



Evaluation of Secondary Metabolites (Antibacterial and Antioxidant Activity) of Amlok (*Diospyros lotus L*) Fruit Extracts of Jammu Region

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Authors' contributions

This work was carried out in collaboration among all authors. Author AA designed the study, wrote the protocol, managed the literature searches and analysis of the study. Author JS also designed the study. Author FH wrote the first draft of the manuscript. Author MM performed the Microbial and statistical analysis. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2020/v32i4231049

Editor(s):

(1) Dr. Takashi Ikeno, National Institute of Mental Health, National Center of Neurology and Psychiatry, Japan.

Reviewers:

(1) Fitriyanti Jumaetri Sami, Sekolah Tinggi Ilmu Farmasi Makassar, Indonesia.

(2) Ali Jahanban-Esfahlan, Tabriz University of Medical Sciences, Iran.

(3) Khoirun Nisa, Indonesia.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/64450>

Original Research Article

Received 02 November 2020

Accepted 08 January 2021

Published 19 January 2021

ABSTRACT

Amlok (*Diospyros lotus L.*) fruit is growing in the hilly areas of jammu region of Jammu and Kashmir India. It is locally known as *amlok*. This fruit has black color and small in size and astringent taste. Analysis of the fruit was done quantitatively and qualitatively for various secondary metabolites such as total phenols, quercetin, flavonoids, alkaloids and tannins with the help of various standard methods. It was analyzed that the fruit contains the abundant amount of the tannins as compared to the rest of the metabolites. The antibacterial activity of the fruit extract shows it has great potential to provide defense against wide spectrum infections because of many pathogenic organisms. This fruit can provide defense against many diseases and carcinogenic infections because it contains the higher amount of the antioxidants as revealed with the help of the antioxidant power assay viz, DPPH Scavenging activity.

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Keywords: *Diospyros lotus*; secondary metabolites; antibacterial activity; antioxidant activity.

1. INTRODUCTION

Human body lives in an environment which is rich in oxygen, contains numerous reactive oxygen (ROS) from endogenous and exogenous sources. During normal operations, various processes like as hydrogen peroxide (H_2O_2) superoxide anion (O_2^-) and hydroxyl radical (OH.) create ROS within the body. Our body is protected with the help of the antioxidants and antioxidant enzymes against these free radicals which causes damage to our body. But if the concentration of the ROS increases than the antioxidants in our body. It causes ageing, irregular physiological functions or different human diseases [1,2]. Oxidative harm protection can be provided by dietary antioxidants. During the recent years most abundant dietary antioxidants are phenolic compounds in ordinary human diets. During recent years, based on promising evidence of their assumed role in preventing many human diseases, phenolics have received considerable attention [3,4]. It is believed that this beneficial effect is mainly due to its chelating activities, radical scavenging properties and antioxidant. Various plants have been examined for their phenolic and antioxidant activities and one of the strongest sources is the date plum persimmon [5,6]. The date plum (amlok) persimmon (*Diospyros lotus L.*) (DPP) is a domestic or native of China and Asia, belonging to the Ebenaceae family. It is cultivated in many countries for because of its edible nature. In traditional Chinese medicine the

fruits are febrifuges and are usually used to facilitate secretions and seeds as a sedative alternative. Nutritional constituents of the fruit have been measured, according to previous studies [7,8].

During the fruit processing of this plant, changes in the phenolic acid content were released [9]. Some antioxidant potential and Phytochemicals of the *Diospyros lotus L* in aqueous methanolic extract have been measured [5,10,11]. The purpose of this study is to determine the antioxidant activity and to evaluate the antibacterial, biochemical and phytochemical activity of the amlok fruit extract.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Samples of the fruit *Diospyros lotus* were collected from the many locations of the Bhaderwah area of the Jammu region for the analysis. Drying of the fruits was done in the shadow followed by the oven drying and finally grinding of the dried fruit samples. The fine powder obtained were dried again in incubator to remove the moisture at temperature of $37^\circ C$. The samples were subjected to the proper packaging in clean bags for preservation purpose, efficient labeling of names of the samples with locations were done. Finally the samples were stored at the low temperature of $4^\circ C$ for further analysis.



Fig. 1. Amlok (*Diospyros lotus*) fruit Fig. 2. Amlok fruit powder

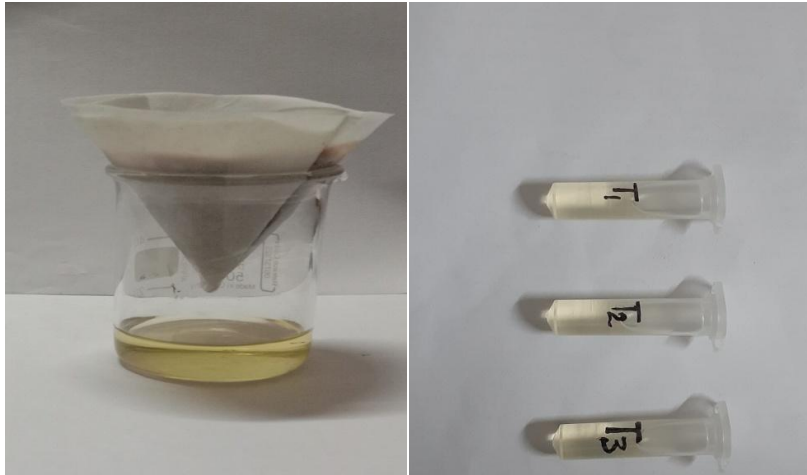


Fig. 3. Amlok (*Diospros lotus*) extract Fig. 4. Amlok extracts of three pulp samples (T₁,T₂, T₃) (T₁=Extract of sample 1, T₂=Extract of sample 2 and T₃= Extract of sample 3)

- Sample 1= Fresh Pulp
- Sample 2= Refrigerated pulp after 7 days of storage at 4°C
- Sample 3= Pulp after storage at room temperature for 7 days

2.2 Extraction of Sample

50 grams of powder form of fruit samples were soaked separately for 48 hours in 200 ml distilled water. 50 percent (v/v) methanol, petroleum ether and acetone for aqueous, acetone, alcoholic and petroleum ether extraction. At periodic time intervals, the soaked material was agitated and the soaked material was filtered using muslin cloth after 48 hours, then followed by filtration using Whatman Filter paper No. 1. The final extract were collected at room temperature and dried. The extracts were stored for further use at 4°C.

2.3 Physico-chemical Analysis

Estimation of (*Diospyros lotus L*) fruit pulp for TSS, Acorbic acid, total proteins, fibre, crude fat, ash, moisture and carbohydrate contents were determined [12].

T.S.S

Using an (Atago refractometer) hand refractometer (0-32°B),(TSS) total soluble solids were measured and expression of the results were indicated as degree Brix (°B). The readings were reported and corrected by adding the temperature variance correction factor.

Crude protein

Using the micro-Kjeldahl process, crude protein was estimated, using factor 6.25 to transform the content of nitrogen into crude protein.

In the Kjeldahl digestion flask, the digestion of (1.0 g) weighed sample with concentrated sulphuric acid (20 ml) and (10.0 g) digestion mixture. The cooling of the substance was done and transfer of the substance into a volumetric flask of 250 ml. Its volume was created with distilled water to the mark and mixed. Distillation flask was used to collect the measured aliquot, then followed by sodium hydroxide 40.0%, and collection of the ammonium borate was done in a flask containing 10 ml of 4% boric acid solution through a condenser. The titration of the distillate was 0.1 N sulphuric acid. Along with the samples, a blank sample was also run.

Per cent Nitrogen= (volume made X Titre value X0.00014)/ (Weight of sample (g)X Aliquot taken (g)) X 100

Carbohydrate [12]

The content of carbohydrates was determined by the differential method. Total sum of the percentage of fat, moisture, protein, ash and fiber content was calculated by subtracting it from 100.

Carbohydrate (%) =100- (protein + fat %+ % moisture % + ash%+ fiber %).

Crude Fat [12]

Using the soxhlet extraction method, crude fat was determined. The sample's fat content was easily extracted at 60°C to 80°C in organic solvent (petroleum ether) and followed for 6 h to reflux. The formula was used to measure the percentage of fat content.

$$\text{Percent fat content} = \frac{\text{Amt. of the ether extract (g)}}{\text{Wt. of the Sample (g)}} \times 100$$

$$W_2 - W_1 / W \times 100$$

Sample Weight-W (g)
Empty beaker Weight-W₁ (g)
Empty beaker Weight + fat content (ether extract) W₂ (g)

Ash [12]

In a pre-weighed silica crucible, one gram of moisture free sample was taken. Slow heating on fire to allow smoking off fat without burning was done by preliminary ashing. It was incinerated in a muffle furnace at 600°C ± 10°C for 8 hours until the smoke stopped evolving from the sample. The crucibles were removed and measured and cooled in a desiccator. The content of ash was measured from the weight in the crucible difference and expressed as a percentage.

Ascorbic acid [12]

The ascorbic acid reduces 2, 6-dichlorophenol indophenol dye into a colour less leuco-base and was calculated by the [13] process. The sample was collected in a 4% oxalic acid solution and titrated for 15 seconds with a regular pink colour dye. The findings were expressed as a sample of mg/100 g. Dye factor (mg of ascorbic acid per ml of dye as follows).

$$\text{Dye Factor} = \frac{0.5}{\text{Titre}}$$

$$\text{Ascorbic acid (mg/100 g)} = \text{Titre} \times \text{Dyefactor} \times \text{volume made up/ Weight of sample x Aliquot of extract taken} \times 100$$

Moisture content

Using an electronic moisture analyzer (Citizeon MB 50 C) at 105°C, the moisture content was measured. An approximately 2 g sample was spread over an aluminum sample holder and put in the analyzer. The sample was heated to 105°C and the loss of evaporative moisture as a

percentage of the moisture content was automatically recorded.

2.4 Secondary Metabolites Analysis

Qualitative and Quantitative analysis of secondary metabolites from fruit samples of *Diospyros louts* were carried out for alkaloids, phenols, flavonoids, and tannins by using methods reported [14].

2.5 Estimation of Quercetin

Qualitative estimation of quercetin from the fruit of *Diospyros lotus* was carried out. 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. Whatman filter paper no.42 were used to filter the solution and falcon tubes with capacity of 15ml were used to collect the filtrate then subjected to storage at -20°C till further analysis.

2.6 DPPH Radical Scavenging Activity Assay

The Radical scavenging behaviour of fruit extract against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was calculated using norm Approach [6]. The radical scavenging operations of DPPH for different extracts of *Diospyros lotus* was examined. A total of 100 µl of sample extract has been added along with the 2.9 ml of DPPH reagent (0.1 mM of methanol) and mixture were vigorously vortexed. At ambient temperature, the mixture absorbance was recorded every 10 minutes at intervals of 60 minutes. As a reference antioxidant compound, gallic acid was utilized. The absorptivity of the Using the UV/Visible spectrophotometer, remaining DPPH radicals were read at 519 nm (Labtronics LT-2900). Triplicate analysis was performed to check the Outcomes. Radical scavenging behavior (DPPH) was evaluated according to the following equations.

$$\text{Redical scavenging activity (\%)} = \frac{\text{Control OD (0min)} - \text{Sample OD (30min)}}{\text{Control OD (0min)}} \times 100$$

2.7 ABTS Free Radical Scavenging Activity

The activity (radical scavenging) of ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-6-sulfonic acid)] was examined according to the [15]

process. At 734 nm, absorbance values were calculated. The norm used was Trolox. The radical scavenging operation of ABTS percent was estimated with the following formula:

$$\text{Radical scavenging activity (Percent ABTS)} \\ = (\text{Acontrol} - \text{Asample}) / \text{Acontrol} \times 100$$

2.8 FRAP Ferric Ion Reducing Ability

Calculation value of FRAP was done spectrophotometrically at 593 nm. The FeSO₄ solution was used to construct a standard curve and the findings are described as $\mu\text{mol Fe (II)}/\text{g FW}$ [16].

2.9 Preparation of Extract for Antimicrobial Activity

With solvents such as n-hexane, chloroform, acetone ethanol, methanol and aqueous media, the ground sample (80 mesh) was extracted. Sample extraction with ethanol (1:10) was performed by shaking for 24 hours, followed by centrifugation for 15 minutes at 10,000 rpm. Supernatants were transferred into pre-weighed falcon tubes and other solvents were further re-extracted with residue. In all solvents, the similar method or procedure was repeated and the extracts were dried in an incubator. For the antimicrobial assay, the dried extracts were dissolved in dimethylsulfoxide (DMSO).

2.10 Microorganism Tested

As mentioned, the bacterial strains were cultivated and retained in Lauria-Broth media. The testing of the fruit extract were done against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherchia coli*, *Salmonella Spp.* and as stated, the standard antibiotic gentamicin [14]. Using the well-diffusion process, the antimicrobial activities of bacterial strains were checked. In Lauria-Broth, preparation of all microbe inoculums was carried out in various test tubes placed in a shaking incubator at 37 °C for 24 hours containing 108 cfu/ml.

2.11 Statistical Analysis

For the Calculation of mean and standard values, the data obtained was subject to ANOVA.

3. RESULTS AND DISCUSSION

Dry fruits (that is, *Diospyros lotus.L*) plays vital role in human population health due to the abundant availability of phytochemicals as shown by the physicochemical analysis presented in the Table 1). So there are a reliable amount of the moisture carbohydrates, ascorbic acid, crude fat, and crude fibre which plays an vital role in the health of the humans and animals. The findings are in accordance with the [9,10].

In the present study, *Diospyros lotus* fruits indicate that it contains higher amounts of the tannins (38.05 ± 0.51) followed by the flavonoids (32.40 ± 2.76) followed by the phenols (16.38 ± 1.2) and alkaloids (2.48 ± 0.22) among all the three samples. Tannins play role in the antimicrobial activity as they don't allow the microorganisms to attach with the cell wall. Tannins got attached with the polysaccharides in the cell membrane and helps in transportation of the proteins. Flavonoids are regarded as the important sources of the antioxidants and has a vital role in the defense against the various diseases. Phenols also play a major role in the defense mechanism against the various infections by acting as the antioxidant. Flavonoids are important secondary metabolites with anti-inflammatory activity and provides remedies for various types of allergies, viruses and infections of tumors [17,18]. Phenols have great potential due to its antioxidant property, receiving great consideration exhibits anticancer, and anti tumor activities [19]. The higher tannin level was found to be among all three samples of the *Diospyros lotus* than rest of the metabolites. Tannins (water-soluble polyphenols) that are involved in blood-acceleration Clotting, lowering blood pressure, decreasing the level of serum lipids, producing liver necrosis and changing immune responses [4].

3.1 Results are Obtained as Triplicate Analysis Mean \pm SD

The higher antioxidant activity of the *Diospyros lotus* fruit extracts is ($12.75 \pm 0.23\%$) of fresh pulp extract as compared to the T₃ which is ($5.26\% \pm 0.01$). The naturally occurring antioxidants are added to the food ingredients or in the various industries related to the food or pharmaceutical which are required for the growth and wellbeing of the humans. Comparison of DPPH free radical Scavenging potential (%) among three samples (T₁, T₂ and T₃) of *Diospyros lotus* for their antioxidant activity shown in Fig. 6.

Table 1. Proximate analysis (%) of *Diospyros lotus* fruit pulps (three samples)

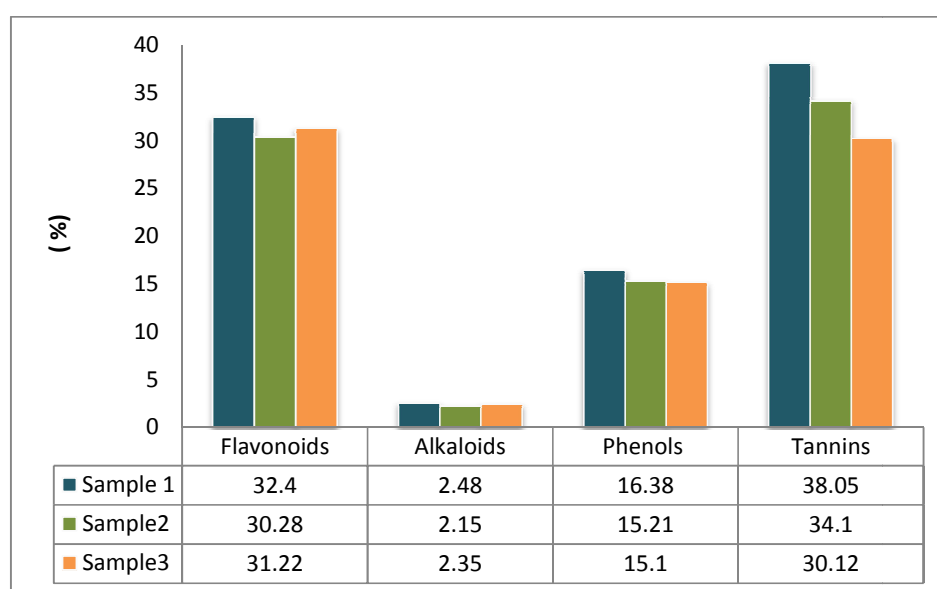
Sr.No.	TSS °B	Protein	Crude Fat	Carbohydrate	Ash	Moisture	Ascorbic Acid mg/100gm	Fibre (g)
(Sample) 1 (Fresh Pulp)	13±0.5	2.0±0.8	4.2±0.5	22±0.3	1.7±0.5	70.5±0.5	12.9±0.5	1.33±0.5
(Sample) 2 (Refrigerated pulp after 7 days of storage at 4°C)	12±0.2	1.90±0.6	4.0±1.0	21±0.2	1.5±0.3	68.4±0.8	11.2±0.2	1.25±0.3
(Sample) 3 (Pulp after storage at room temperature for 7 days)	10±0.6	2.5±0.4	3.90±0.2	18±0.6	1.2±0.2	65.2±0.6	13.6±1.3	1.05±0.2

Mean ±SD, Triplicate analysis (n=3)

Table 2. Flavonoids, Alkaloids, Phenols and Tannins content in the three samples of the (*Diospyros lotus.L*)

Sr.No.	Flavonoids	Alkaloids	Phenols	Tannins
Sample 1 (T ₁) (Extract of Fresh Pulp)	32.40±2.76	2.48±0.22	16.38±1.2	38.05±0.51
Sample 2 (T ₂) (Extract of refrigerated pulp after 7 days of storage at 4°C)	30.28±1.90	2.15±0.18	15.21±1.0	34.10±0.21
Sample 3 (T ₃) (Extract of pulp after storage at room temperature for 7 days)	31.22±1.80	2.35±0.16	15.10±1.1	30.12±0.41

Values are expressed in terms of Mean ± SD after Triplicate analysis

**Fig. 5. Comparison of Flavonoids, Alkaloids, Phenols and Tannins content in the three samples of the (*Diospyros lotus.L*)****Table 3. Analysis of DPPH free radical Scavenging potential (%) of *Diospyros lotus* (T₁ is extract from fresh pulp, T₂ is extract from refrigerated pulp after 7 days of storage at 4°C and T₃ is extract of pulp after storage at room temperature for 7 days) fruit extracts at 519 nm**

Samples	Fruit extract Conc.(µg/ml)	DPPH free radical Scavenging potential (%)
T ₁	30	12.75 ±0.23
T ₂	30	7.20 ± 0.11
T ₃	30	5.26±0.01

The scavenging potential of DPPH reflects the antioxidant values of different fruit extracts, Confirmed values of *Diospyros lotus* after the antioxidant power assay, where similar findings have also been reported by [6] [20]. Analysis showed that the fruit of *Diospyros lotus* has

antioxidant compounds that are useful for human health.

The amount of the quercetin is (0.050±0.02 to 0.045±0.01 mg/ml) among all the three samples the (*Diospyros lotus L*) fruit samples as shown in

the Table 4. Quercetin plays an important in the many biochemical reactions.

Quercetin, being an antioxidant, inhibits lipoproteins of low density by means of In-Vitro oxidation. It has previously been reported that quercetin intake is not directly associated with mortality from coronary heart disease [21].

Table 4. Quercetin estimation from *Diospyros lotus* fruit samples (T₁ is extract from fresh pulp, T₂ is extract from refrigerated pulp after 7 days of storage at 4°C and T₃ is extract of pulp after storage at room temperature for 7 days)

S.no.	Quercetin (mg/ml)
Sample 1 (T ₁)	0.050±0.02
Sample 2 (T ₂)	0.048±0.04
Sample 3 (T ₃)	0.045±0.01

Mean values± SD, after triplicate analysis

Largest FRAP and ABTS values of *Diospyros lotus* in the current analysis were found to be 552.6 µmol/g in sample 3 (T₃) and 50.6% respectively than rest of the samples [22] these findings are related to the dry powder.

Antibacterial activity of the extracts of the *Diospyros lotus* were examined indifferent solvents against Gram-Positive and Gram-negative bacterial strains as shown in the Table 6. As depicted from the results that different extracts of the *Diospyros lotus* fruit shown antibacterial activity. The better results were shown by the methanolic extracts. It was seen that the extracts show the inhibition of the growth of the bacterial strains. The zones of the inhibition of all the samples for (Gram-positive and Gram-negative) bacterial strains were compared with the genatamycin. Genatamycin is a broad spectrum antibiotic.

Table 5. Analysis of ABTS and FRAP among three samples (T₁ is extract from fresh pulp, T₂ is extract from refrigerated pulp after 7 days of storage at 4°C and T₃ is extract of pulp after storage at room temperature for 7 days)

S.no	ABTS (%)	FRAP (µmol/gm)
Sample 1 (T ₁)	42.2±0.06	540.2±18.0
Sample 2 (T ₂)	48.4±0.04	545.5±14.0
Sample 3 (T ₃)	50.6±0.08	552.6±12.0

Mean values± SD, after triplicate analysis

DMSO, dimethyl sulfoxide. -, no inhibition.

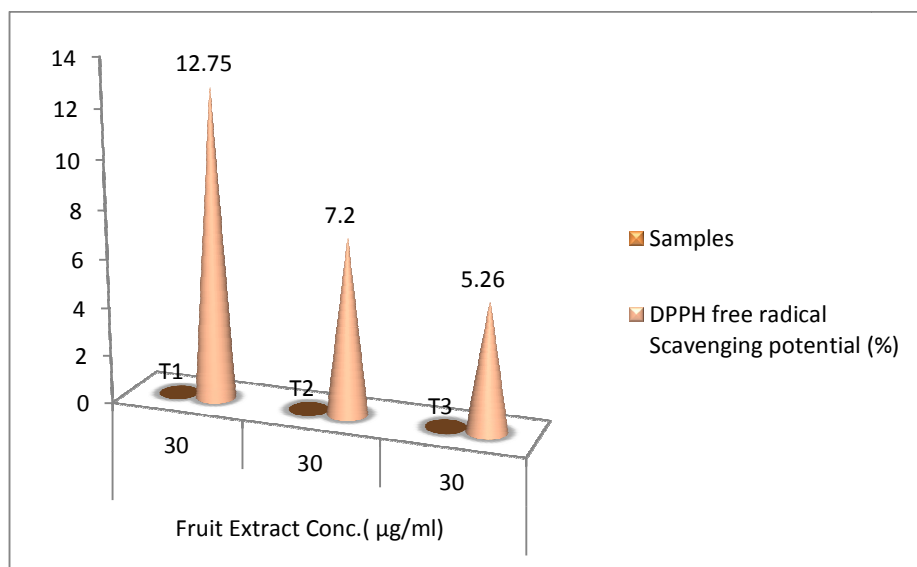


Fig. 6. Comparison of Three samples of *Diospyros lotus* for their (antioxidant activity) DPPH free radical Scavenging potential (%) (T₁ is extract from fresh pulp, T₂ is extract from refrigerated pulp after 7 days of storage at 4°C and T₃ is extract of pulp after storage at room temperature for 7 days) at 517 nm

Table 6. Antibacterial activity of *Diospyros lotus* fruit extracts

Diameter of Zone of inhibition in mm							
<i>Diospyros lotus</i> fruit extracts (20 mg/ml)							
Microbial Strains	n hexane	Chloroform	Ethanol	Methanol	Acetone	Gentamycine	DMSO
<i>S.aureus</i>	11.0±1.2	12.2±0.6	20.0±1.14	22.67±0.90	17.1±0.40	24.1±0.40	-
<i>S.pyrogenes</i>	10.6±2.0	13.1±0.11	11.0±3.48	11.0±1.20	4.80±4.14	22.70±0.45	-
<i>Salmonella spp.</i>	18.5±1.4	-	16.5±0.45	17.0±0.50	-	22.1±0.80	-
<i>E.Coli</i>	10.2±0.1	11.21±1.1	16.5±0.61	20.0±1.50	7.5±1.30	16.2±1.70	-

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

Table 7. Minimum inhibitory concentration (mg/mL) of various extracts of *Diospyros lotus*

Microorganisms	Inhibition zone diameter in (%)				
	Extract Concentration (mg/ml)	Growth inhibition zone diameter (%)			
		n hexane	Chloroform	Ethanol	Methanol
<i>S.aureus</i>	20	-	11±0.90	-	8 ±0.8
	15	-	0.7±1.21	-	7±0.20
	10	-	0.3±1.17	-	0.2±0.1
<i>S.pyrogenes</i>	20	11.4±0.1	12±1.8	18±0.4	0.7±0.04
	15	-	-	12±0.50	0.3±0.01
	10	-	-	-	-
<i>Salmonella Spp.</i>	20	-	-	14±0.20	-
	15	-	-	11±0.10	-
	10	-	-	-	-
<i>E.Coli</i>	20	12±1.2	18.2±1.1	16±0.70	17±1.50
	15	10±1.1	-	14±0.70	-
	10	-	-	8±0.60	-

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

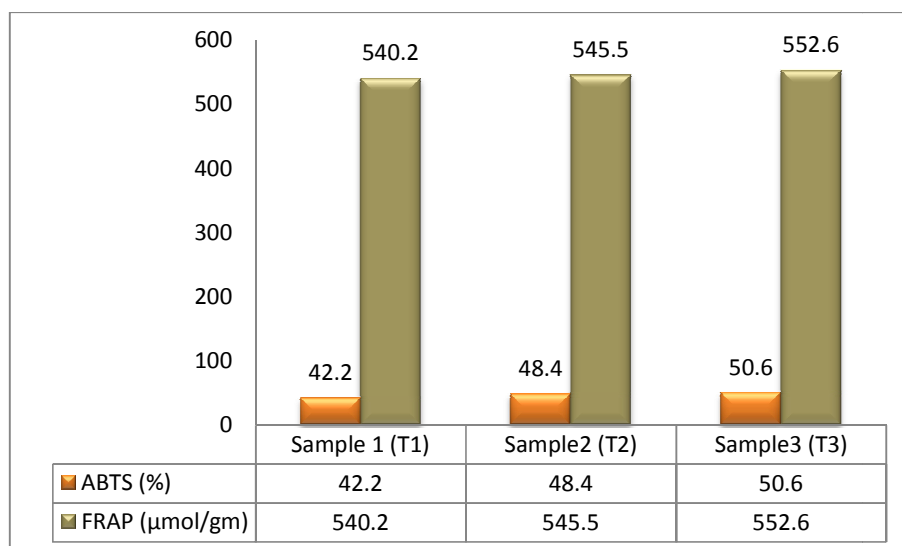


Fig. 7. comparison of ABTS and FRAP among three samples (T₁ is extract from fresh pulp, T₂ is extract from refrigerated pulp after 7 days of storage at 4°C and T₃ is extract of pulp after storage at room temperature for 7 days)

The findings indicate that there are antibacterial properties of *Diospyros lotus* fruit. In order to protect the human population from various infections, this fruit can provide valuable nutrients. Dried fruits are magnificent source of phenolic acids and polyphenols. The largest group of phytochemicals in these fruits can be accountable for the potential benefit of the human population. Scientific basis for the recommendation to Increasing the consumption of fruit in the diet by health authorities is epidemiological evidence that people eat generously on a regular basis. Such foods have lower rates of obesity, cardiovascular disease, diabetes, various cancers and other chronic diseases. Fruits dried, fiber and bioactive compounds are a convenient step towards healthier eating with unique combinations of essential nutrients. Consequently, more for the assessment of the health benefits of *Diospyros lotus*, studies are recommended. At molecular-level *Diospyros lotus* fruit is a good source of active mixtures or compounds that could be appropriate for the development of new drugs to control human and animal infectious diseases in the pharmaceutical industry.

4. CONCLUSION

In the present study, it is apparent that (*Diospyros lotus L*) fruits of Jammu region contain several secondary metabolites, like flavonoids, phenols, tannin, alkaloids. Among all the three samples the T₁ sample (fresh pulp

sample) has the highest content of the tannins then flavonoids followed by phenol and alkaloids. This sample also shows the highest DPPH free radical Scavenging potential (%). These findings showed that the fruit possess good antioxidant activity. Tannins play role in the antimicrobial activity, flavonoids are regarded as the important sources of the antioxidants and play an important role in the defense against the various diseases. Phenols also impart a magnificent role in the defense mechanism against the various infections by acting as the antioxidant. The scavenging potential of DPPH reflects the antioxidant values of different fruit extracts showed that this fruit has great potential in fighting against various infections and diseases. Quercetin, being an antioxidant, inhibits lipoproteins of low density by means of In-Vitro oxidation. The FRAP and ABTS values are also in effective range. *Diospyros lotus* contains Antibacterial activity and is effective against Gram +ve and Gram -ve bacterial strains. So this fruit has the highest potential to become a well and cost effective source of the natural antioxidants. Hence *Diospyros lotus* fruit is contains ample active compounds that could be appropriate for the development of new drugs to control human and animal infectious diseases in the pharmaceutical industry.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

We acknowledge financial support from the CSIR (Council of Scientific and Industrial Research (CSIR) fellowship with file number mentioned in the award letter 09/1238/ (0002)2K19 EMR-I. Authors would like to thank division of food science and technology and school of biotechnology SKUAST-Jammu, for their cooperation and technical assistance in this research. We also thank regional horticulture research substation SKUAST-Jammu, bhaderwah (J & K) India for providing the fruits.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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