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Phytochemical and comparative biological studies of *Diospyros kaki* extracts

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Abstract

Many plants attained popularity as medicinal plants but still various horizons require analyst's interest. Amongst, *Diospyros kaki* is one of these medicinal plants granted with potent antioxidant activity. This study was focused to elucidate antioxidant status and biological activities of *D. kaki* with special reference to its bioactive molecules linked to various health claims. Phytochemicals like saponins, terpenoids, flavonoids and tannins were screened qualitatively in different organs by successive extraction with solvents of increasing polarity from less polar to more polar solvents along with determination of total phenolic and flavonoid contents quantitatively. Moreover, with respect to phytopharmaceutical potential *In vitro* antibacterial, antifungal, anti-inflammatory, anticancer and anti-diabetic activities were also evaluated. Phytochemical screening reflected presence of active constituents in all organs but abundance of these was found in ethanol and methanol extracts of leaves and unripe fruit. Similarly, methanol extract of leaves revealed significant anticancer, anti-inflammatory and antibacterial activity against gram negative bacteria whereas anti fungal, anti-diabetic and antibacterial activity against gram positive bacteria were shown in ethanol extract of unripe fruit. These findings concluded that *D. kaki* could be capitalized in pharmacology due to presence of different phytochemicals comprising of antioxidant characteristics but resulted remarkable phytochemicals in leaves and unripe fruit extracts in addition to significant biological activities make them more remarkable and profound amongst other extracts. Methanol and ethanol extracts due to good purity level as compared to other solvents found to be more dynamic against biological activities. Keeping in mind the above facts, further biological and pharmaceutical investigations are required to characterize new bioactive components.

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Introduction

Plants are a rich source of different classes of bioactive secondary metabolites which are used for promoting public health or to combat various disorders (Jang *et al.*, 2007; Céspedes *et al.*, 2008). From the mid of 19th century, various bioactive chemical constituents have been reported from plants and many of them are being utilized as potential constituents of the present day medicine (Uddin *et al.*, 2012). The important bioactive secondary metabolites are flavonoids, steroids, alkaloids, terpenes, coumarins, saponins, tannins and phenolic compounds for the medicinal purposes (Edeoga *et al.*, 2005). These secondary metabolites are reported to reveal numerous biological activities including antimicrobial, antihypertensive, antioxidant, ant-iinflammatory and anticancer activity (Savithramma *et al.*, 2012; Kuppusamy *et al.*, 2015). Plants can quench free radical as antioxidants that can be used in the form of vitamins, minerals and neutraceuticals (Patil *et al.*, 2009).

Diospyros kaki (*D. kaki*) which belongs to the *Ebenaceae* family is a deciduous small tree commonly known as Japanese fruit is native to China and many other regions (Zhou *et al.*, 2016). It is nutritious plant enriched with many bioactive components like proteins, ascorbic acid (AsA), lipids, sugar, vitamins, polyphenols (especially tannin), flavonoids, dietary fibers and minerals (Butt *et al.*, 2015).

Traditionally, this plant is used to cure different skin disorders such as skin eruptions, eczema and pimples (Kashif *et al.*, 2017). In traditional medicines *D. kaki* is used to treat diarrhea, arteriosclerosis, cough and apoplexy (Kim *et al.*, 2009). It is taken as antitussive, sedative agent, carminative and to cure bronchial complaints in many traditional medicinal systems (Singh and Joshi, 2011). Previously, it has been reported that *D. kaki* contains many pharmacological activities including potent radical scavenging and antigen toxicity of seed (Jang *et al.*, 2010), anti-inflammatory action of leaves (Kim *et al.*, 2016), anti-carcinogenic, antihypertensive (Kawase *et al.*, 2003), anti-diabetic (Li *et al.*, 2007) and antioxidant properties of peel and pulp *(Jang et al.,* 2010). Different studies have been found about reported pharmacological activities and phyto-constituents profile on *D. kaki* fruit in dermatology and cosmetics (Giordani *et al.*, 2011; Xie *et al.*, 2015). The current study was focused to explore *D. kaki* plant due to its reported phytochemical and pharmacological importance. For this, we speculated on potential use of different organs of *D. kaki* where these may have possible applications in pharmaceuticals and stimulating the drug finding work. So, *D. kaki* organs were screened chemically along with evaluated *in vitro* anti-inflammation, anti-diabetic, anticancer, antibacterial and antifungal activities to exploit its antioxidant potential and function in disease prevention.

Material and methods

Collection of plant material

Different plant organs including young green leaves, black bark, stem, ripe and unripe fruit of *D. kaki* were collected In July 2017 to Sep, 2017 from their natural habitat from *plant* nursery, Lahore, Pakistan. The investigated organs of *D. kaki* were carefully washed with faucet water to remove soil particles then dipped with distilled water to save quality. The plant material was then stored in clean labeled small plastic containers to keep integrity for analysis. The analysis was carried out at NIAB (MAB Lab-1), Faisalabad, Pakistan.

Drying and grinding of plant material

The studied and cleaned plant organs were then air dried at room temperature for 72 hours. The dried plant material was then finely ground into powder form and packed in labeled neat airtight small plastic bottles at room temperature for further experiments.

Phytochemical screening

The phytochemical screening was carried out based on coloration or precipitation reactions. According to (Houghton and Raman, 2012) method, plant material was directly converted into powdered form. Phytochemical analysis and biological activities like antibacterial and antifungal were conducted at

laboratory of Applied chemistry, Govt College University, Faisalabad, Pakistan. Similarly, anti diabetic and anti-inflammatory activities were executed at Plant Breeding and Genetics Division (MAB Lab-1) Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan while anticancer activity was performed at Hussain Ebrahim Jamal Research Institute of Chemistry, Karachi, Pakistan.

Preparation of plant extracts

The plant organs were extracted with solvents from less polar solvents to more polar solvents using sequential extraction (Das and Sharma, 2001; Das *et al.*, 2010) to extract various chemical constituents based on polarity.

The powdered plant material (50 g) was dissolved in 500 mL solvent petroleum ether (40 - 60 °C) and put in an airtight container to stand at room temperature for three days until the soluble matter was dissolved. To filter extract, Whatmann filter paper No. 42 was used. The marc left after extraction was then air dried and it was again extracted with solvent dichloromethane (CH_2Cl_2) for another 3 days. This was followed by the extraction with ethyl acetate and finally methanol. The six extracts of 6 organs of *D. kaki* were observed (Jeyaseelan *et al.,* 2012).

Determination of extracts yield

The ratio between the observed mass of the dry plant extract and total mass of plant material used in experiment is called the performance of crude extract (Baxter *et al.,* 1998). This yield can be calculated using following formula:

% yield = Weight of extract / Weight of dry Powder × 100

Qualitative determination of phytochemicals

The presence of saponins, tannins, flavonoids, terpenoids, total phenolic and flavonoid contents were determined qualitatively by preliminary phytochemical screening of the crude extracts using following standard protocols (Evans, 2002).

Test for tannins

500 mg of each dry plant extract was boiled in 5 mL H_2O and filtered. Then few drops of $FeCl_3$ solution was added in filtrate and blue-black coloration indicated the presence of tannins (Sofowora, 1982; Evans, 2002).

Test for saponins

200 mg of the powdered extract was boiled in 500 mL distilled H_2O and filtered. 10 mL of this filtrate was then mixed with 5 mL deionized H_2O and shaken utterly to obtain strong determined lather. Then this lather was mixed with three drops of olive oil and shaken enormously till development of emulsion (Sofowora, 1982; Evans, 2002).

Test for flavonoids

5 mL diluted ammonia solution was mixed in a little portion of the aqueous filtrate of each extract, followed by adding five to six drops of concentrated H_2SO_4. The observed yellow color was an indication of flavonoids in extract (Madaan *et al.,* 2011).

Test for terpenoids

5 mL of each plant extract was mixed vigorously with 2 mL chloroform. Then concentrated H_2SO_4 (3mL) was added carefully to have a layer. The reddish brown color on the inner surface confirmed the presence of terpenoids (Caceres *et al.,* 1992).

Qunatitative determination of phytochemicals

Total phenolics and flavonoids contents of *D. kaki* were studied quantitatively for roots, bark, stem, leave, ripe and unripe fruit separately.

Test for total flavonoid contents (TFC)

Total flavonoid contents of each crude extract was determined following the calorimetric method. 4 mL distilled H_2O was mixed in 1mL plant sample. Then 0.15 mL of 10% $NaNO_3$ was added in this reaction mixture. After five minutes, 0.15 mL of $AlCl_3$ (10%) was then added in this reaction solution. This mixture was then incubated for 6 minutes followed by the addition of 1 mL sodium hydroxide. Then absorbance was measured at 510 nm using UV-Vis

spectrophotometer. Total flavonoid contents were calculated using the plotted standard calibration curve with quercetin. The data found was the mean of three readings. Total flavonoid contents were expressed as quercetin equivalents (mg quercetin/g dried sample) (Singleton *et al.,* 1999).

Test for total phenolics contents (TPC)

A micro colorimetric method as explained by (Ainsworth and Gillespie, 2007) was followed for the determination of phenolic compounds with some modifications consisting of Folin-Ciocalteau (F-C) reagent. To homogenize 500 mg plant material was added in 95% ice cold methanol (0.5 mL) using an ice cold pestle and mortar. Then plant samples were allowed to stand at room temperature for 48 hrs in the dark. The plant extracts were centrifuged at 14,462 × g for 5 min. The supernatant was then separated and used for TPC determination. The 100 µL supernatant and 100 µL of F-C reagent (10% (v/v)) were vortexed utterly and then 800 µL of 0.7 M Na_2CO_3 was put in all tubes and incubated at lab temperature for one hour. Then absorbance was measured at 765 nm. The total phenolics content was calculated using the plotted standard calibration curve with gallic acid.

Invitro biological activities

Anti-inflammatory activity (Inhibition of albumin denaturation)

The anti-inflammation was determined by following the inhibition method of albumin denaturation. Methods as (Sakat *et al.*, 2010) and (Mizushima and Kobayashi, 1968) were used to test this activity with minor modifications. An aqueous solution of 1% bovine albumin serum was prepared and then its pH was adjusted at 6.0 with 1M HCL. The reaction solution was mixture of test extracts with concentration of 5 mg /mL of DMSO to prepare stock solutions. Diclofenac sodium (10mg) as stanadard drug was also used to prepare stock solution as test samples. These stock solutions of test extracts and standard drug were further used to get two final concentrations of 100 and 400 µg /mL. The standard and all the extract solutions were incubated for 20 min at 37^0 C and then heated at 70 °C in water bath for 5 min. The turbidity was measured at 660 nm with spectrophotometer after cooling reaction mixtures. The readings were taken in triplicates (Leelaprakash and Dass, 2011).

$$\% \text{ inhibition} = \frac{\text{Control}_{Abs} - \text{sample}_{Abs} \times 100}{\text{Control}_{Abs}}$$

Where sample_{Abs} is the absorbance of plant extract / standard and Control_{Abs} is the absorbance without sample.

Anti cancer activity

Standard 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) colorimetric assay was used to evaluate anticancer activity of compounds in 96-well flat-bottomed microplates (Mosmann, 1983). Minimum Essential Medium Eagle was used as culture for HeLa cells, supplemented with 5% fetal bovine serum (FBS), penicillin (100 IU / mL) and ostreptomycin (100 µg/ mL) in flasks (75 cm^2) and kept in carbon dioxide (5%) incubator at 37 °C. Then exponentially harvested growing cells were counted with haemocytometer and diluted with specific medium.

The cell culture was prepared with concentration of 6 $x10^4$ cells/ mL and introduced into 96-well plates (100 µL /well). After keeping whole night on incubation, medium was detached and fresh medium 200 µL was added having different concentrations (1-30 µM) of compounds. After 2 days, 200 µL of 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide (0.5 mg /mL) was put in wells and again incubated for 4 hrs. Afterwards, 100 µL of dimethyl sulfoxide was added in all wells. The extent of 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide reduction to formazan in cells was recorded by absorbance at 570 nm, using Micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). Anticancer activity was calculated as concentration causing growth inhibition of 50% (IC_{50}) for HeLa cell lines. The % inhibition was explained by following formula:

$$\% \text{ inhibition} = \frac{\text{Control}_{Abs} - \text{Sample}_{Abs} \times 10}{\text{Control}_{Abs}}$$

% inhibition = the results were processed by using Soft- Max Pro software (Molecular Device, USA).

Anti-diabetic activity (α-amylase inhibitory studies)

The α-amylase inhibition was determined by following 3,5-dinitrosalicylic acid (DNSA) method (Miller, 1959). The *D. kaki* extracts were dissolved in lowest amount of 10% DMSO and was then dissolved in 0.02 M Na_2SO_4 buffer, 6 mM sodium chloride at pH 6.9 to get two final concentrations of 100 and 400 µg /mL. The reaction solution comprised 200 µL α-amylase solution and 200 µL of the plant material which was further incubated at 30 °C for 10 min. After that 200 µL of 1% starch solution in distilled H_2O (w/v) was mixed with all extracts and incubated for 3 min. 200 µL DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8 mL of 2M NaOH and 20 mL of 0.096 M of 3,5 DNSA solution) was further added to bring reaction at end and then boiled at 90 °C in a water bath for 10 min. All the reaction solutions were diluted with 5 mL distilled H_2O and the absorbance was measured at 540 nm with UV-Visible spectrophotometer. The blank of 100% enzyme activity was prepared using 200 µL of buffer instead of plant extract. Another blank reaction was also prepared without enzymatic solution at all concentration using the plant extract. Acarbose was used as standard drug and reaction was executed similarly as mentioned above with both concentrations of 100 and 400 µg /mL. The α-amylase inhibition was measured as % inhibition using the following equation:

$$\% \alpha \text{ amylase inhibition} = \frac{\text{Control (100\%)}_{Abs} - \text{Sampl}_{Abs} \times 100}{\text{Control (100\%)}_{Abs}}$$

Antibacterial activity

The antibacterial property was determined by the disc diffusion method with minor modifications (Bauer *et al.*, 1966; Rios *et al.*, 1988; Alzoreky and Nakahara, 2003). A bacterial strain of two bacteria like *streptococcus and E. coli* was used. The barium sulphate turbidity method was carried out to regulate density of the bacterial colony. Nutrient agar (28 g) was dissolved in 1000 mL distilled water and was autoclaved at 121 °C for thirty minutes along with the Petri dishes. Agar solution was dropped rapidly into the Petri dishes after autoclave and was laid out equally that it extends over about 4 mm of the dish and was then left for coagulation in open air. In the meantime, the extracts under study were let fall vertically drop wise with µL pipit on a 9 mm filter paper disc and were allowed to stand for drying. Now the bacterial culture was spread equally over the culture plates with purified cotton swab and the dried filter paper disc was put over the bacterial cultural plates with tweezers and was kept in the incubator for one day. The diameter of the inhibition zone was measured after 24 hours and the results were calculated.

Minimum inhibitory concentration (MIC) measurements

The lowest concentration which is obtained to retain inoculums feasibility is called minimum inhibitory concentration (Carson *et al.*, 1995).The MIC of the extracts against gram-positive and gram-negative bacteria was found by periodic dilution method (Iwaki *et al.*, 2006; Khan *et al.*, 2007). To find out stock solution, 10 mg of the plant extract was dissolved in 1 mL distilled water. After that, it was diluted 10 times by serial dilution method in which 9.5 mL H_2O and 0.5 mL plant extract was mixed and then from this mixture 0.5 mL was taken and put into another test tube having 9.5 mL H_2O. This process was repeated 10 folds and the solutions of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% were made. Then 10%, 40%, 70% and 90% were selected from this solution to find out antibacterial test.

Antifungal activity

The antifungal activity was tested by disc diffusion method with some modifications (Bauer *et al.*, 1966; Rios *et al.*, 1988; Alzoreky and Nakahara, 2003). A fungal strain of *A. flavus* (*Aspergillus flavus*) and *A. niger* (*Aspergillus niger*) was used. Potato dextrose agar (28 g) was dissolved in 1000 mL H_2O. Then it

was placed for autoclave along with the Petri dishes at 121°C for 30 min. This agar solution was then dropped rapidly into the Petri dishes soon after autoclave and was spread equally that it occupies about 4 mm of the dish and was then kept for coagulation in open air. The extracts under study was put drop wise by µL pipit on 9 mm filter paper discs and was allowed to stand for drying. The culture of fungus was placed equally over the culture plates with purified cotton swab and the dried filter paper disc was put in the bacterial cultural plates using purified tweezers and was incubated for one day. The diameter of the inhibition zone was measured after incubation of 24 hours and the results were calculated.

Statistical analysis

All the data was reported as mean ± SD. For analysis and organization of resulting data, descriptive statistics was applied. Two-way ANOVA with replications was used to analyze data. Significance of data was tested by analysis of variance and Tukey (HSD) Test at $p < 0.05$ and where applicable at $p < 0.01$ using XL-STAT software.

Data was also subjected to principal component analysis using computer software Microsoft Excel along with XLSTAT Version 2012.1.02, Copyright Addinsoft 1995-2012 (http://www.xlstat.com).

Results and discussion

The extraction performance

The fine powder of plant extracts (stem, leaves, bark, ripe and unripe fruit) was weighed. Ethanol and methanol extracts showed the highest % yield in all organs of *D. kaki* whereas the lowest yield (%) was observed with the petroleum ether extract (Table 1).

Table 1. Extracts yield (%) from *D. kaki* extracts according to the solvent polarity.

Sr No.	Solvents	Extract	Powdered mass (g)	Extracted mass (g)	Yield (%)
1	Petroleum ether	Root	50	0.5	1
2	Dichloro methane	Root	50	0.6	1.2
3	Ethyl acetate	Root	50	0.97	1.9
4	Ethanol	Root	50	3.4	6.8
5	Methanol	Root	50	4.07	8.14
6	Aqueous	Root	50	1.9	3.8
7	Petroleum ether	Bark	50	1.2	2.4
8	Dichloro methane	Bark	50	1.3	2.6
9	Ethyl acetate	Bark	50	1.7	3.4
10	Ethanol	Bark	50	3.9	7.8
11	Methanol	Bark	50	4.3	8.6
12	Aqueous	Bark	50	2.1	4.2
13	Petroleum ether	Stem	50	0.17	0.34
14	Dichloro methane	Stem	50	0.5	1
15	Ethyl acetate	Stem	50	0.2	0.4
16	Ethanol	Stem	50	2.1	4.2
17	Methanol	Stem	50	3.3	6.6
18	Aqueous	Stem	50	1	2
19	Petroleum ether	Leave	50	0.9	1.8
20	Dichloro methane	Leave	50	1.2	2.4
21	Ethyl acetate	Leave	50	1.4	2.8
22	Ethanol	Leave	50	6.2	12.40
23	Methanol	Leave	50	6.4	12.8
24	Aqueous	Leave	50	3.1	6.2
25	Petroleum ether	Unripe fruit	50	1.17	2.34
26	Dichloro methane	Unripe fruit	50	1.23	2.46
27	Ethyl acetate	Unripe fruit	50	1.4	2.8
28	Ethanol	Unripe fruit	50	6.84	13.68
29	Methanol	Unripe fruit	50	8.92	17.84
30	Aqueous	Unripe fruit	50	3.45	6.9
31	Petroleum ether	Ripe fruit	50	0.9	1.8
32	Dichloro methane	Ripe fruit	50	1	2
33	Ethyl acetate	Ripe fruit	50	1.1	2.2
34	Ethanol	Ripe fruit	50	3.64	7.28
35	Methanol	Ripe fruit	50	4.2	8.4
36	Aqueous	Ripe fruit	50	2.1	4.2

The extraction analysis showed capacity of solvent to extract maximum number of compounds may have biological activities is more in methanol and ethanol, the most polar solvents after water (Nounagon *et al.*, 2017).

Phytochemical screening

Qualitative analysis

In the last few years, search for biologically active constituents from natural origin has been extensively increased. Plants comprise a large number of phytochemicals like tannins, saponins, flavonoids and terpenoids that have direct link with medicinal value of particular plant.

These active constituents after isolation and formulations in many drug dosage forms are used to cure various diseases (Tiwari *et al.*, 2011). The present study on *D. kaki also* indicated the presence of active components (Table 2).

Table 2. Phytochemical constituents for *D. kaki* extracts powder.

Sr No.	Solvents	Extract	Tannin	Saponin	Flavonoid	Terpenoid
1	Petroleum ether	Root	+	-	+	-
2	Dichloro methane	Root	-	+	+	+
3	Ethyl acetate	Root	+	+	+	+
4	Ethanol	Root	+	+	+	+
5	Methanol	Root	+	+	+	+
6	Aqueous	Root	+	+	+	+
7	Petroleum ether	Bark	+	+	-	-
8	Dichloro methane	Bark	+	+	+	-
9	Ethyl acetate	Bark	+	+	+	+
10	Ethanol	Bark	+	+	+	+
11	Methanol	Bark	+	+	+	+
12	Aqueous	Bark	+	+	+	+
13	Petroleum ether	Stem	+	+	+	+
14	Dichloro methane	Stem	+	+	-	-
15	Ethyl acetate	Stem	+	++	+	+
16	Ethanol	Stem	+	+	+	+
17	Methanol	Stem	+	+	+	+
18	Aqueous	Stem	+	+	+	+
19	Petroleum ether	Leave	+	+	+	+
20	Dichloro methane	Leave	+	+	+	+
21	Ethyl acetate	Leave	+	+	++	+
22	Ethanol	Leave	+	+	++	++
23	Methanol	Leave	++	++	+++	+++
24	Aqueous	Leave	+	+	+	+
25	Petroleum ether	Unripe fruit	+	-	+	-
26	Dichloro methane	Unripe fruit	-	-	+	+
27	Ethyl acetate	Unripe fruit	+	+	+	+
28	Ethanol	Unripe fruit	+	+	++	+
29	Methanol	Unripe fruit	++	++	+++	++
30	Aqueous	Unripe fruit	+	+	+	+
31	Petroleum ether	Ripe fruit	-	+	+	+
32	Dichloro methane	Ripe fruit	-	-	+	-
33	Ethyl acetate	Ripe fruit	+	+	+	+
34	Ethanol	Ripe fruit	+	+	+	+
35	Methanol	Ripe fruit	+	+	++	++
36	Aqueous	Ripe fruit	+	+	+	+

+: Low color intensity, +++: high color intensity, - : absence of coloration.

In these screening process tannins, saponins, flavonoids and terpenoids were almost found in all organs of *D. kaki*. The results for the phytochemical screening indicated the presence of tannins with verification of blue-black color. Result for saponins was also found positive with the forth formation of about 1.1 cm. The appearance of yellow color was found in test for flavonoid, confirms that the plant also comprises flavonoid group of phytochemicals. The successive appearance of reddish brown color on

inner face confirmed terpenoids presence in the plant. It has been reported that *D. kaki* is a great source of carotenoids, sugars, saponins, tannins, amin acids, flavonoids, terpenoids, and phenols (Thuong *et al.*, 2008).

Qunatitaive analysis

Total polyphenols and flavonides contents (TPC and TFC)

Total polyphenolic and flavonoid contents were possessed by linear regression equation using standards as gallic acid and quercetin respectively. TFC and TPC were found (Table 3) in all parts of *D. kaki* but the methanolic extract of *D. kaki* leaves has the highest (840 ± 0.01 µg EAG/mg of extract) total polyphenols and (670±1.21 µg EQ /mg of extract) of total flavonoids. More than 77.4% flavonoids have been reported from leaves extract of *D. kaki* (Bei *et al.*, 2009) reinforced our findings. Biological properties including endothelial function improvement, anti-inflammation, anti-ageing, anti-atherosclerosis, anti-apoptosis, cardiovascular protection and anti-carcinogen have been possessed by phenolic components (Han *et al.*, 2007).

Table 3. Total polyphenols and flavonoids content of *D. kaki* extracts.

Extracts	Total Polyphenols (µgEAG/mg)	Total Flavonoïds (µg EQ/mg)
Ethanol root	196 ±0.002	402±0.7
Methanol root	204±0.002	485±0.9
Ethanol bark	312±0.003	432±0.8
Methanol bark	409±0.004	455±0.9
Ethanol stem	166±0.002	376±0.7
Methanol stem	181±0.005	406±0.7
Ethanol leave	783±0.02	598±1.1
Methanol leave	840±0.01	670±1.2
Ethanol unripe fruit	616±0.07	520±1.1
Methanol unripe fruit	667±0.08	611±1.2
Ethanol ripe fruit	397±0.004	537±1.0
Methanol ripe fruit	450±0.005	601±1.1

EAG: Equivalent Gallic acid; EQ: Equivalent Quercetin.

Polyphenols out of all phytochemicals are appreciably recognized as anti-inflammatory, antioxidant, antimicrobial, and antiviral agents (Steinmetz and Potter, 1996).

A large number of studies have focused on the phenolics for their biological activities being strong antioxidants and free radical scavengers (Rice-evans *et al.*, 1995; Kähkönen *et al.*, 1999; Sugihara *et al.*, 1999). TFC and TPC have also been reported to prevent DNA from oxidative harm such as inhibition of tumor cells growth and reflected anti-inflammatory and antimicrobial activities (Torel *et al.*, 1986; Yao *et al.*, 2010) which encouraged our resulted polyphenols and flavonoids contents as future perspective to prevent diseases.

Biological activities

Drugs finding work from plants has been improved worldwide to get a daily extended demand of public for these drugs reveal various biological effects like antidiabetic, antiviral and anticancer (Dahanukar *et al.*, 2000). The components of *D. kaki* possess a large number of pharmacological activities as anti-inflammatory, antibacterial, blood pressure-lowering, anti-cancer and lipid lowering effect (Yin *et al.*, 2010).

While performing our work, the phytoconstituents which we have observed are well known in various pharmacological activities.

Table 4. Anti-bacterial effects and minimum inhibitory concentration in *D. kaki*.

Sr. No.	Sample name	E. Coli Mean ± SD	MIC Concentration	MIC Mean	Streptococcus Mean ± SD	MIC concentration	MIC Mean
1	Standard	39.5 ± 2.3			45.50 ± 0.9		
2	Leaves Methanol	29.25± 0.8	90%	12.25	13.00 ± 1.6	90%	12
			70%	9.75		70%	10.25
			40%	9.00		40%	9.25
			10%	Nil		10%	Nil
3	Leaves Ethanol	11.75± 1.2	90%	9.75	16.75 ± 1.5	90%	12.75
			70%	Nil		70%	10.00
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
4	Bark methanol	9.75 ± 0.9	90%	9.75	12.75 ± 1.5	90%	9.25
			70%	Nil		70%	Nil
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
5	Bark ethanol	11.5 ± 2.0	90%	9.50	9.75 ± 0.9	90%	9.00
			70%	Nil		70%	Nil
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
6	Stem methanol	12 ± 2.1602	90%	9.25	12.00 ± 1.4142	90%	9.5
			70%	Nil		70%	Nil
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
7	Stem ethanol	16.25± 1.2	90%	12.50	10.25 ± 1.2	90%	9.25
			70%	10.25		70%	Nil
			40%	9.25		40%	Nil
			10%	Nil		10%	Nil
8	Unripe fruit methanol	9.75 ± 0.9	90%	9.00	15.75 ± 0.9	90%	13.25
			70%	Nil		70%	9.75
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
9	Unripe fruit ethanol	10.25± 0.9	90%	9.5	32.5 ± 0.8	90%	14.50
			70%	Nil		70%	12.25
			40%	Nil		40%	9.75
			10%	Nil		10%	Nil
10	Ripe fruit methanol	11.00± 1.2	90%	10.25	20.25 ± 1.7	90%	13.75
			70%	Nil		70%	11.25
			40%	Nil		40%	9.25
			10%	Nil		10%	Nil
11	Ripe Fruit ethanol	12.25± 1.2	90%	9.25	18.75 ± 1.7	90%	12.75
			70%	Nil		70%	9.00
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
12	Roots methanol	14.75± 1.7	90%	10.5	19.00 ± 1.8	90%	13.75
			70%	9.00		70%	9.5
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil
13	Roots ethanol	15.5 ± 1.2	90%	13.25	17 ± 1.6	90%	12.5
			70%	10.75		70%	9.00
			40%	9.25		40%	Nil
			10%	Nil		10%	Nil

Antibacterial activity

This study showed that antibacterial activity has been found in all organs of *D. kaki* (Table 4). As compared to standard drugs, the results revealed that in both the extracts bacterial activity for the *streptococcus* was more sensitive as compared to *E. coli*. Ethanol extract of unripe fruit showed the strongest zone of inhibition 32.50 mm against gram positive bacteria *"the streptococcus"* as compared to other organs which was found nearly equal to standard (ciprofloxacin 250 mg) zone of inhibition (45.5 mm). Methanol extract of leaves revealed 29.25 mm inhibitory action against the gram negative bacteria "*E. coli"* which was found not more lower than standard (39.50 mm) inhibitory zones. In this study, we observed that the ethanolic extract showed better antibacterial activity against the gram-positive bacteria in comparison to the gram-negative bacteria. The reported antibacterial activity by many authors in different medicinal plant extracts (El Astal *et al.*, 2005; Khan *et al.*, 2007; Jain *et al.*, 2008) encouraged our present findings.

Table 5. Anti-fungal potential in extracts of *D. kaki.*

Sr. No.	Extracts	*A. Niger* Mean ± SD	*A. Flavus* Mean ± SD
1	Standard	20.25 ± 2.5	20.50 ± 2.0
2	Leaves Methanol	14.75 ± 2.7	13.00 ± 1.6
3	Leaves Ethanol	11.5 ± 2.0	14.25 ± 1.7
4	Bark methanol	11.75 ± 1.2	12.75 ± 2.5
5	Bark ethanol	12.75 ± 0.9	13.00 ± 2.1
6	Stem methanol	13.00 ± 2.1	13.25 ± 1.7
7	Stem ethanol	12.25 ± 1.2	9.75 ± 0.9
8	Unripe fruit methanol	9.25 ± 0.9	15.50 ± 2.0
9	Unripe fruit ethanol	17.50 ± 0.8	16.25 ± 2.0
10	Ripe fruit methanol	12.25 ± 1.7	13.25 ± 2.0
11	Ripe Fruit ethanol	10.00 ± 2.6	13.50 ± 1.7
12	Roots methanol	14.50 ± 1.2	11.25 ± 0.9
13	Roots ethanol	16.75 ± 2.0	14.75 ± 1.5

The MIC values of different parts of *D. kaki* were observed (Table 4). MIC of all extracts was found in 90%, 70%, 40% and 10% concentrated samples against the same bacterial strains *E. coli* and *streptococcus*. Every sample showed inhibitory zone at 90% concentration and no inhibition was found at 10% concentration. Total activity range against the *E. coli* and *streptococcus* was 9 mm-14.50 mm.

Antifungal Activity

Methanol and ethanol extracts of different parts of *D. kaki* were assessed for their antifungal activities against a fungal strain of *A. flavus* and *A. niger* (Table 5). In this study , the *D. kaki extracts* showed appreciable activity against fungal strains. In comaprison to standard antibiotic drugs, *D. kaki* extracts were found not to be inactive against *A. flavus and A. niger* . Ethanol extract of unripe fruit showed the highest inhibition zone of 17.50 mm against *A. niger* and 16.25 mm against *A. flavus* which was found almost near to standard drug (20.25 mm) (Table 5) even at very low concentrations. The ethanolic unripe fruit extract contained antifungal activity against microbes could be due to significant presence of phenolic compounds, tannins, terpeneoids saponins and flavonoids. The phytochemicals such as phenolic compounds and flavonoids present in plants as secondary metabolites may be accountable for antimicrobial activity (Shahidi Bonjar *et al.*, 2004; Thirumurugan, 2010; Hosamani *et al.*, 2011) which could assist our present findings. The polyphenolic compounds as flavonoids

consist of many biological activities according to their strength to form complex with microbial extracellular wall (Wuyep *et al.*, 2017). Therefore, the antifungal activity in unripe fruit extract of *D.kaki* agreed with reported work in literature.

Anti-diabetic activity (α- amylase inhibition)

In present study methanol and ethanol extracts of all organs showed anti-diabetic activity with both concentration of 100 µg/mL and 400 µg/mL but ethanol extract of unripe fruit with concentration 400µg/mL showed significant α- amylase inhibition of 67% which was found less than 82% inhibition of standard drug (Acarbose). Although resulted inhibition was less than standard drug but unripe fruit reflected appreciable anti-diabetic activity (Fig 1) which could be due to large presence of polyphenols. The Antioxidant potential of the greater number of biologically active polyphenols such as tannins and flavonoids of *D. kaki* has been reported to control the diabetes resulted from oxidative damage (Hosny and Rosazza, 2002).

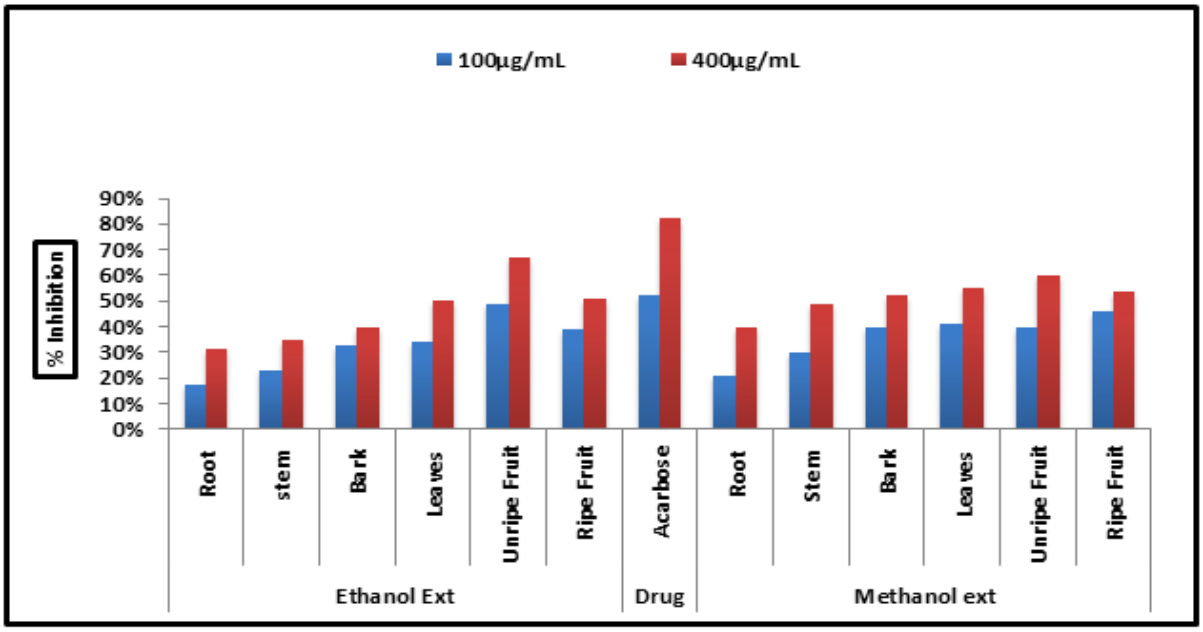

Fig. 1. α- Amylase inhibitory activity of different extracts of *D. kaki*.

It has also been reported that unripe fruit extract of *D. kaki* prevent hypercholesterolemia and its role as antioxidant, anti-diabetic, hypotensive, anticancer, antif-ebril and demulcent as well as juice from unripe fruit for treatment of hypertension (Singh and Joshi, 2011; Yaqub *et al.*, 2016).

Another report investigated the major water-soluble polyphenols like proanthocyanidin in *D. kaki* leaves with α-amylase inhibition (Kawakami *et al.*, 2010) which could assist resulted anti-diabetic activity of methanolic extract of unripe fruit in this study. So *D. kaki* raw fruit being rich in phytochemicals as reported above can play crucial role in treatment and prevention of diabetes mellitus further reinforced our research work on *D. kaki* plant.

Anticancer activity

The current study indicated less anticancer potential in almost all parts of *D. kaki* but methanol extract of *D. kaki* leaves showed anticancer activity 40% against Hela cell lines but found less than standard drug (Doxorubicin) (70%) as shown in Fig.2. Even though resulted anticancer activity in leaves extract of *D. kaki* was quite less than standard but found not to be inactive even at very low concentration (30 ug /mL) which could be the result of remarkable polyphenols including total flavonoids. Flavonoids in association vitamin C has also been reported to show a protective effect on cancer of esophagus using data from retrospective study organized in northern Italy (Rossi *et al.*, 2010). In another report on *D. kaki*, flavonoids showed a positive impact on human health as anti-

carcinogenic agent (Suzuki *et al.*, 2005) motivated our resulted carcinogenic effects of *D. kaki* leaves. Previously, *D. kaki* leaves have also been reported to consist of biologically active constituents comprising to large number of biological activities such as anti-cancer , anti-inflammatory, lowering of blood pressure and anti-bacterial effect (Zhang *et al.*, 1983).

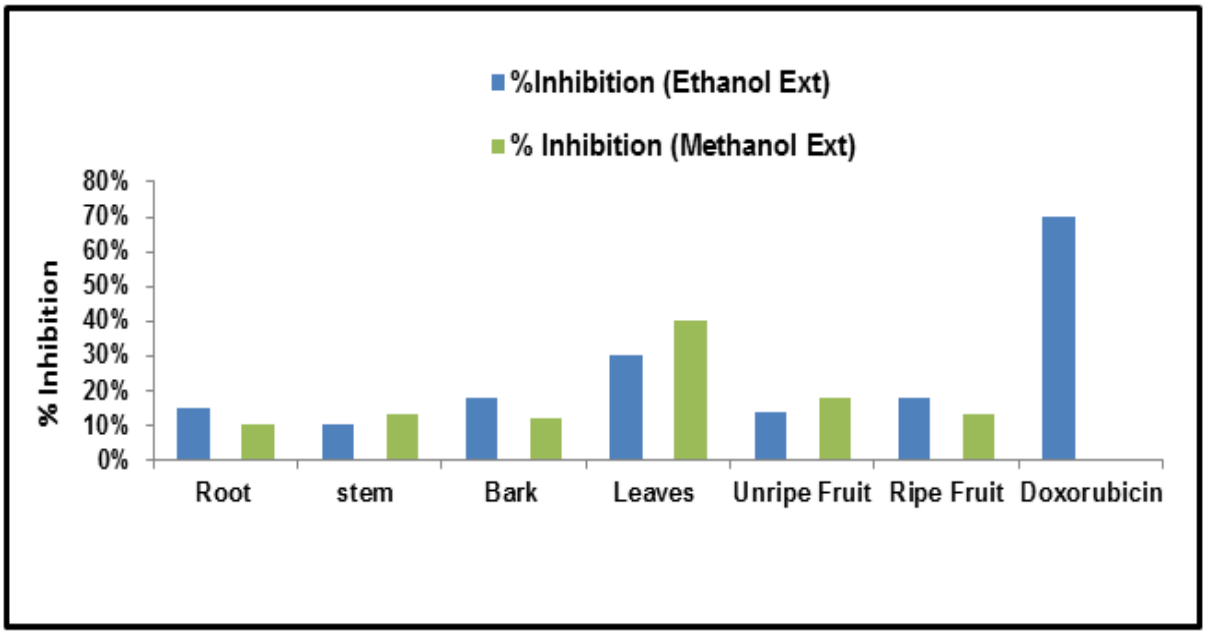

Fig. 2. Anticancer activity of different extracts of *D. kaki*.

It have been provided by extensive evidence in many preclinical effective studies that synthetic as well as naturally occurring derivatives of terpenoids possess both therapeutic and chemo protective effects against breast, skin, colon and prostate cancer (Liby *et al.*, 2007; Chaturvedi *et al.*, 2008; Rabi and Gupta, 2008; Mullauer *et al.*, 2010; Bishayee *et al.*, 2011; Patlolla and Rao, 2012). Keeping in mind above reported carcinogenic effects, methanol leave extract of *D. kaki* could show remarkable carcinogenic effects by increasing extract concentrations.

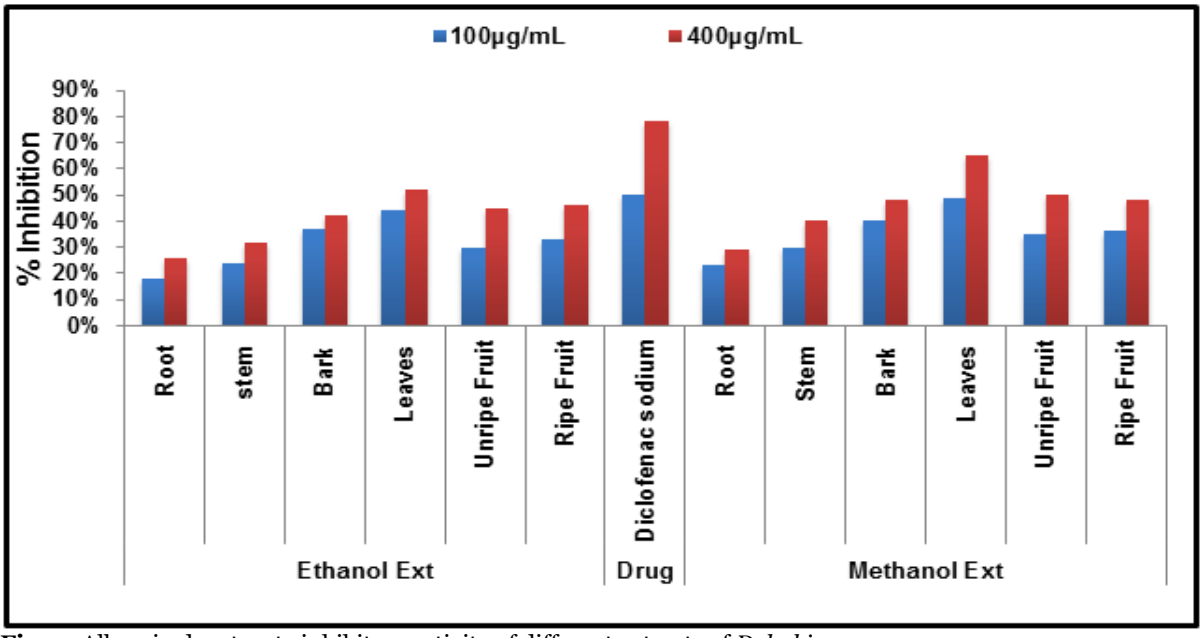

Fig. 3. Albumin denaturate inhibitory activity of different extracts of *D. kaki*.

Anti-inflammatory activity (Inhibition of albumin denaturation)

Mostly biological proteins do not function properly when denatured and protein denaturation is one of distinct reason of inflammation (Leelaprakash and Dass, 2011). In this research work, ethanol and methanol extracts of all organs demonstrated inhibition of albumin denaturation at 100µg/mL and 400µg/mL concentrations but the methanol extract of leaves with 400 µg/mL showed highest inhibition of 65% which was less than 78% inhibition of Diclofenac sodium (standard drug) (Fig 3). This accountable anti-inflammation of methanol leave extract of *D. kaki* could be due to the presence of bioactive compounds including flavonoids, saponins, tannins, terpenoids and phenolics. A wide range of phytochemicals in *D. kaki* have been known to comprise a large number of physiological benefits such as anti-inflammatory, anti-carcinogenic and antioxidant activities (Wang *et al.*, 1996; Hollman and Katan, 1999).

Conclusion

In this study, biological activities were shown by all extracts of *D. kaki* but methanol extract of *D. kaki* leaves demonstrated remarkable anti-inflammatory and anticancer activities as well as antibacterial activity against the gram negative bacteria *E. coli*. Similarly, the appreciable anti diabetic, antibacterial and antifungal activities were reflected by ethanol extract of unripe fruit along with considerable antifungal activity by methanol extract of unripe fruit also even at lowest possible concentration (µg/mL). Potent biological activities reflected by unripe fruit and leaves extracts could be due to the significant presence of polyphenolic compounds including flavonoids, saponins, tannins, terpenoids, phenols and other biologically active compounds comprising of antioxidant characteristics. These extract might be free radical inhibitors or possibly serve as primary oxidants to inhibit the albumin denaturation, microbes, carcinogenic effects and diabetes. Therefore, leaves and unripe fruit could be selected at wide spectrum screens towards emerging a broad range of therapeutic and pharmaceutical drugs. Moreover, *D. kaki* may assist isolation, purification and characterization of new phytochemicals in the development of pharmaceutical herbal drugs. So, we may take this plant as an excellent natural source to treat many diseases and may be effective in the activity guided isolation of its active natural products.

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