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PHYTOCHEMICAL SCREENING, ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF *DOUEPIA TORTUOSA* CAMB., A CRUCIFER ENDEMIC TO PAKISTAN

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Abstract

Douepia tortuosa (*D. tortuosa*) has not been evaluated for its pharmacological significance prior to this. This study was aimed to identify bioactive compounds occurring in water (AQE), methanol (ME), ethyl acetate (EAE) and *n*-hexane (HE) extracts of *D. tortuosa* and to evaluate its antimicrobial and antioxidant potential. The microscopic analysis of the plant powder presented characteristic starch granules and calcium oxalate crystals. The ME presented the best extraction yield value campared to the other solvents. Maximum soluble phenolics content, 793.24 µg GAE/g was found in EAE. The extracts were screened out for the presence of bioactive compounds by using Fourier-Transform Infrared Spectroscopy (FT-IR) and Gas Chromatography–Mass Spectrometry (GC-MS). The FT-IR spectrum of various extracts indicated the dominancy of N-H, C-H, C=O, N-O and C-N functional groups. The GC-MS profiling indicated the presence of a spectrum of substantial medicinal compounds in various extracts including Squalene, Heptacosane, Cis-vaccenic acid, Octadecanoic acid, γ-Sitosterol, vitamin E, Tetracontane, Palmitoleic acid and Dotriacontane. The ME was highly effective antibacterial agent against *K. pneumoniae* whereas, EAE showed strong antibacterial activity against *A. baumannii* and *E. coli*. The EAE and AQE were the best antifungal agents against *A. flavus* and *A. niger*. According to IC50 values the AQE (32.422 µg/mL) and EAE (31.6895 µg/mL) has significantly comparable antioxidant activity with standard ascorbic acid (26.917 µg/mL). The plant can be explored in future for the isolation of antimicrobial, antioxidant and anticancer compounds with promising effects.

Key words: Medicinal plants; *Douepia tortuosa;* GC-MS; FT-IR; Phytochemicals; Microscopic analysis.

Introduction

The synthetic antibiotics are extensively used for treating infectious diseases. However, recently it has been identified that microbial strains can develop resistance to these antibiotics (Miethke *et al*., 2021). Therefore, the use of plant-derived therapeutic agents for treating pathogenic diseases is a plausible approach (Aschale *et al*., 2022). Medicinal plants contain secondary metabolites ensuring their survival under harsh climatic conditions and preventing them from the attack of pathogens. These secondary metabolites can be exploited as potential therapeutic agents against pathogenic microbes (Aryal *et al*., 2022). Plant extracts have been used as antibacterial, antifungal, antioxidative and anticancer drugs in a number of investigations (Ramasamy *et al*., 2022). Moreover, phytochemicals in medicinal plants have strong antioxidative properties and can be used as alternatives to synthetic antioxidants (Yu *et al*., 2021). Parts of medicinal plant such as roots, stems, leaves and seeds have been shown to possess antibacterial and antioxidant activities (Sadaf *et al*., 2021).

Pathogenic bacteria cause harmful infections and widespread problems such as abdominal and extra abdominal conditions in human beings (Sarowska *et al*., 2019). The *Acinetobacter baumannii* is reported to be a notorious microbe causing blood stream infection and pneumonia in human (Naing *et al*., 2022). The *Klebsiella pneumoniae* has been reported to cause infections of respiratory passages, gastrointestinal tract and urinary tract (Effah *et al*., 2020). The *Staphylococcus epidermidis* is reported to cause bronchopulmonary dysplasia and white matter injury (Joubert *et al*., 2022). Fungi of the genus *Aspergillus* are associated with a large variety of clinical manifestations ranging from allergic reactions to life-threatening infections. Moreover, there are reports of *Aspergillus niger* to cause respiratory tract infections. The *Aspergillus flavus* has been reported to results in aspergillosis in human being (Palmieri *et al*., 2022).

Oxidation is a basic process of energy production controlled by cells through different strategies called antioxidant defense system (Halliwell & Gutteridge, 2015). However, the higher production of reactive oxygen species (ROS) inside the cells results in many diseases such as cancer, heart problems and nerve disorders (Lai *et al*., 2010). Human body is unable to synthesize antioxidants and obtain them from external sources along with food. To fulfill the demand of antioxidants many synthetic compounds like butylated hydroxyl toluene and butylated hydroxy anisole are extensively used as synthetic antioxidants. These synthetic antioxidants are thought to be carcinogenic causing liver cancer (Lin *et al*., 2007; Song *et al*., 2010). Therefore, antioxidants, found in natural sources (plants) are of great interest to researchers. Recent research has demonstrated that phenolics can behave as powerful plant-derived anti-oxidants (Hossain *et al*., 2022). It's worth noting that phenolic content and antioxidant potential in medicinal plants have a strong relationship (Lyu *et al*., 2022).

On the basis of ethno-pharmacological information, scientists have been working on the discovery of new compounds from highly medicinal plants. Such compounds are then tested in laboratories for potential antioxidant, antimicrobial and cytotoxic activities in order to link traditional herbal therapeutic system with modern scientific evidences (Kumar *et al*., 2019; Zahir *et al*., 2021). Pakistan is home of more than 6000 species of higher plants of which 600-700 are highly medicinal

(Shinwari, 2010; Ahmed *et al*., 2014). Due to occurrence of sufficient number of medicinal plants in the region traditional therapeutic system is well established in Pakistan. However, no sufficient scientific data is available about the systematic investigation of medicinal plants by using modern and advanced scientific tools.

The plant species *D. tortuosa* is endemic to Pakistan and not listed either threatened or endangered. It belongs to family Brassicacea. It is a perennial plant species found in Khyber Pakhtunkhwa and Punjab provinces of Pakistan. Plant body is 30-75 cm tall, glabrous, glaucous perennial with woody root. Upper leaves are small, narrowly oblong, 2-5 cm long, 0.5-2.5 cm broad, sessile or sub-sessile. Lower leaves are 5-15 cm long, 2-6 cm broad, elliptic-oblong, shortly stalked, usually acute, entire fleshy, glabrous. Flowers are pinkish or violet in color and are 10-15 mm across. Flowering season is July to October (Jafri, 1973). Very little information is available about the medicinal value of this plant species. Therefore, this study was aimed to determine phytochemical composition, antimicrobial and antioxidant significance of *D. tortuosa*.

Material and Methods

Plant collection: The plant, *D. tortuosa* was collected from its natural habitat in Speena Moor Tehsil Banda Daud Shah (Latitude: 33.4 N, Longitude: 71.45 E and Altitude: 415.00 m/1361.55 ft) District Karak, KP, Pakistan and authenticated taxonomically at the herbarium of University of Science and Technology Bannu, Pakistan. The voucher specimen number Dt-D2 was assigned to it for future reference. This plant species is abundant in this locality and not declared either threatened or vulnerable. The whole plant material was first washed with tape water in order to remove dust particles and then spread onto blotting papers for air drying in shade at 35ºC for 15 days. After complete dryness to constant weight, it was powdered with an electric blender. The fine powder material was passed through a 1 mm sieve, stored in dark bottles at 4ºC.

Powder microscopy: The powder drug microscopy was performed with slight modifications to the method of Pal *et al*. (2018). A small quantity of the fine powder was applied to a glass slide and wet with distilled water. The sample was stained with equal quantity of phloroglucinol-HCl and safranin. A drop of glycerin was applied on the glass slide and the sample was observed using light microscope (Labomed, USA).

Preparation of crude extracts: The crude extracts for water or aqueous (AQE), methanol (ME), *n*-hexane (HE) and ethyl acetate (EAE) were prepared by dissolving 50g of plant powder in 1L of each solvent in dark plastic bottles for three days with constant shaking. The extracts were sieved with muslin cloth followed by further filtration using filter paper (Whatman #1). The solvents were rotary-evaporated (Rotary Model; RE301, Japan) to obtain the respective crude extracts (Ain *et al*., 2022).

Plant extraction yield: The AQE, HE, ME and EAE were weighed and their yield percentages were determined.

$$\text{Extract yield (\%)} = \frac{\text{Weight of dry extract}}{\text{Weight of leaf powder}} \times 100$$

Qualitative analysis of phytochemicals: Various extracts of *D. tortousa* were analyzed for the presence of phytochemicals like flavonoids, coumarins, saponins, tannins, terpenoids, alkaloids and anthraquinones according to standard protocols (Harborne, 1973; Wagner *et al*., 1984; Trease & Evans, 1985; Sofowora, 1993; Fahad & Bano, 2012).

Determination of total phenolic (tsp) content: The method of Swain & Hillis (1959) was implemented to determine the total phenolics content of various organic extracts of *D. tortuosa.* A 1 mg/mL of each extract was dissolved in respective solvent. Folin-Ciocalteau reagent was prepared in distilled water according to the standard protocol. In the next step a 250 µL from 1mg/mL of each extract prepared in respective solvent was mixed with 250 µL of Folin-Ciocalteau reagent and 2.5 mL of 7.5% of Sodium bicarbonate. The whole mixture was kept 90 minutes in dark. A blank was prepared by mixing Folin-Ciocalteau reagent with 7.5% of sodium bicarbonate and pure solvent in which the plant extract was prepared. The absorbance was taken at 765 nm of Spectrophotometer (SP-300, Japan) against the blank.

FT-IR analysis of extracts: The FT-IR analysis was performed for methanol, *n*-hexane and ethyl acetate extracts. The 10mg of each extract was compressed with 100mg of KBr pellet, to obtain luminous sample discs. The respective discs were separately loaded in FT-IR spectroscope (Shimadzu, Japan). The scan range was maintained at 400 to 4000 cm^{1} with a resolution of 4 cm^{-1} (Visveshwari *et al*., 2017).

GC-MS analysis: Methanol, *n*-hexane and ethyl acetate extracts were analyzed by GC-MS. The 20 mg from each extract were dissolved in 2 mL dichloromethane and filtered with 0.45 µm PTFE filter. Thermo Scientific Trace 1310 GC system was used for chromatographic separation. N5 grade Helium gas at the rate of 1.41 mL/min was used as career gas; splitless injection for 1.0 min; split and purge flow were 5.6 and 30 mL/min respectively; sample injection volume and injector temperature were 1 µL and 270°C respectively. Agilent HP-5MS (5%-phenyl)-methyl-polysiloxane) column (30m length × 0.25 mm internal diameter and 0.25 µm film thickness; GC temperature program was 40°C for 1 min, 10°C/ min ramping, 300°C final temperature and hold for 7 min. Thermo Scientific ISQ 7000 LT Single Quadrupole, MS system was used for mass spectral analysis. Ionization mode was Electronic Impact (EI); transfer line temp was 300°C; solvent delay time 7.5 min; Electron energy 70 eV; Ionization polarity was positive; Mass resolution was one mass unit; scan range/ time was 75- 550 m/z in 0.2 sec. The percent peak area and retention time of respective phytochemical contents of the extracts were noted. The spectra were matched for identification with already available NIST-20 library.

Antibacterial activity: Antibacterial tests were performed using the agar well diffusion method (Mbata *et al*., 2008). The nutrient agar media, pipette tips, glass plates (9 cm diameter) and wire-loop were autoclaved as per standard procedure. A 15 mL of the hot agar was solidified per Petri

plate and the bacterial pathogens viz. *Klebsiella pneumoniae* (MTCC 618), *Escherichia coli* (ATCC 25922) and *Acinetobacter baumannii* (ATCC 17978) were speckled uniformly on the surface of agar with sterile wire-loop. Six (6) bores were pierced in each media plate using autoclaved borers (6 mm diameter). A 67 µL of each extract solution (30 mg/mL concentration) prepared in Dimethyl Sulfoxide (DMSO) was added to the respective well. Erythromycin (0.5 mg/mL in DMSO) and pure DMSO were specified as positive and negative control respectively. The whole procedure was preceded in a laminar flow cabinet. All the Petri plates were incubated at 37°C for 24 hours. The bacterial growth inhibition was measured in mm.

We also determined relative bacterial growth inhibition percentage of the extracts with respect to tested antibiotic (Naz *et al*., 2011; Naz *et al*., 2013).

$$\text{Relative percentage inhibition} = \frac{X - Y}{Z - Y} \times 100$$

X represents total area of bacterial growth inhibition by the extract, Y denotes total area of bacterial growth inhibition by the solvent (negative control) and Z represents total area of bacterial growth inhibition by the antibiotic (positive control).

$$\text{Total area of bacterial growth inhibition} = \pi r^2$$

R represents radius of inhibition zone.

Antifungal activity: The antifungal test was carried out according to the approach of Washington & Sutter (1980); Naz *et al*., (2017) which involve the use of an agar tube dilution method. Twelve mg/mL of each extract was prepared in Dimethyl Sulfoxide (DMSO). Glass tubes and Malt Agar media were autoclaved. Six (6) mL of the Malt agar was dispensed in to each glass tube containing 1mL of the respective extract solution. The Malt agar was let to solidify with oblique slants and the fungal spores were inoculated on the uppermost 3 mm surface of the media. The tubes were incubated at 37°C for 6 days. Terbinafine was used as standards antifungal agent for comparison. The following formula was implemented to estimate the antifungal activity;

$$\text{Inhibition of fungal growth (\%)} = \frac{(100 - \text{Linear growth in treatment (mm)})}{(\text{Linear growth in control (mm)})} \times 100$$

DPPH antioxidant activity: The DPPH (1, 1-biphenyl, 2-picrylhydrazyl) assay was implemented to determine the total antioxidant capacity of the extracts (Brand-Williams *et al*., 1995). A solution of DPPH (5 mg/100 mL of methanol) was prepared. Its OD was adjusted in the rage of 0.7-0.9 at 517 nm. Stock solutions of 500 µg/mL of the plant extracts were prepared in respective solvents. Each stock solution was subjected to serial dilutions of 400, 300, 200 and 100 µg/mL. A 100 µL of the plant extract (prepared in respective solvent) with 3 mL of the DPPH solution at concentrations of (100–500 µg/mL) were subjected to evaluation for antioxidant potential. The pure respective solvents were correspondingly used as blanks. The solutions were shaken well and kept in dark for half an hour, followed by taking its absorbance at 517nm of Spectrophotometer (SP-300, Japan).

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Absorbance of control} - \text{Sample absorbance})}{(\text{Absorbance of control})} \times 100$$

Data analysis: The analysis of variance (ANOVA) and least significant difference tests were used to analyze the data. For each treatment replicate, the standard error values were recorded. For calculating IC50 values linear regression analysis was done using an internet tool (MLA-"Quest Graph IC50 calculator". AAT Bioquest, Inc., May 23, 2022, https://www.aatbio.com/tools/ic50-calculator) (Lisowska *et al*., 2019).

Results and Discussion

Powder microscopy: This is the first ever study reporting *D. tortuosa* as a crude drug based upon the evaluation of its biological activities and phytochemical profiling. The microscopic analysis of whole plant powder indicated the presence of a large number of starch granules, mostly rounded in outline and stained bluish with iodine. Higher density of transparent and prismatic calcium oxalate crystals was observed. Straight trichome rods, broken xylem vessels and epidermal tissues with intact stomatal apparatus were the characteristic features of powder drug material of *D. tortuosa* (Fig. 1). Evaluation of organoleptic, macroscopic and microscopic characteristics are pivotal for the correct identification and standardization of crude drugs (Nafees *et al*., 2022).

Plants extraction yield: Extraction is an important step to recognize, isolate and recover compounds found in plants. The extraction method, phytochemicals present and solvent used strongly effects extraction yield of a plant material (Ngo *et al*., 2017). Therefore, the aqueous (AQE), *n*-hexane (HE), methanol (ME) and ethyl acetate (EAE) extracts of *D. tortuosa* were weighed and their yield percentages were determined (Fig. 2). The maximum extraction yield (4.32%) was obtained in methanol and the minimum extraction yield 3.4% was obtained in water. Out of the four solvents tested methanol resulted in higher extraction yield which might be because of differences in polarity of the tested solvents (Truong *et al*., 2019). This study also indicated that *D. tortuosa* contains a reasonable amount of polar compounds which were readily soluble in methanol. These findings are in consistent with extraction yield of other medicinal plants (Do *et al*., 2014; Kuppusamy *et al*., 2015).

Preliminary phytochemicals analysis of the extracts: Phytochemicals tests of the ME, AQE and EAE showed the

presence of flavonoids, coumarins, saponins, tannins and terpenoids. The anthraquinones and alkaloids were absent in all the extracts. The HE was positive only for the presence of terpenoids and lacked flavonoids, coumarins, saponins, tannins, alkaloids and anthraquinones (Table 1). Phytochemicals like flavonoids, phenolics, saponins, coumarins, tannins and terpenoids are of prime importance while evaluating the quality of plant extracts for their medicinal value (Moreira *et al*., 2008; Naz *et al*., 2020; Duraipandiyan & Ignacimuthu, 2011).

Total soluble phenolics content: Maximum total soluble phenolics content was found in EAE 793.24 µg GAE/g followed by ME 645.123 µg GAE/g, AQE 554.7 µg GAE/g and HE 474.521 µg GAE/g (Fig. 3). The higher content of total soluble phenolics in ethyl acetate and methanol extracts could be attributed to their higher polarity and greater solubility for polyphenols in plant extracts (Zhao *et al*., 2006). The amount of phenols is considered as a significant parameter while determining the quality and biological significance of the extracts (Moreira *et al*., 2008). The phenols rich crude extracts of plants are used in food industry as natural antioxidants and improving quality of food products (Kahkonen *et al*., 1999). This research study reported that *D. tortuosa* is well equipped with natural phenolics which could be exploited on a large scale for industrial utilization.

FT-IR analysis of the extracts: To identify the presence of various functional group in different samples, FT-IR spectra were recorded (Fig. 4). The data of peak values and functional groups as identified by FT-IR analysis is given in (Table 2). The methanol extract showed the presence of characteristic absorption bands at position 3218 cm^{-1}, 2922.76-2852 cm^{-1}, 1709.39, 1613 cm^{-1}, 1572 cm^{-1} which were assigned to N-H, C-H (alkanes), (C=O representing esters), (N-H representing primary amine), N-O asymmetric stretch, N-O symmetric stretch and C-N (aliphatic amines) functional groups respectively. The aqueous extract showed characteristic absorption bands at 3225.20 cm^{-1} (for N-H), 1568.64 cm^{-1} (for N-O asymmetric stretch), 1388.89 cm^{-1} (for N-O symmetric stretch), 1261.81 cm^{-1} (C-F representing alkyl halide)) and 1089.28 cm^{-1} (C-N representing aliphatic amines) respectively. The ethyl acetate extract showed the presence of characteristic absorption bands at 3788 cm^{-1} (O-H stretching), 2920-2852 cm^{-1} (C-H stretching showing alkanes), 2003 cm^{-1} (C=C=C stretching representing allene), 1718 cm^{-1} (C=O representing conjugated aldehyde and conjugated acid), 1640 cm^{-1} (C=C stretching of alkene) and 1444 cm^{-1} (C-H bending for alkane and methyl group). The signals appearing at the various stretching frequencies in FT-IR spectrum are showing various functional groups of biological compounds present in *D. tortuosa* which is further supported by GC-MS data.

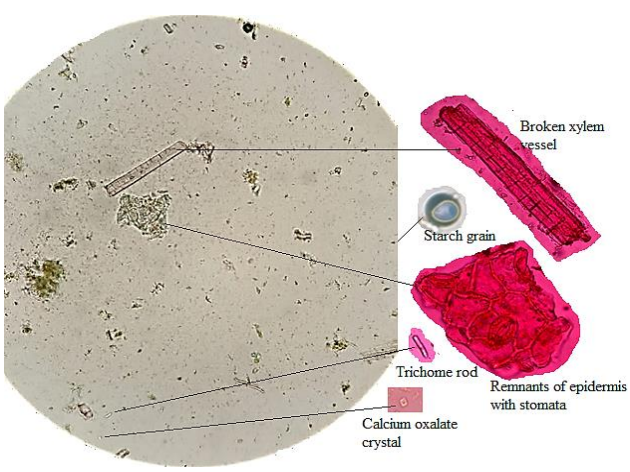

Fig. 1. Powder drug microscopy of *D. tortuosa* at 20 and 100 X.

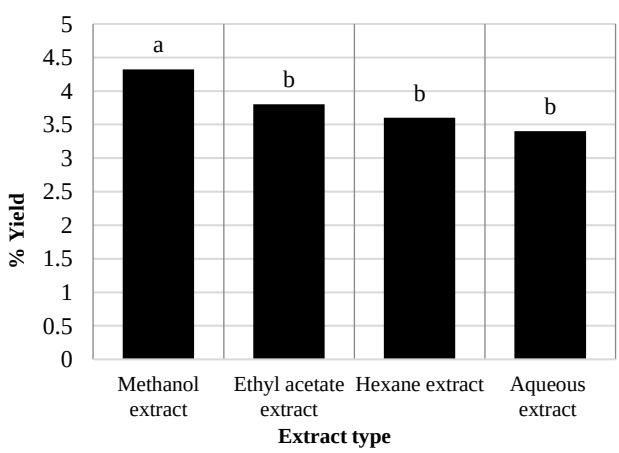

Fig. 2. Percent (%) extract yield of *D. tortuosa* in various solvents.

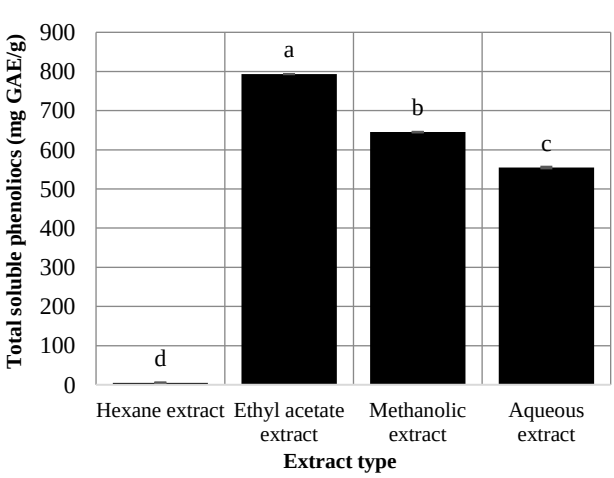

Fig. 3. Total soluble phenolics contents of *D. tortuosa*.

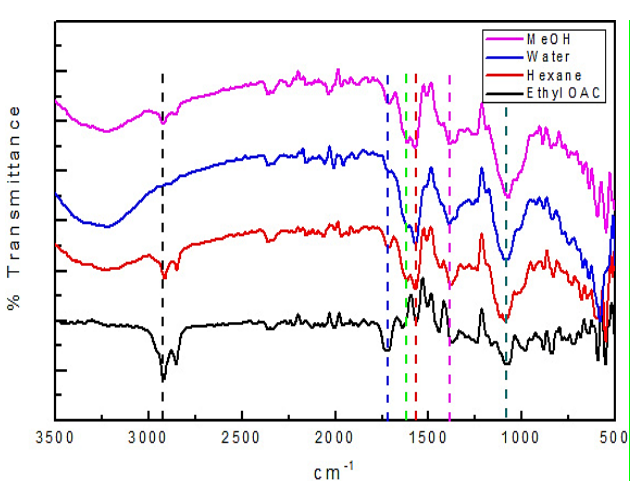

Fig. 4. FTIR spectra of the *D. tortuosa* extracts.

Table 1. Phytochemical screening of *Douepia tortousa* extracts.

Phytochemical	Methanol	Aqueous (Water)	n-hexane	Ethyl acetate
Flavonoids	+	+	_	+
Coumarins	+	+	_	+
Saponin	+	+	_	+
Tannin	+	+	_	+
Terpenoid	+	+	+	+
Alkaloid	_	_	_	_
Anthraquinone	_	_	_	_

GC-MS profiling: GC-MS profiling of methanol, aqueous (water), ethyl acetate and *n*-hexane extracts of *D. tortuosa*. The GC-MS identified a total of 11 bioactive compounds in methanol extract of *D. tortuosa* (Table 3). The major compounds in methanol extract having higher peak area % above 3% were 2, 3-Dimethoxytoluene, Octadecanoic acid, Cis-vaccenic acid, Squalene, γ-Sitosterol, Heptacene, Eicosanic acid methyl ester and Vitamin E.

In *n*-hexane extract (Table 4), the GC-MS identified 10 compounds of which Octadecanoic acid, Linoleic acid, Squalene and Tetracontane were major compounds having greater peak area % whereas, compounds like Mono (2-ethyhexyl) phthalate, Heptacosane, α-Tocopheryl acetate, β-Sitosterol were minor compounds with lesser peak area (%).

In ethyl acetate extract, (Table 5) the major compounds identified by GC-MS included Octadecanoic acid, Palmitoleic acid, Squalene, Heptacosane, Doctriaconate, γ –Sitosterol while, the compounds Octadecane,3-ethyl-5-(2-ethylbutyl)-, Behenic alcohol, Tetratetracontane, Heptacosane, Vitamin E, Octadecanal, 2-bromo- had lesser peak area %.

Table 2. The FTIR spectra peak values and functional groups data for the leaf extracts of *D. tortousa.*

Extract	Peak value (cm^{-1})	Functional group
Methanolic	3218	N-H
	2922.76-2852	C-H
	1709.39-1613	R−COOH
	1572.18	N-O asymmetric stretch
	1384	N-O symmetric stretch
	1072.85	C-N (aliphatic amines)
Aqueous (water) extract	3225.20	N-H
	1568.64	N-O asymmetric stretch
	1388.89	N-O symmetric stretch
	1261.81	C-F (Alkyl halide)
	1089.28	C-N (aliphatic amines)
n-Hexane extract	3223	N-H
	2915-2849	C-H (alkanes)
	1709	C=O (esters)
	1614	N-H (primary amine)
	1569	N-O asymmetric stretch
	1380	N-O symmetric stretch
	1239	CN Aromatic amine
	1089	C-N (aliphatic amines)
Ethyl acetate extract	3788	O-H stretching
	2920-2852	C-H stretching (alkanes)
	2003	C=C=C stretching (allene)
	1718	C=O (conjugated aldehyde and conjugated acid)
	1640	C=C stretching (alkene)
	1444	C-H bending (alkane, methyl group)

Antibacterial activity: The antibacterial properties of different solvent extracts of *D. tortuosa* i.e., aqueous (AQE), methanolic (ME), ethyl acetate (EAE) and *n*-Hexane (HE) against *K. pneumoniae*, *A. baumannii*, *E. coli* and *S. aureus* have been evaluated in this study (Fig. 5). The ME resulted the maximum (p<0.05) antibacterial activity against *K. pneumoniae* showing 29 mm zone of growth inhibition. Next to ME, AQE was more effective antibacterial agent showing 26 mm zone of growth inhibition against *K. pneumoniae*. The EAE and HE extracts showed statistically minimum antibacterial activity against *K. pneumoniae*. The EAE resulted the maximum antibacterial activity against *A. baumannii* and *E. coli*. The EAE showed 29 and 33 mm zones of growth inhibition against *A. baumannii* and *E. coli* respectively. The least effective antibacterial agent against *A. baumannii* and *E. coli* was HE. Against *S. aureus* significantly largest (p<0.05) zone of growth inhibition i.e., 37 mm was shown by the AQE. The ranking of various extracts according to their antibacterial activity against *S. aureus* was AQE>ME=EAE>HE.

The antibiotic erythromycin was used in this study in order to calculate the relative percentage inhibition of antibacterial activity of various solvent extracts of *D. tortuosa* (Fig. 6). The ME showed maximum relative percentage inhibition 77.88% against *K. pneumoniae*. The EAE exhibited maximum relative percentage inhibition 62.40% and 93.47% against *A. baumannii* and *E. coli*. The AQE exhibited highest relative percentage inhibition 79.13% against *S. aureus*.

Table. 3. GC-MS profiling of the methanolic extract of *D. tortuosa*.

S. No.	Name of compound	Molecular formula	Molecular weight	Nature of compound	Start retention time	End retention time	% Area	Pharmacological activities
1.	2,3-Dimethoxytoluene	$C_9H_{12}O_2$	152.19	Phenol	14.89	15.22	11.91	Not reported
2.	Trans-Ferulic acid	$C_{10}H_{10}O_4$	194.18	Derivative of hydroxycinnamic acids	18.34	18.48	1.48	antioxidant, antimicrobial, antifungal and anti-inflammation (Srinivasan *et al*., 2007; Doss *et al*., 2018)
3.	Eicosanic acid, methyl ester	$C_{21}H_{42}O_2$	326.6	Fatty acid ester	18.82	19.03	3.31	Alpha-glucosidase inhibitors activity. (Elaiyaraja & Chandramohan, 2016)
4.	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	Fatty acid	19.17	19.37	16.35	Antimicrobial activity (Rahuman *et al*., 2000)
5.	Gamolenic acid	$C_{18}H_{30}O_2$	278.4	Fatty acid	20.52	20.69	2.3	anti-inflammatory, antithrombotic, and mastalgia (breast pain) and atopic eczema (Murrin, 2007)
6.	Cis-vaccenic acid	$C_{18}H_{34}O_2$	282.5	Isomer of oleic acid	20.78	21.21	32.22	antibacterial and hypolipidemic (Hamazaki *et al*., 2016; Semwal *et al*., 2018)
7.	Squalene	$C_{30}H_{50}$	410.7	Triterpene	26.34	26.56	11.66	Antioxidant, antibacterial Chemopreventive and anticancer (Newmark, 1997; Güneş *et al*., 2013
8.	Heptacosane	$C_{27}H_{56}$	380.7	Straight-chain alkane	26.77	26.97	3.28	Antibacterial, Antioxidant (Yogeswari *et al*., 2012; Mihailovi *et al*., 2011)
9.	Vitamin E	$C_{29}H_{50}O_2$	430.71	Lipophilic molecule	28.69	28.93	3.94	Immune-modulatory properties, antioxidant (Nowicka and Nowicka & Kruk, 2017; Jehad *et al*., 2013)
10.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436.633	Prenol lipids	29.79	29.91	0.88	Antimicrobial Malathi *et al*., (2016)
11.	γ-Sitosterol	$C_{29}H_{50}O$	414.7067	Sterol	30.75	31.11	12.63	Antidiabetic Balamurugan *et al*., 2011

The antibacterial potential of *D. tortuosa* was experimentally understood from the current studies. The extract of *D. tortuosa* offered higher antibacterial activity against the tested pathogenic bacteria identifying it as a highly medicinal plant. The medicinal value of a plants lies in its phytochemical constituents (Tabassum *et al*., 2022). Our study demonstrated that *D. tortuosa* is equipped with biologically active compounds as identified by GC-MS with proven antibacterial properties (Naz *et al*., 2020).

There were differences among the efficacy of different extracts against the tested bacteria. It has been accepted worldwide that pharmacological significance of medicinal plants lies in their phytochemical constituents who are soluble in different solvents (Cowan, 1999). The development of multidrug resistant bacterial strains of *K. pneumoniae* has appeared as potential threat to human beings (Manandhar *et al*., 2019). The relative percentage inhibition of antibacterial activity shown by methanol extract of *D. tortuosa* against *K. pneumoniae* reveals that it could be a potential alternative in order to fight this bacterial pathogen. The current research work was a basic approach in order to explore antimicrobial properties of *D. tortuosa* in various solvents. Therefore, we recommend more research work should be carried out to identify the highly active compounds and also exploring their mechanism of action against pathogenic bacteria.

Antifungal activity: As compared with standard drug Terbinafine, the results of this study revealed that in the various extracts for antifungal activity, *A. niger* was more sensitive than *A. flavus* (Fig. 7). The % inhibition in linear growth of fungi ranged from 45-82% for the tested fungal strains. Among the various extracts, the EAE showed a promising antifungal activity against *A. flavus*. It caused 85% inhibition in linear growth of *A. flavus*. The ranking of different solvent extracts of *D. tortuosa* according to their antifungal properties against *A. flavus* was EAE>AQE=HE>ME. The AQE showed the best antifungal activity against *A. niger* resulting 82 % inhibition of its growth. Next to AQE the ME was more effective antifungal agent against *A. niger* and showed 70% inhibition in its hyphal growth. The antifungal activity of EAE and HE against *A. niger* was only 57% and 60 % but it was significant as compared with negative control (p<0.05).

It has been observed that the *In vitro* sensitivity of the two fungal pathogens to the various extracts of *D. tortuosa* was highly variable. The EAE was highly effective antifungal agent against *A. flavus* while AQE was highly effective against *A. niger*. This higher antifungal activity of *D. tortuosa* may be attributed to its phytochemical composition. The EAE had octadecanoic acid which is a powerful antimicrobial agent (Rahuman *et al*., 2000). It has been found that there exists a close relationship between the antifungal properties and bioactive compounds in plant extracts (Abdelghani *et al*., 2008). Moreover, phytochemicals like phenols found in the extracts of *D. tortuosa* have been reported for strong antifungal properties (Banso & Adeyemo, 2007). Saponins have soapy properties and is therefore, considered as a strong antifungal agent (Barile *et al*., 2007). Bioactive compounds like phenolic compounds, flavonoids, alkaloids, coumarins and terpenoids showed strong antifungal activity against fungal pathogens (Duraipandiyan & Ignacimuthu, 2011).

Table. 4. GC-MS profiling of the n-hexane extract of *D. tortuosa.*

S. No	Name of compound	Molecular formula	Molecular weight	Nature of compound	Start RT	End RT	% Area	Pharmacological activities
1.	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	Fatty acid	19.2	19.41	6.31	Antimicrobial activity (Rahuman *et al*., 2000)
2.	Palmitoleic acid	$C_{16}H_{30}O_2$	254.41	Fatty acid	20.76	21.2	19.11	Antioxidant (Zadeh-Hashem *et al*., 2016)
3.	Octadecane,3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366.7	Alkane	23.83	24	0.22	Not known (Amudha *et al*., 2018)
4.	Behenic alcohol	$C_{22}H_{46}O$	326.60	Fatty alcohol	24.62	24.72	0.44	Antiviral (Abdel-Haq *et al*., 2006)
5.	Tetratetracontane	$C_{40}H_{82}$	563.1	Alkane	25.35	25.59	2.44	Antibacterial (Gumgumjee & Hajar, 2015)
6.	Heptacosane	$C_{27}H_{56}$	380.7	Straight-chain alkane	26.03	26.24	3.91	Antibacterial, Antioxidant (Yogeswari *et al*., 2012; Mihailovi *et al*., 2011)
7.	Squalene	$C_{30}H_{50}$	410.7	Triterpene	26.36	26.65	6.79	Antioxidant, antibacterial Chemopreventive and anticancer (Newmark, 1997; Güneş *et al*., 2013
8.	Dotriacontane	$C_{32}H_{66}$	450.9	Long chain alkane	26.77	27.26	54.31	Cytotoxic (Giri *et al*., 2016)
10.	Vitamin E	$C_{29}H_{50}O_2$	430.71	Lipophilic molecule	28.73	28.85	1.22	Immune-modulatory properties, antioxidant (Nowicka & Kruk, 2017; Jehad *et al*., 2013)
11.	Octadecanal, 2-bromo-	$C_{18}H_{35}BrO$	347.4		29.65	29.79	0.6	Antimicrobial (Kadhim *et al*., 2016)
12.	γ -Sitosterol	$C_{29}H_{50}O$	414.7067	Sterol	30.81	31.17	3.29	Antidiabetic Balamurugan *et al*., 2011

Table 5. GC-MS profiling of the Ethyl acetate extract of *D. tortuosa.*

S. No.	Name of compound	Molecular formula	Molecular weight	Nature of compound	Start RT	End RT	% Area	Pharmacological activities
1.	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	Fatty acid	19.14	19.29	4.48	Antimicrobial activity (Rahuman *et al*., 2000)
2.	Linoleic acid	$C_{18}H_{32}O_2$	280.4	Fatty acid	20.8	21	8.55	neuroprotective, anti-inflammatory, and antidepressant Blondeau *et al*., 2015
3.	Mono (2-ethyhexyl) phthalate	$C_{16}H_{21}O_4$	277.33	Metabolite of di-(2-ethylhexyl) phthalate	24.31	24.39	0.67	Increase visceral fat tissue and body weight Schmidt *et al*., 2012
5.	Heptacosane	$C_{27}H_{56}$	380.7	Straight-chain alkane	26.04	26.18	2.54	Antibacterial, Antioxidant (Yogeswari *et al*., 2012; Mihailovi *et al*., 2011)
6.	Squalene	$C_{30}H_{50}$	410.7	Triterpene	26.36	26.45	7.67	Antioxidant, antibacterial Chemopreventive and anticancer (Newmark, 1997; Güneş *et al*., 2013
7.	Tetracontane	$C_{40}H_{82}$	563.1	Alkane	26.76	27.01	70.15	Antibacterial (Gumgumjee & Hajar, AS 2015)
9.	α-Tocopheryl acetate	$C_{31}H_{52}O_3$	472.7	Vitamin	28.71	28.91	1.46	Antioxidant, antibacterial (Bidossi *et al*., 2017)
10.	β-Sitosterol	$C_{29}H_{50}O$	414.7067	Sterol	30.78	30.91	1.99	Antidiabetic Balamurugan *et al*., 2011

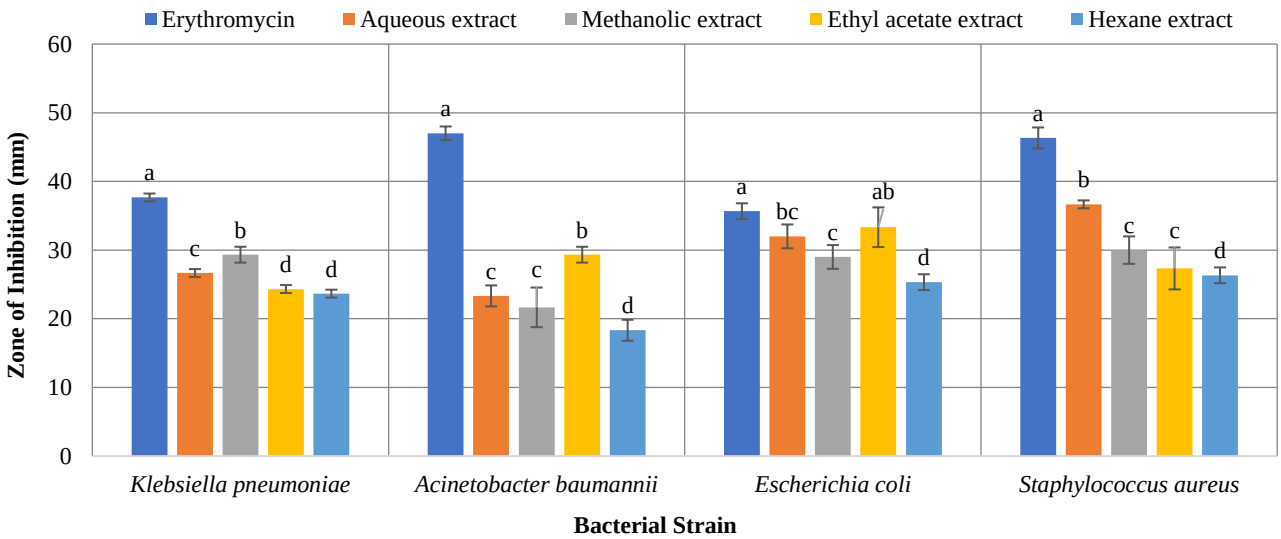

Fig. 5. Antibacterial activity of *D. tortuosa* aqueous (AQE), methanolic (ME), ethyl acetate (EAE) and *n*-Hexane (HE) extracts. Data represented is mean of three separate replicates.

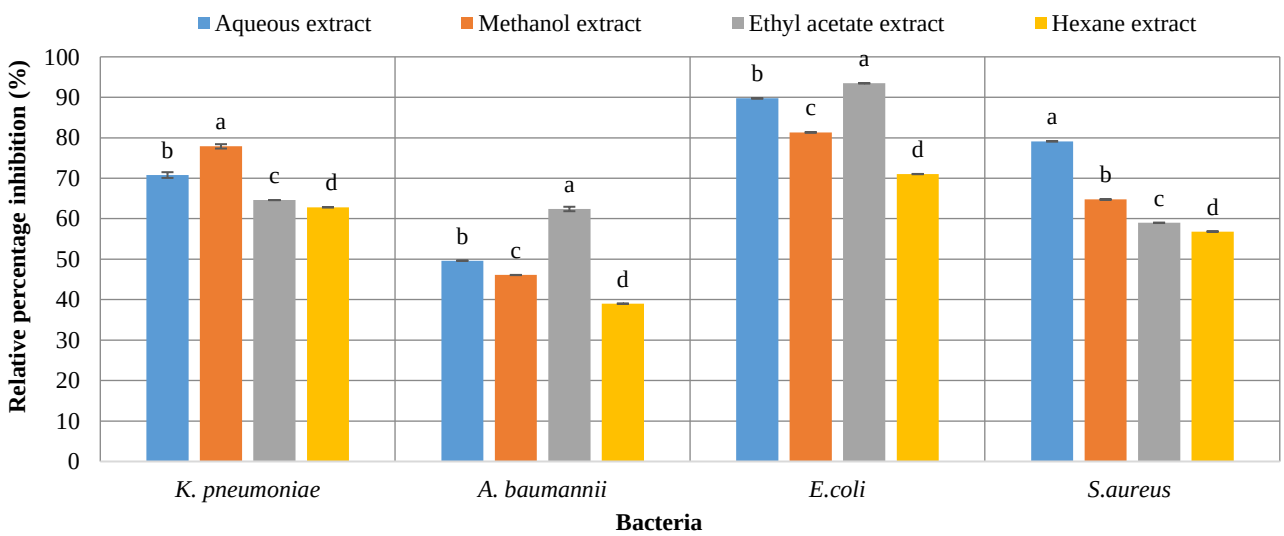

Fig. 6. Relative percentage inhibition of *D. tortuosa* aqueous (AQE), methanolic (ME), ethyl acetate (EAE) and *n*-Hexane (HE) extracts compared to antibiotic erythromycin. AQE: aqueous (water) extract, ME: methanol extract, EAE: ethyl acetate extract, HE: hexane extract.

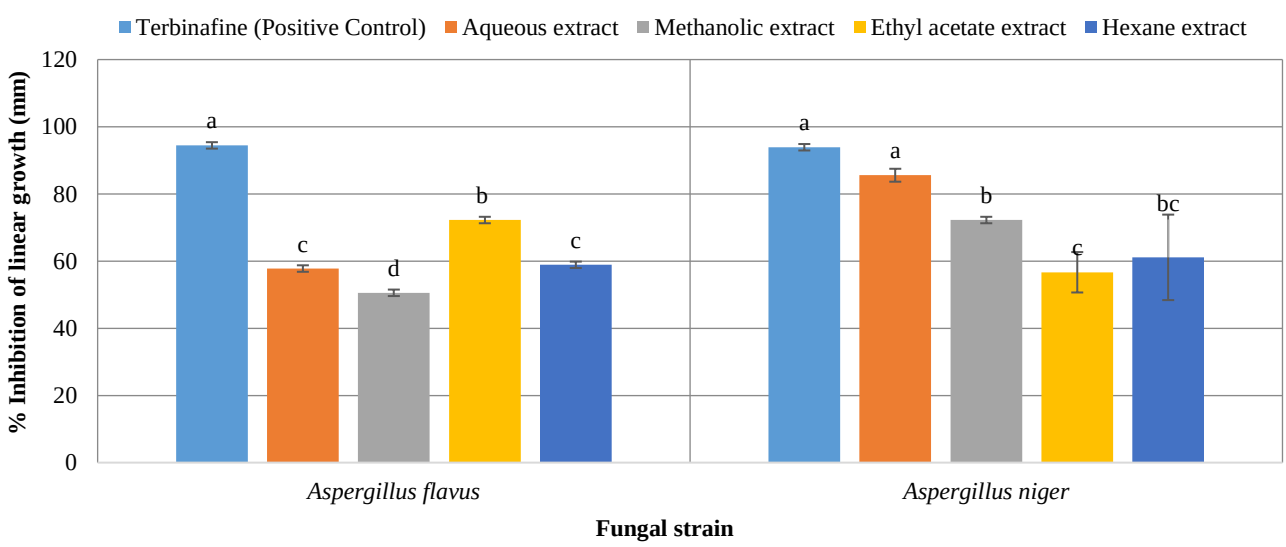

Fig. 7. Antifungal activity of *D. tortuosa*.

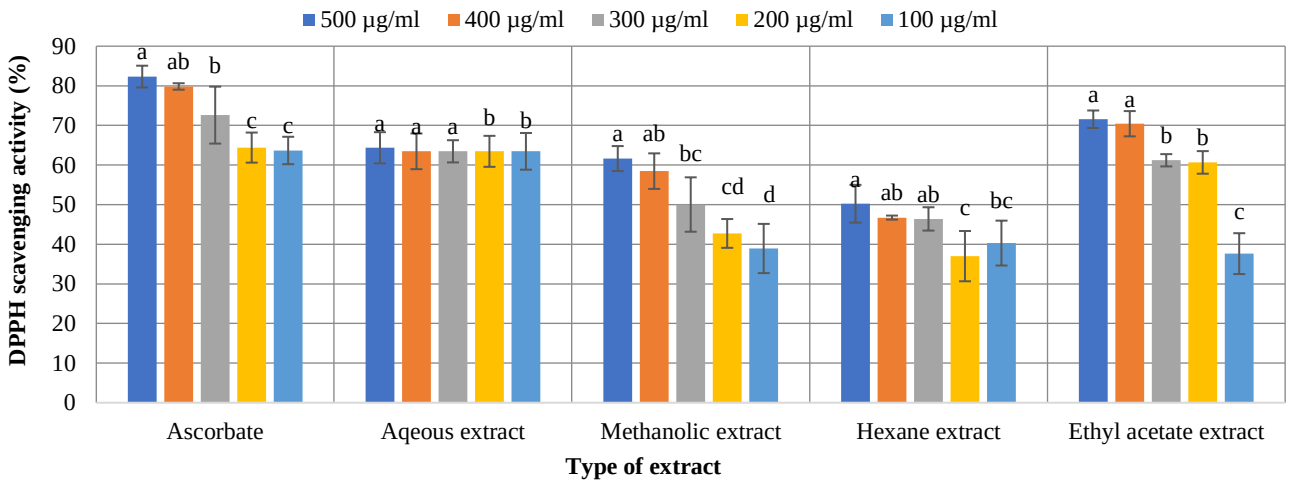

Fig. 8. DPPH scavenging activity of *D. tortuosa.*

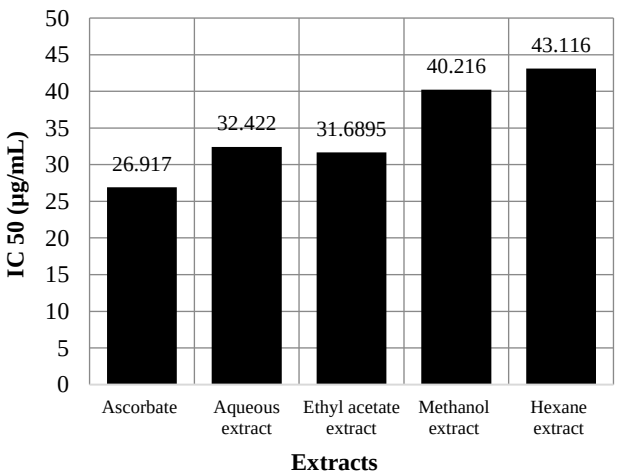

Fig. 9. IC50 values for DPPH antioxidant activity.

Antioxidants activity: Results revealed that the AQE, ME, EAE and HE of *D. tortuosa* showed strong scavenging activity for free radicals as assayed by DPPH free radical scavenging activity (Fig. 8). It was observed that AQE and EAE have higher antioxidant activity than the other extracts. At 500 µg/mL the scavenging activity of the EAE and AQE reached 71 % and 63 %, whereas at the same concentration, the scavenging activity of ascorbic acid was 82%. Next to the AQE and EAE, the ME was more effective scavenger of free radicals. According to antioxidant activity the various extracts were ranked as EAE>AQE>ME>HE.

According to IC50 values, the AQE (32.422 µg/mL) and EAE (31.6895 µg/mL) has significantly comparable antioxidant activity with ascorbic acid (26.917 µg/mL). The IC50 value recorded for ME and HE were 294.08 µg/mL and 228.44 µg/Ml respectively (Fig. 9).

Phenolic compounds found in plant extracts act as antioxidants due to the hydrogen donating property of their OH- groups (Aberoumand & Deokule, 2008). Therefore, it could be concluded that these phenolic compounds are responsible for the antioxidant activity of *D. tortuosa* extracts. Antioxidants found in medicinal plants are explored for developing natural antioxidants formulations for use in food, herbal medicines and cosmetic (Miliauskas *et al*., 2004). We recommend that the higher antioxidant activity of aqueous and ethyl acetate extracts of *D. tortuosa* could be highly beneficial for application in food industry after proper investigation of its cytotoxicity in animal models.

Conclusion

The current research concluded that *D. tortuosa* is equipped with a variety of bioactive compounds including vitamin E. The various extracts of *D. tortuosa* were antibacterial, antifungal and antioxidant agents. The plant may be consumed as a natural source of antioxidants and as a crude drug against bacterial and fungal infections. Moreover, the plant may be further investigated for its potential phytochemicals specifically active against cancer. Moreover, powder microscopy could be successfully applied for identification and control of adulteration in drug powder of this plant species.

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