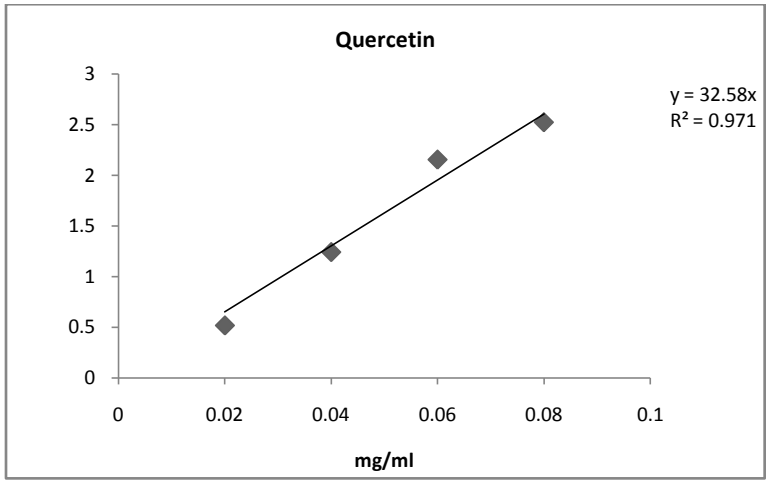


Figure 2.Comparsion of *D. Lotus* and Gallic acid for their antioxidant activity

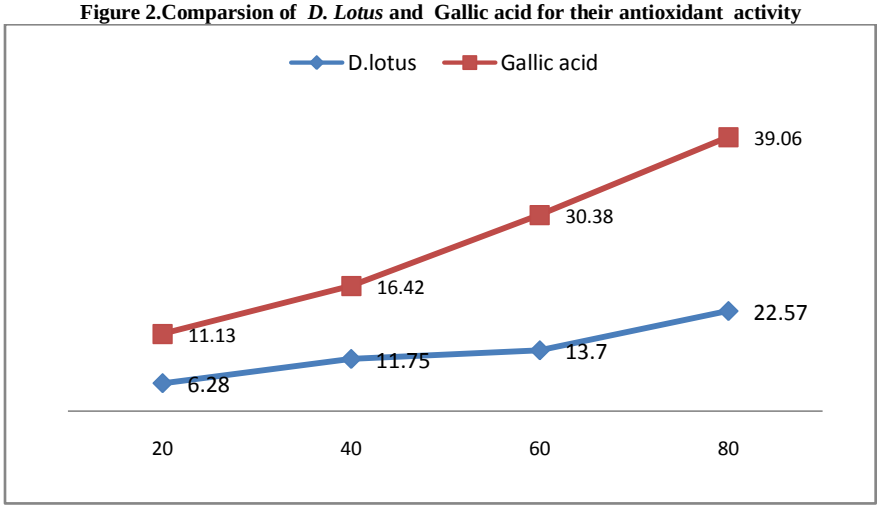
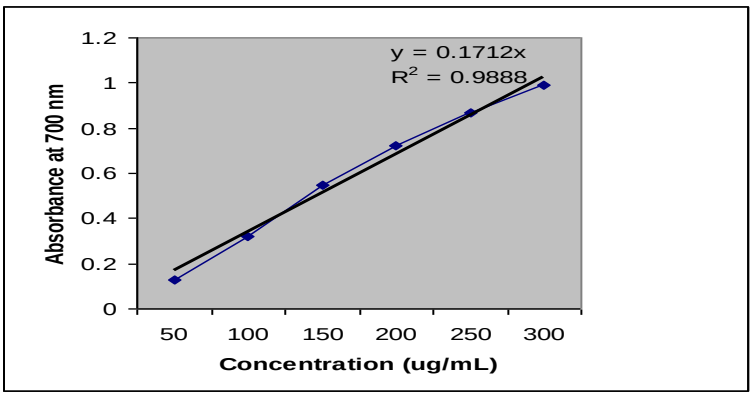


Figure 3. Reducing power assay of Methanolic extract of fruit extracts. Gallic acid was taken as standard. Values are in terms of Mean ±SD, after triplicate analysis.



3.5 Antimicrobial activity of fruit extracts

Antimicrobial activity of the extracts of *D lotus* in five different organic solvents and aqueous media were tested against Gram +ve and Gram –ve bacterial strains (Table 6). These results indicate that the different extracts of *D lotus* exhibit antibacterial activity, however, methanolic extracs have shown better results as compared to other extracts applied in this experiment Bacterial strains were tested by applying 50 µg/ml n-hexane ,chloroform, acetone ethanol and methanolic extracts for 24 hours by well diffusion assays. It was observed that extracts showed inhibition of growth of bacterial strains. Zone of inhibition of all tested samples for Gram +ve and Gram –ve bacterial strains were compared to gentamycin which is broad spectrum antibiotic. *Klebsiella pneumoniae* were found to be less susceptible as compared to other strains (Table 6). Water extract was found to be less effective nearly against all bacterial strains and exhibit less zone of inhibition for *Klebsiella pneumonia.*

3.6 Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of various *D. Lotus* fruit extracts against bacterial strains was determined .It was observed that most susceptible bacterial strain was *Staphyllococcus aureus* (0.3± 0.1mm) followed by *Staphyllococcus proggenes* (0.5±0.01 mm) against the concentration of 1mg/ mL of methanolic extracts of *D. Lotus* fruit (Table 7). All other bacterial strains showed comparatively higher susceptibility against fruit extracts.

Table 06: Antibacterial activity of *D. Lotus* fruit extracts

Strain	Diameter of zone of inhibition in mm							
	E.sativa seeds extracts (mg/ml)						Gentamycine	DMSO
	n-hexane	chloroform	Acetone	Ethanol	Methanol	Aqueous		
S.aureus (ATCC 25923	13.0±1.4	13.4±0.4	18.2±0.44	21.0±1.16	24.67±0.98	-	25.2±0.55	-
P. aeruginosa (ATCC 9027)	19.3±1.7	-	-	17.7±0.47	18.0±0.54	-	23.1±0.97	-
E. coli (ATCC 8739)	12.4±0.1	12.3±1.1	9.5±1.33	17.5±0.71	21.0±1.53	-	18.2±1.78	-
K.pneumonia (ATCC 10031)	-	-			11.0±1.56	-	21.1±2.56	-
S. pyogenes (ATCC 19615	11.6±2.1	14.1±0.34	5.89±2.14	12.0±4.48	12.5±1.21	13.0±1.2	24.76±0.55	-

DMSO, dimethyl sulfoxide. -, no inhibition.

Values are in terms of Mean±SD after triplicate analysis.

Table.7: Minimum inhibitory concentration (mg/mL) of various extracts of *D. Lotus*

Microorganisms	Diameter of zone of inhibition in %				
	Extract concentration (mg/ml)	Growth inhibition zone diameter (%)			
		n-hexane	chloroform	Ethanol	Methanol
S. aureus	10	-	12±0.99	-	9±0.9
	5	-	0.9±1.3	-	8±0.23
	1	-	0.5±1.22	-	0.3±0.1
P. aeruginosa	10	-	-	15±0.22	-
	5	-	-	12±0.11	-
	1	-	-	-	-
E. coli	10	13±1.4	20.3±1.1	17±0.71	18±1.53
	5	11±1.1	-	15±0.71	-
	1	-	-	10±0.66	-
K. pneumonia	10	19.3±1.7	-	12±4.48	11±1.56
	5	11±1.1	-	0.7±0.45	0.7±0.8
	1	0.9±0.7	-	-	0.6±0.2
S. proggenes	10	12.4±0.1	13±1.9	19±0.6	0.8± 0.06
	5	-	-	14±0.55	0.5± 0.01
	1	-	-	-	-

Values are in terms of Mean±SD after triplicate analysis.

4. Discussion

Dry fruits (ie *D.Lotus* plays important role in the health of human population might be due to availability of phyto chemicals like flavonoids, phenols, alkaloids tannin, saponins etc. Analysis of *D. Lotus* fruits in present study indicates that this fruit contained higher amounts of proximate parameters and secondary metabolites (Tables 1 and 2). Flavonoids as important secondary metabolites posses anti-inflammatory activity and provides remedy against different types of allergies, viruses and tumor infections [18, 19]. Studies on phytochemical analysis of *D. lotus* shows that it is rich in flavonoids, as reported[20]. Significant amount of phenols was found in fruit extract of *D. lotus* (Table 2). Phenols are receiving great consideration due to its antioxidant property exhibits anticancer, and anti tumor activities[20]. The higher level of tannins was found in *D Lotus* (Table 2) and values were comparable with those reported[20]. Tannins (water-soluble polyphenols), involved in accelerating blood clotting, decreases blood pressure, reduces serum lipid level, generate liver necrosis, and changing immune responses [21].

The values of saponins found in *D.lotus* fruit was lower than values of saponins reported[22]. Saponins are surface active phytochemical and important to reduce risk of coronary heart disease if present in diets . Quercetin, being a antioxidant inhibits low-density lipoproteins by oxidation in-vitro. Previously reported that ingestion of quercetin is not directly linked with mortality of coronary heart diseases[23].

DPPH scavenging potential (Table 4, Figs 3 and 4) represents antioxidants values of various fruits extracts, which was also confirmed after antioxidant power assay (Fig 5) and IC_{50} values of *D. lotus* (Table 5) where as similar results were also reported[11, 24].The analysis revealed that *D.lotus* fruit have antioxidants compounds and useful for human health .

The antimicrobial activities of n-hexane, chloroform acetone, ethanol, methanol and aqueous extracts of *D.Lotus* fruits, against selected ATCC strains of bacteria are presented in table 6. According to results higher inhibitory zones of *Stphylococcus aureus* (ATCC 25923) was obtained for methanolic fruit extracts (24.67 ±0.98 mm) followed by ethanol extract (21.0 ±1.13 mm) .,Where as lowest MIC value (Table 7) was obtained for *Stphylococcus aureus* (0.3 ±0.01 %) when 1mg/ml of methanol fruit extract was used . The results indicates antibacterial potential of *D. lotus* fruit that can provide valuable nutrients to protect human population from different infections.

Dried fruits are excellent source of polyphenol and phenolic acids. These compounds make up the largest group of phytochemcials in the diet and responsible for the potential benefit for human population after consumption. The scientific basis for the recommendation to increase fruit consumption in the diet by health authorities is the epidemiological evidence that individuals who regularly eat generous amounts of these foods have lower rates of cardiovascular disease, obesity , several cancers, diabetes and other chronic diseases. Dried fruits, with unique combination of essential nutrients, fiber and bioactive compounds are a convenient step toward healthier eating. Therefore more studies are recommended for the assessment of health benefits of *D. Lotus* fruit in molecular level so that active compounds could be isolated that might be suitable for pharmaceutical industries to develop new drug to control the human and animal infectious disease.

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