Antibacterial activities of *Diospyros blancoi, Phoenix dactylifera* and *Morus nigra* against dental caries causing pathogens: An *in vitro* study

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Abstract: The study was undertaken to determine the in-vitro antibacterial potential of *Diospyrosblancoi*, *Phoenix dactylifera* and *Morusnigra* leaf extracts in hexane, chloroform, methanol, ethyl-acetate and aqueous extracts against dental caries causing bacteria. Disc diffusion assay was used to determine the antibacterial efficacy; the extracts were further separated using Thin Layer Chromatography and the anti-biofilm activity of the extracts was also determined. The preliminary phytochemical screening of the extracts revealed the presences of flavonoids, saponins, alkaloids and tannins because of which the extracts showed strong antibacterial activity against the selected pathogens. The ethyl-acetate extracts showed maximum inhibitory effect on biofilm formation by *S. mutans.*96% inhibition was observed in methanol extract of *Diospyrosblancoi*, and 95% in ethyl acetate extract. The results evidenced that the plants inhibit the growth of oral bacteria responsible for dental caries with their abundance source of secondary metabolites and can be used as an alternative treatment for caries, thus minimizing the antibiotics used to treat the disease in local medicine.

Keywords: Antibacterial, dental caries, plant extracts

INTRODUCTION

Due to high occurrence and effecting individual's quality of life oral diseases is the major public health problem (Butt *et al*., 2009). Dental caries, a very common oral condition caused by a biofilm of microorganisms that are present on the tooth surface (Allaker and Douglas, 2009; Ambrosio *et al*., 2008). The available published data exhibit that caries condition varies among population and from country to country among developing and developed ones (Cummins, 2013). Dental caries is the most common childhood disease; five-times more common than childhood asthma. It is also the primary pathological cause of tooth loss in children (Tatintcyan and Minalyan, 2005). For treating caries, the usual procedure is tooth extraction (Petersen *et al*., 2005), and use of antibiotics (e.g. tetracycline, erythromycin, vancomycin and penicillin) to prevent tooth decay (Joshi and Joshi, 2005; Amadi *et al*., 2007) but their use is associated with side effects. The use of antibiotics also leads to resistant bacteria (Diazgranados *et al*., 2008) which are itself a threatening situation. This situation diverted the efforts towards finding natural products as the potential medicine for treating dental caries that are safe to use. Presence of wide variety of secondary metabolites in medicinal plants having *in vitro* antimicrobial activities provide a hope for novel drug compounds (Lewis and Ausubel, 2006).

Diospyrosblancoi (Family: Ebenaceae), *Phoenix dactylifera* (Family: Arecaceae) and *Morusnigra* (Family: Moraceae) were selected for present investigation as these plants possess a range of medicinal properties. Traditionally *Diospyrosblancoi* is used to treat fungal diseases, insomnia, internal hemorrhage, vermifuge and vermicide, astringent and as bactericidal (Tezuka *et al*., 1973; Thomas *et al*., 2006) and also has antidiarrheal activity. Juice of bark and seed is used to treat snakebite, dysentery (Ghani, 2003). The anti-inflammatory activity is due to triterpenoids (Chopra *et al*., 1956). *Phoenix dactylifera* has applications like antidiarrheal, anti-inflammatory, anti-proliferative (Elberry *et al*., 2011), antibacterial (Al-Daihan and Bhat, 2012; Perveen *et al*., 2012), anti-mutagenic, antioxidant (Saddiq and Bawazir, 2010; Bokhari and Perveen, 2012) anti-diabetic (Zangiabadi *et al*., 2011; Fatima *et al*., 2012;Michael *et al*., 2013). *Morusnigra* also has many applications like antibacterial, antifungal, antiviral, anti-nematodal, anti-oxidant, anti-cancer, cytotoxic, anti-inflammatory and immune-regulating activities (Kim *et al*., 1999; Zhang *et al*., 2009; Ozygen *et al*., 2009). In view of these reported medicinal applications, the present work was carried out to evaluate the antibacterial potential of five different leave extract against six dental caries causing microorganisms.

MATERIALS AND METHOD

Collection, Drying and extraction of plant material

Plants were collected from different areas of Rawalpindi, Islamabad and Lahore, Pakistan from March-May, 2010 respectively and they were identified by Dr. Abdul Nasir from Department of Plant Sciences, Punjab University Lahore. The shade-dried and powdered aerial parts of selected plants (500g each) were then subjected for extraction by cold maceration method successively with methanol occasionally shaking for a week. This was then

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filtered and same process was repeated twice using same volume of solvent each time, it was further subjected to fractionation successively with n-hexane, chloroform, ethyl acetate and water These extracts were concentrated under vacuum by Rotavapor-R20 at 40ºC to obtain crude semi solid mass. The quantity of semi-solid extract obtained after drying is given in table 1.

Test microorganisms

Six bacterial strains were tested**.** *Streptococcus mutans* (ATCC 25175) *Streptococcus mitis*, *Staphylococcus aureus* (ATCC 12600) *Pseudomonas aeruginosa* (ATCC 29999) *Bacillus subtilis* (ATCC 11774) and *Escherichia coli* were procured from Microbiologics Pakistan. These were then sub cultured on Nutrient agar, LB and Brain Heart plates and incubated aerobically at 37°C for 24 hours

Antibacterial assay of crude extract

Preparation of inocula using mcfarland 0.5 turbidity standard: Overnight bacterial cultures in nutrient broth was vortexed and turbidity was adjusted by adding sterile saline, while using 0.5 McFarland turbidity standard as reference until 10^6 colony forming unit (CFU) per ml was obtained and was used as inocula.

Preparation of agar plates: Nutrient agar and Brain heart infusion medium was used for antibacterial activity. Media was sterilized by autoclaving with pH 7. When it was cooled up to 45°C, it was seeded with 10ml of bacterial inoculum and it was poured in sterilized petri plates. Plates were then allowed to dry and then with sterile borer wells of 6mm were made in each plate

Assay: 100µL of each extract was propelled in the wells of already inoculated specific media agar plates for each organism. The plates were allowed to stand for 10-15 minutes for proper diffusion of the extract, after this it was incubated at 37ºC for 24h (Aneja *et al*., 2010;Khokra *et al*., 2008). The diameter of zone of inhibition for each concentration was measured and compared with standard antibiotic (ciprofloxacin) and negative control DMSO. Assay was done in duplicate.

Phytochemical analysis

The crud extract of the plants were screened for the presences of different groups of secondary metabolites like saponins, alkaloids, tannins and flavonoids (Parekh and Chanda, 2007).

Test for alkaloids

0.5 g of the extract was stirred with 5mL of 1% aqueous HCL on water bath. Divide it in 2 parts. Treat 1mL of filtrate with few drops of Mayer's reagent and the other 1mL with Dragendorff's reagent. Turbidity or precipitation is the confirmation of the presences of alkaloids

Test for saponins

The ability of saponins to produce frothing in aqueous solution is used as screening for this compound. 0.5g of plant extract was dissolved in water and shaken vigorously. Appearance of forth indicate the presences of saponins.

Test for tannins

0.5g of extract was dissolved in 10mL of distilled water in the test tube. After filtration 0.1% $FeCl_3$ was added to the filtrate. Appearance of blue-black, green or blue-green precipitate showed the presences of tannins.

Test for flavonoids

0.5g of extract was dissolved in ethanol, warmed and filtered. After this 3 pieces of magnesium turnings was added to the filtrate and then few drops of conc. HCl was added. Appearance of pink, red or orange colour indicates the presences of flavonoids.

Phytochemical analysis by thin layer chromatography (TLC) of crude extracts

TLC separation was carried out on silica gel 60 F_{254} plates (Merck) of 20×20cm size and 0.25mm thickness. Different solvent system were tested, with final solvent system of Hexane: Chloroform: Methanol (2:7:1) for *Diospyrosblancoi*, Chloroform: Methanol (7:3) for *Morusnigra* and (5:5) with same solvents for *Phoenix dactylifera*. The plates were observed under UV light at 254 and 365nm and their Rfvalues are noted.

Table 1: Total yield obtained after fractionation of crude extract

Plant methanol n-hexane chloroform ethyl acetate aqueous
Fraction (g)
*Morisnigra*401012103
*Phoenix dactylifera*306884
*Diospyrosblancoi*255882

Detection of biofilm formation in streptococci and Biofilm inhibition assay

BHI broth was prepared according to given instructions with 1% D-glucose. 3ml of this broth was then shifted to sterilize screw cap tubes under aseptic conditions and autoclaved. The broth was then inoculated with overnight culture of *S. mutans* and *S. mitis*. Selected extracts (inhibitor of biofilm formation) were also added in different concentrations to give the desired concentration of extract from stock solution (100mg/mL of DMSO). The controls are without the extracts. They were then incubated at 37ºC for 18 hours at an angle of 30º. After incubation, pH was noted and supernatant was removed. Wash the tube with biofilm with .85% normal saline, then add 3 ml of saline in the tube and shake the tube well to separate the cells adhered with wall (Shivani *et al*., 2012).

OD was measured at 550nm using Varian-fluorescence spectrophotometer (Model: SYS-FL-FCI). The effect of an inhibitor measured as the percentage decrease in reaction rate. Percent inhibition is calculated as:

Percent Inhibition = Rate without inhibitor - rate with inhibitor / Rate without inhibitor × 100

STATISTICAL ANALYSIS

All experiments were conducted in duplicate. Data was analyzed statistically using standard deviation and excel (2010 version).

RESULTS

Since decades the activity of plants against bacteria has been studied but it increased during the last three decade. There is enough published data on several antimicrobial evaluations based on the traditional African, Asian and Chinese plant-based drugs (Suffredini *et al*., 2004). The antibacterial assay clearly indicates that the selected plants have antibacterial activity but it varies from solvent to solvent against different oral pathogens. Antibacterial activity of *Phoenix dactylifera* extarcts indicates that n-hexane frcation was inactive against all the bacterial strains. The aqueous, methanol and ethyl acetate fraction was active against three bacterial strains, while the chloroform fraction was active against *B. subtilis* only (fig. 1). Antibacterial activity was found to be moderate in chloroform and ethyl acetate crude extract of *Diospyros blancoi* against all tested bacteria with maximum inhibition zone of 20mm against *S. aureus* and *S. mutans* in ethyl acetate extract. Aqueous and n-hexane fraction was almost in-active against all the bacteria (fig. 2). In case of *Moris nigra*, the ethylacetate fraction was found active against four bacterial strains, with no activity in n-hexane and aqueous fractions (fig. 3).

Phytochemical analysis

Analysis of *Moris nigra* (table 2) revealed the presences of moderate quantities of saponins in chloroform and methanol fraction. Moderate quantities of tannins were also present in methanol fraction. Tannins and flavonoids were found absent from hexane fraction. Saponins are absent in aqueous fraction. The phytochemical analysis of *Phoenix dactylifera* revealed the presences of alkaloids and tannins in all the extracts (table 3). High concentrations of alkaloids are present in methanol and ethyl acetate fraction. Low quantities of tannins are found in all the extracts. Saponins and flavonoids were found absent in hexane fraction.

Phytochemical analysis of *Diospyros blancoi* (table 4.8) indicated the presences of saponins in high quantities in hexane and ethyl acetate fraction. Moderate quantities of alkaloids, tannins were found in hexane, ethyl acetate and methanol fraction. Flavonoids were found absent in hexane fraction. Tannins were also absent in chloroform fraction.

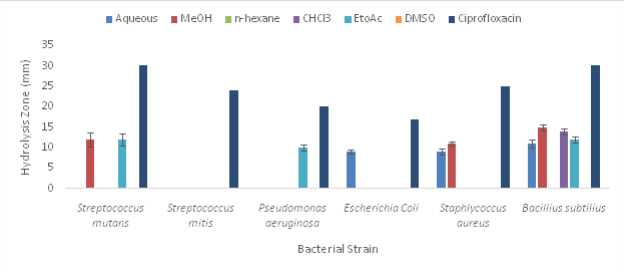

Fig. 1: Antibacterial activities of crude fractions of leaves of *Phoenix dactylifera*

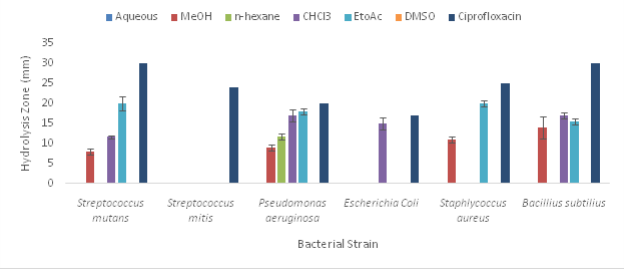

Fig. 2: Antibacterial activities of crude fractions of leaves of *Diospyros blancoi*

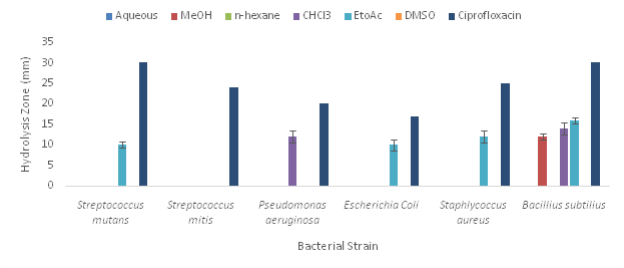

Fig. 3: Antibacterial activity of crude fractions of leaves of *Morus nigra*

Thin layer chromatography (TLC) separation

Results of TLC are depicted in table 5 and fig. 4. With Ethyl acetate: chloro form and methanol solvent system for *Diospyrosblancoi* we obtained 6 spots with highest Rf value of 0.85. In chloroform and methanol solvent system (7:3) for *Morusnigra* we obtained 2 spots and with same solvent system (5:5) for *Phoenix dactylifera* we obtained 3 spots with 0.14 the highest Rf value.

Anti-biofilm formation (using 1% dextrose) by plant extracts

The extracts with antibacterial activity showed positive anti-adherence effect on streptococcal biofilm formation on the glass surface with 1% dextrose. The extracts also inhibited the biofilm formation with decrease turbidity at 550nm. Maximum inhibition was observed in methanol and ethyl acetate extracts of *Disopyrosblancoi* (table 6). The ethyl acetate and aqueous extract of *Phoenix*

Table 2: Phytochemical analysis of *Morusnigra*

Biochemical Alkaloid Tannins Saponins Flavonoid Test Mayer's test Dragendorff test ($FeCl_3$)(Mg-ribbon)					
Hexane fraction	-	+	-	+	-
Chloroform fraction	+	+	+	++	+
Ethyl acetate fraction	+	+	+	+	-
Methanol fraction	+	+	++	++	+
Aqueous fraction	-	+	+	-	+

-, absent,+ present,++ Highly present

Table 3: Phytochemical analysis of *Phoenix dactylifera*

Biochemical Alkaloid Tannins Saponins Flavonoids Test Mayer's test Dragendorff test ($FeCl_3$)(Mg-ribbon)					
Hexane fraction	+	+	+	-	-
Chloroform fraction	+	+	+	+	+
Ethyl acetate fraction	+	++	+	+	+
Methanol fraction	++	+	+	+	+
Aqueous fraction	+	+	+	+	+

-, absent,+ present,++ Highly present

Table 4: Phytochemical analysis of *Diospyrosblancoi*

Biochemical Alkaloid Tannins Saponins Flavonoids Test Mayer's test Dragendorff test ($FeCl_3$)(Mg-ribbon)					
Hexane fraction	++	-	+	+++	-
Chloroform fraction	+	+	-	+	+
Ethyl acetate fraction	-	+	++	+++	+
Methanol fraction	++	++	++	+	+
Aqueous fraction	+	+	+	+	+

Table 5: Results of TLC of crude extracts

Solvent system	Number of spots obtained	Rf value
Ethyl acetate: Chloroform: Methanol	Compound 1	0.21
	Compound 2	0.56
	Compound 3	0.75
	Compound 4	0.85
	Compound 5	0.53
	Compound 6	0.45
Chloroform: Methanol	Compound 1	0.46
	Compound 2	0.45
Chloroform: Methanol	Compound 1	0.14
	Compound 2	0.13
	Compound 3	0.12

dactylifera also had strong anti-adherence activity. The ethyl acetate extract of *Morusnigra* also shoed 87% of inhibition. The decrease in pH was also observed as is shown in table 6.

DISCUSSION

Nature produces a great array of natural products, with most diversity seen in the plants and microorganisms (König, *et al.*, 2006, Wink, 2008). Plants are regarded as libraries of small molecules with great diversity in structure, which would otherwise be unavailable in synthetic chemistry. In present study the crude extracts of *Diospyros blancoi*, *Morus nigra* and *Phoenix dactylifera* were obtained by using methanol as main extraction solvent and then it was fractionated using solvent comibinations of variying polarity starting from non-polar to polar solvents. The fractions hence collected as well as the crude extracts were evaluated for their antibacterial potential using Agar well and disc diffusion assay against six oral bacterial strains i.e. *Streptococcus mutans* (ATCC 25175) *Streptococcus mitis*, *Staphylococcus aureus* (ATCC 12600), *Pseudomonas aeruginosa* (ATCC 29999), *Bacillus subtilis*, and *Escherichia coli*.

The antibacterial activities observed in *Phoenix dactylfera* are due to presences of different secondary metabolites. Abbas and Ateya, 2011 reported the presences of, Cerotic

Table 6: Anti-adherence effect of plant extracts (with 1% dextrose) at 37ºC

Plant extract Fraction Concentration pHOD % age Inhibition (µg/ml)				
Phoenix Ethyl acetate	100	6	0.145	93%
dactylifera Aqueous	100	6	0.100	90%
Control	-	7	0.912	-
Diospyros Ethyl acetate	100	6	0.025	95%
Blancoi Methanol	100	6	0.145	96%
Chloroform	100	6	0.154	76%
Control	-	7	0.822	-
Morus Ethyl acetate	100	6	0.12	87%
Nigra Control	-	7	0.78	-

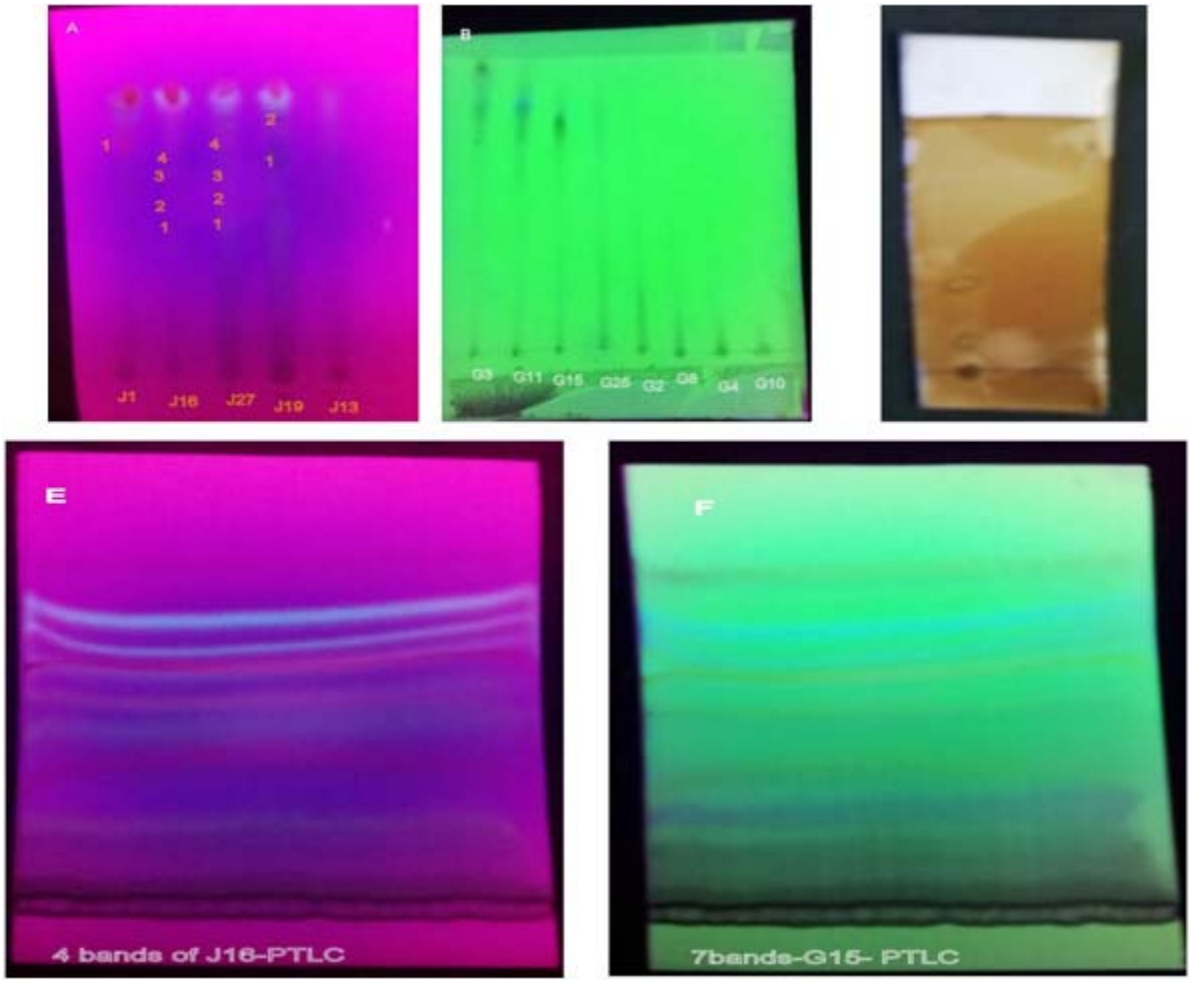

Fig. 4: The TLC plates of crude extracts of ethyl acetate extract of *Diospyrosblancoi* (A), *Morusnigra* (B), *Phoenix dactylifera* (C). E &F are the TLC fingerprinting of A and B

acid, Lignoceric acid, Behenic acid, in n-hexane fraction and Luteolin-7-O-glucoside, Isorhamnetin-3-O-glucoside, Naringin, Apigenin and Rutin in ethyl acetate fraction of *Phoenix dactylifera*. Aqueous and n-hexane fraction of *Diospyros blancoi* was almost in-active against all the bacteria, while moderate activity was observed in methanol, ethyl acetate and chloroform fractions. This supports the studies conducted by (Maridass, et al., 2008) which confers the antimicrobial activity of Diospyros blancoi. Diosquinone, might be the reason of activity we observed against *Staph. aureus* (Watt, 1980). In case of *Morus nigra*, the ethylacetate fraction was found active against four bacterial strains which is in accordance with the findings of (Ali *et al*., 2013; Malik *et al*., 2012)with no activity in *n*-hexane and aqueous fractions.

Phytochemical analysis revealed the presences of large number of secondary metabolites that are responsible for all the biological activities of the plants. Coumarins, flavonoids, phenols are reported in leaves of *Morus nigra* (Zhang *et al*., 2009). Alkaloids, phenols and flavonoids

are also reported in this plant (Ali *et al*., 2013; Malik *et al*., 2012; Ozgen *et al*., 2009). Cinnamic acids, flavonoid glycosides, flavanols are reported phenolic compounds (Mukherjee *et al*., 1998) in *Phoenix dactylifera***.** The phytochemical analysis of *Diospyros blancoi* revealed the presences of tannin (Shoba and Thomas, 2001), alkaloids (Galvez *et al*., 1993), flavonoids (Otshudi *et al*., 2000), sterols, saponins, terpenes and reducing sugars (Maj *et al*., 2010).

As a result of TLC, different spots were identified on the basis of Rf values.The variation in different extracts might be due to difference in polarity of solvent systems, which determines the type of reactions and solubility of compounds. Chloroform and methanol have the ability to extract the antimicrobial compounds like flavonoids, alkaloids, tannins. Anti-biofilm formation assay was performed and for present study the leaf parts of the plants were selected because leaves contain more secondary metabolites which are responsible for antimicrobial activity (Duarte *et al*., 2006). The polyphenolic compounds isolated from plants have anti-caries activity, which is due to growth inhibition against oral bacteria.

CONCLUSION

Our study results showed the scientific proof for the use of these indigenous plants for oral care and also for treating the multi drug resistant bacteria and for dealing with the side effects of synthetic drugs that are used to treat dental caries locally. Little literature is available for use of these plants in anti- biofilm formation study. We are reporting for the first time the anti-caries activity of these three plants. The results evidenced that the plants inhibit the growth of oral bacteria responsible for dental caries with their abundance source of secondary metabolites and can be used as an alternative treatment for caries, thus minimizing the antibiotics used to treat the disease in local medicine.

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