Abstract

The various plant parts (stem, leaf, bark, fruit and sap) extract of *Ficus benghalensis* had been known for their anathematic, analgesic, anti-inflammatory, antioxidants, antidiabetic, immuno-modulatory and antimicrobial activity for ancient time. These plant part extracts are used in various ayurvedic medicines to treat diarrhea, dysentery and piles, teeth disorders, rheumatism, skin disorders like sores and to boost immune system. The present study was focused on the prop root extracts (hydro & ethanolic) of *F. benghalensis* against the pure bacterial strains of human oral cavity which inhibits gingival and supragingival diseases and was also analyzed for the bioactive phytochemicals compounds. The qualitative analysis of the *F. benghalensis* prop roots crude hydroextract revealed the presence of flavonoids, saponins, steroids and reducing sugars while ethanolic extracts showed the presence of alkaloids, tannins, and glycosides additionally. The Liquid Chromatography Mass Spectroscopy (LCMS) showed presence of \( \alpha \)-ethyl-\( \alpha \)-phenyl glutarimide, 1, 2- Dihexanoyl-sn-Glycero-3-Phosphocholine, Antimony trisulfide, 2, 2, 2-trichloroethanol, Antimony trioxide at retention time of 1.586 min., 12.377 min., 13.646 min., 14.376 min., and 22.185 min respectively in crude hydroextract of prop roots of *F. benghalensis*. The Thin Layer Chromatography (TLC) product as bioactive compound was identified as 2-chloroethyl phosphonic acid. The antibacterial activity of crude hydroextract were also determined and found the encouraging results against bacterial strain *Streptococcus* sp. (8 mm zone of inhibition), *Lactobacillus* sp. (9 mm), *Actinobacillus* sp. (7 mm), *Fusobacterium* sp. (9 mm) but TLC separated product (2-chloroethyl phosphonic acid) showed antibacterial activity only against *Lactobacillus* sp. as 17 mm zone of inhibition.

Key words: *Ficus benghalensis*, TLC, prop roots, immuno-modulatory, phytochemicals, LCMS.

Introduction

More than 700 species of bacteria inhabit the oral cavity and many of these are associated with oral diseases (1). The development of dental caries are due to the acidogenic and aciduric Gram-positive bacteria (*Streptococcus mutans* and *S. sobrinus*), *lactobacilli* and *actinomycetes*, that metabolize sucrose to organic acids mainly lactic acid which dissolve the calcium phosphate in teeth, causing decalcification and eventual decay. Dental caries is therefore a supragingival stage (2) and periodontal diseases are subjected as subgingival stage that is linked to anaerobic Gram-negative bacteria (*Porphyromonas gingivalis*, *Actinobacillus* sp., *Prevotella* sp. and *Fusobacterium* sp.) (1, 3). Various parts of *F. benghalensis* is used as ayurvedic medicine against various microbial diseases. These plant
parts are acrid, astringent, anodyne, sweet, refrigerant, depurative, vulnerary, anti-inflammatory, antiarthritic, anti-diarrhoeal, anti-emetic, ophthalmic, stypic, diaphoretic and tonic traditionally (4). Mukherjee et al, 1998 (5); and Husain et al., 1992 (6) used some plant parts of *F. benghalensis* to treat diarrhea, dysentery and piles, Warrier et al, 1993 (7) used as rheumatism, skin disorders like sores and to boost immune system (8), as a hypoglycemic in ayurveda (9, 10, 11, 12). It is found to show antibacterial activity against dental caries causing bacteria *Actinomyces vicosus* (13).

*F. benghalensis* is the world's largest tree in terms of its spread and some of old trees covers over an acre of ground. It is also the national tree of India. Its name banyan is named for the merchants who set up their shop under the spreading trees. *F. benghalensis* is an epiphyte, in the beginning of its life, growing on another tree where some fig-eating bird deposited a seed. As it grows, it produces prop roots from its horizontal branches which then take root where they touch the ground. These prop roots create a forest on their own. This tree can get 100 feet tall and with its massive limbs supported by prop roots can spread over an area of several acres. It has large and thick leathery leaves (14). Plant-derived substances have become of great interest due to their availability, cellular totipotency, and various natural applications. These medicinal plants are the richest bioresource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (15). Natural constituents of plants can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components (16). The chemical constituents in plants may be therapeutically active or inactive. The bioactive compounds of plants are alkaloids, flavonoids, tannins, glycosides, steroids, reducing sugars, saponins etc.

The phytochemicals research based on the use of prop roots extract of *F. benghalensis* as an herbal remedy could be considered an effective and novel approach in the discovery of new anti-infective agents. The knowledge of the prop root chemical constituents of *F. benghalensis* could open the doors, not only for the discovery of economic materials (tannins, oils, gums, precursors for the synthesis of complex chemical substances) but also may be valuable in disclosing new sources of therapeutic agents such as anathematic, analgesic, anti-inflammatory, antioxidants, antidiabetic, immunomodulatory and antimicrobial activity and could be use cure various diseases like subgingival and supragingival of tooth disorders.

**Material and Methods**

The sample of prop roots of *F. benghalensis* was collected from Surajpur, Baddi, Himachal Pradesh, India-13205 in the month of February when the atmospheric temperature was 18°C. The specific locations were 30°53′15.44″N, 76°48′29.26″E and 1935 feet elevation. Sample was washed under running tap water to remove surface impurities and then was cut into small pieces of 2-3 cm, dried under reduced pressure, grinds to prepare a fine powder and stored in polypropylene container at 4°C for future use.

**Aqueous Extraction:** The 10% w/v aqueous prop root extract was subjected to extraction in a Soxhlet apparatus at 100°C for 48 hours in distilled water. Ten grams of the prop roots powder were loaded in the thimble of Soxhlet apparatus. It was fitted with appropriate size round bottom flask with 100 ml distilled water, and upper part was fitted with condenser. Constant heat was provided by heater for recycling of the solvent. After complete extraction, extracted material was evaporated to 20 ml at 50°C under reduced pressure. Furthermore, the extract in round bottom flask was transferred into clean boiling tubes and stored at 4°C.

**Ethanolic Extraction:** The 10% w/v ethanolic prop root extract was subjected to extraction in
a Soxhlet apparatus at 78.3°C for 48 hours in ethanol. After complete extraction, extracted material was evaporated to 20 ml at 35°C under reduced pressure. Furthermore, the extract in round bottom flask was transferred into clean boiling tubes and stored at 4°C. The percentage yield of extraction was calculated as:

Percentage yield of extract = \( \frac{\text{mass of extract (mg)}}{\text{mass of sample taken (mg)}} \times 100 \)

**Bioactive compound analysis:** The qualitative experiments were performed on both of the extracts to confirm the presence of the bioactive chemical constituents such as alkaloids, flavonoids, tannins, saponins, glycosides, steroids and reducing sugar by standard procedures. As for alkaloid detection, 10 ml of extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer’s reagent (Dissolve 1.358 g of HgCl₂ in 60 ml of H₂O and pour into a solution of 5g of KI in 10 ml of H₂O, final volume was made to 100 ml with distilled water). The sample was then observed for the presence of turbidity or yellow precipitation (17). For flavonoids, 1 ml of 10% NaOH was added to 3 ml of the extract. The sample was then observed for the formation of yellow color (17). For tannins, 4 ml of 5% FeCl₃ was added to 2 ml of extract. Then the sample was observed for the development of a dark green precipitate. In the second test, 4ml of freshly prepared 10% KOH was added to 4 ml of extract. It was then observed for the formation of a dirty white precipitate (18). For saponins, Frothing Test: 2 ml of extract was taken in a test tube and vigorously shaken for 2 minutes. The sample was then observed for the presence of froth at least 1 cm in height. Emulsion Test: Five drops of olive oil were added to 3 ml of concentration extract in a test tube and the mixture was vigorously shaken. Formation of stable emulsion was then observed in the sample (19). For glycosides estimation, 20 ml of 50% H₂SO₄ was added to 2 ml of conc. extract in a test tube. The mixture was heated in a water bath for 15 minutes. 10 ml of Fehling’s solution was then added and the mixture was boiled. The sample was then observed for the development of brick red precipitate. For steroids, 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. Concentrated H₂SO₄ was carefully added to the filtrate to form lower layer and observed for the formation of reddish brown color at the interface (17). And for the confirmation of reducing sugar, 0.5 ml of extract, 1 ml of water and 5-8 drops of Fehling’s solution was added at hot. It was observed for development of brick red precipitate (20).

**Preparative TLC Separation of bioactive compounds:** The TLC plates were cleaned and dried in hot air oven. Slurry was prepared by mixing silica gel G with distilled water in clean beaker with continuous stirring. Then slurry was poured over the glass plates with the help of TLC plate spreader to make film of 2.0 mm thick. Then the plate was allowed to cool at room temperature then marked about 2.0 cm from the bottom as the baseline. 10 µl aqueous extract was loaded at the center of the baseline of the plate.

**Development of Chromatogram:** The development tank was saturated with solvent system of chloroform, methanol and water (10:10:3) for the analysis of bioactive compounds. The plate was kept in the tank without touching baseline by solvent and left for development. After running the solvent about 1 hour, the final solvent front was marked and the plate was air dried. Few pieces of iodine crystals were kept in the tank. The tank was covered with glass plate to saturate the tank with iodine vapor. Then the plate was kept in iodine vapor saturated tank and left for few minutes till the spot appear onto the TLC plate. Rf value was calculated by measuring the distance traveled by the each bioactive compound to the total distance traveled by solvent in cm and each compound was also compared with respective controls.

**Collection of the TLC products:** Spots on the Phytochemicals from Prop Root extracts
preparative TLC silica gel G plates were scratched with the help of clean and dry spatula and collected in a beaker containing 10 ml of methanol and left overnight. Then the content in the beaker was stirred and filtered through Whatman no. 42 filter paper. The filtrates of each spot were collected in clean and dry test tubes. The filtrates were then concentrated. The filtrates containing bioactive compound were used for the LCMS identification (20, 21).

**Antibacterial activity determination:** Four different pure bacterial strains were isolated from the oral cavity of human mouth. These strains were identified as *Streptococcus* sp., *Lactobacillus* sp., *Actinobacillus* sp., and *Fusobacterium* sp. Discs were made out of absorbent paper. The sterilized discs were then soaked in 1 ml of bioactive compound of preparative TLC product, crude aqueous extract, and crude ethanolic extract. These discs were left overnight in the respective solutions of natural compounds. Next day, nutrient agar (peptone 5.0 g, beef extract 3.0 g, sodium chloride 5.0 g, pH 7.0, agar 15 g in one liter distilled water) plates were prepared. The 100µl inoculum of each bacterial strain was spreaded on to the agar plates. The discs of appropriate natural compounds were then placed on the agar plates after that the plates were incubated at 37°C for 24 hours. Then plates were observed for any inhibition of bacterial growth as indicated by a clear zone around the discs. The size of each the zone of inhibition was measured and expressed in mm.

**LCMS identification of the compounds involved:** The prop roots hydro-extract and TLC products of *F. benghalensis* were concentrated under reduced pressure with the help of rotatory evaporator and dried to powdered extracts. Dried extracts were dissolved in 10 ml of methanol and filtered through Whatman filter paper no. 42. Then these samples were passed through Na₂SO₃ column, collected and concentrated to 1ml. Liquid chromatography/mass spectrometry (LC/MS) with a quadruple ion trap MS was done. The column used was Symmetry (Walters) C18 column (250 x 4.6 mm). A 25µl sample volume was injected using the system’s autosampler. Solvent A contained 5% formic acid in water and solvent B consisted of HPLC-grade methanol. The flow coming from the LC was split 1:28 and introduced to the ESI (electrospray ionization) turbo spray. A scan rate of 4.000 amu/s was performed under negative ionization in the enhanced scan mode. The UV response during LC/MS was monitored at 360 nm. The LC/MS was operated in the positive- ion mode using Diode array detector and electrospray ionization (ESI) source: Ion spray voltage: -4.500 V, Declustering potential: -50 V, Entrance potential: -10 V, Collision energy: -90 to 10 V, Curtain gas: 40 psi, Nebulizer gas: 45 psi, Turbo gas: 80 psi. Finally compounds identification was corroborated based on the relative retention time and mass fragmentation pattern spectrums with those of standards and the NIST147 library database of the LCMS system (22).

**Results and Discussion**
During detection of phytochemicals in sample extracts showed the variation in type of phytochemicals present in different solvents (hydro and ethanol). The analysis for the phytochemicals of the *F. benghalensis* prop roots hydroextract revealed the presence of flavonoids, saponins, steroids and reducing sugars as well as ethanolic extract confirmed the detection of alkaloids, tannins and glycosides. Kawo (23); Yusha’u et al. (24); and Kawo et al. (25), supports the present study after concluding the result that these phytochemicals having the ability to dissolve into specific type of solvents. For rest of the nondetectable phytochemicals in both (hydro & ethanol) extracts of the prop root, Adoum et al. (26) and World Health Organization (WHO 2003) report explained the seasonal variations and the geographical location of a plant can affect the presence active constituents and chemical composition of the plants which may be induced the biological activity by many factors like climate, soil, and propagation method. Odugbemi (27), well explained the time of collection of plant sample for these detections also affects its efficiency.

Arun Kumar et al
The preparative TLC, Liquid chromatography and mass spectrosopy were performed for the analysis of bioactive chemical compounds present in hydroextract of the sample. As the Fig. 1.0 showed the TLC product at $R_f$ value of separated bioactive compound found as 0.7005. However this compound was confirmed as 2-chloroethyl phosphonic acid by compared with control compound $R_f$ value and Liquid chromatography and mass spectrosopy techniques (Fig. 3.0 to Fig. 3.1). The Liquid Chromatography Mass Spectrosopy (LCMS) also showed presence of $\alpha$-ethyl-$\alpha$-phenyl glutarimide (Doriden), 1, 2- Dihexanoyl-sn-
Fig. 2.2. MS of LC peak having retention time 12.377, 1, 2- Dihexanoyl-sn-Glycero-3-Phosphocholine (C_{20}H_{40}NO_{8}P) having molecular weight 453.5 was identified.

Fig. 2.3. MS of LC peak having retention time 13.646, Antimony trisulfide (S_{6}Sb_{4}) having molecular weight 679.4 was identified.

Fig. 2.4. MS of LC peak having retention time 14.376, 2, 2, 2-trichloroethanol (C_{2}H_{5}OCl_{3}) having molecular weight 149.4 was identified.

Arun Kumar et al
Glycero-3-Phosphocholine, Antimony trisulfide, 2, 2, 2-trichloroethanol, Antimony trioxide at retention time of 1.586 min., 12.377 min., 13.646 min., 14.376 min., and 22.185 min respectively in crude hydroextract of prop roots of *F. benghalensis* (Fig. 2.0 to 2.5). The LC pattern of active compounds separated after TLC is shown in Fig. 3.0.

As so many researchers isolated and purified many plant part extracts of variety of plants for the confirmed the presence of various phytochemicals for the exploiting of the pharmacological importance such as analgesic, anti-diabetic, antibacterial, and antibacterial properties. Jothy *et al.* (22) carried out to isolate active compounds in four steps using multiple extractions, fractionation using column chromatography and purification using preparative thin-layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS) from *Cassia fistula* seed extract. HPLC and LC/MS tests on this distinct peak showed the presence major bioactive compound with anti-yeast activity in the active fraction isolated from crude methanolic extract of *C. fistula* seeds.

In the present work, the bioactive compound (2-chloroethyl phosphoric acid) separated by TLC was investigated for their antibacterial activity. The antimicrobial activity was found maximum against *Lactobacillus* sp. as 17 mm zone of inhibition at 0.15 mg/ml concentration of compound. The antibacterial activity of crude hydroextract were also determined and found against bacterial strain *Streptococcus* sp. (8 mm zone of inhibition), *Lactobacillus* sp. (9 mm), *Actinobacillus* sp. (7 mm), *Fusobacterium* sp. (9 mm). The mass spectrum pattern of active compounds separated after TLC is shown in Fig. 3.1.

The antibacterial activity of *F. benghalensis* Linn bark was reported by Bhangale *et al.* (13) on *Actinomyces viscosus*. The hydro alcoholic extract of plant was used and found to be effective against *Actinomyces viscosus*. The maximum zone of inhibition was found to be 15.2 mm at 0.10 mg/ml concentration of extract. Study concluded the seven bioactive phytochemicals (flavonoids, saponins, steroids, reducing sugars, alkaloids, tannins, and glycosides) were detected during qualitative analysis of both crude (ethanolic and hydro) extracts of *F. benghalensis*. Crude hydroextract was having varying degree of antibacterial activity against pure oral dental bacterial (Streptococcus sp., Lactobacillus sp., Actinobacillus sp., and Fusobacterium sp.) strains. Furthermore efforts were made for the identification chemical compounds of crude hydroextract of *Fusobacterium* sp. 2-chloroethyl phosphonic acid was purified on TLC followed by LCMS. The compounds $\alpha$-ethyl-$\alpha$-phenyl...
Fig. 2.5. MS of LC peak having retention time 22.185, Antimony trioxide (Sb2O3) having molecular weight 291.6 was identified.

Fig. 3.0. LC chromatograph of a preparative TLC product.

Fig. 3.1. MS of LC peak having retention time of 2.049, 2-chloroethyl phosphonic acid (C2H6ClO3P) having molecular weight 144.5 was identified.
glutarimide, 1, 2- Dihexanoyl-sn-Glycero-3-Phosphocholine, Antimony trisulfide, 2, 2, 2-trichloroethanol, and Antimony trioxide were also identified by LCMS. These pure chemicals could be use as an ayurvedic medicine for the treatment of various teeth disorders (gingival, subgingival and supragingival stages) and to boost immune system.

Acknowledgments
The authors gratefully acknowledge the financial and laboratory support for to extend this research by Bhojia Institute of Life Sciences, Bhud, Baddi, and District-Solan (H.P.) -173205 and grateful to the Sophisticated Analytical Instrumentation Facility Laboratory of Punjab University for providing the Chromatographic and Mass Spectroscopic techniques in this research.

References


