ABSTRACT

Objective: The medicinal plants are loaded with numerous effective antimicrobial, antioxidants and anticancer agents which provide an alternative means of therapy to various infections caused by drug resistant bacteria, oxidative stress and dreadful diseases like cancer and other physiological disorders. The present study is designed to evaluate the biological properties of ethanolic extracts of bark, fruits and leaves of *Ficus racemosa* in terms of its antibacterial, antioxidant, cytotoxic activities and phytochemical analysis to find out the active compounds responsible for these activities.

Methods: Standard methods were adopted for the analysis of secondary metabolites and antioxidant activities. Antibacterial activity was determined by using standard agar well diffusion method. Anticancer activity of the extracts was assayed by XTT assay.

Results: Phytochemical investigation of ethanolic extract revealed the presence of phenols, flavonoids and alkaloids. Among all the extracts studied leaf extract showed good activity against all the test bacteria. In DPPH assay, the IC$_{50}$ value of crude ethanol bark extract was 52.04 µg/mL whereas IC$_{50}$ value for reference ascorbic acid was 42.17 µg/mL. Cell viability assay on Calu6 cell line demonstrated that all the extracts at 160 µg/ml were effective and the highest percentage of inhibition of 58.8% and 46.6% was exhibited by hot ethanolic extracts of bark and fruits, respectively.

Conclusion: Detailed analysis of phytochemical properties of *F. racemosa* determines its significance as a rich source of molecules with high pharmaceutical value. In addition, this study has also explored the anticancer properties of these extracts against lung anaplastic carcinoma cell line Calu6, which has shown promising results. From this we can conclude the plant to be a natural source of antioxidants and phytochemicals with potent antimicrobial and anticancer properties.

Keywords: *Ficus racemosa*, Antioxidant activity, Antibacterial activity, Cytotoxic activity, DPPH, ABTS, XTT-assay.

INTRODUCTION

India is known for its rich diversity of medicinal plants and hence called botanical garden of the world [1]. Many of the natural products in plants of medicinal value offer us new sources of drugs which have been used effectively in traditional medicine. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional system of medicine [2]. A large number of medicinal plants are used in several formulations for the treatment of various diseases caused by microbes. Several plant species are used by many ethnic groups for the treatment of various ailments ranging from minor infections to dysentery, skin diseases, asthma, malaria etc. [3]. The drugs obtained from plants are less toxic; side effects are scanty and also cost effective.

Oxidative stress leads to many diseases, such as brain dysfunction, cancer, heart diseases, age related degenerative conditions, declination of the immune system, cancer, coronary arteriosclerosis, ageing processes, carcinogenesis, gastric ulcer and DNA damage [4, 5]. Phytochemicals are a rich source of antioxidants such as polyphenols and flavonoids, which can delay or inhibit the oxidation of biomolecules by regulation of oxidative chain reactions [6]. Polyphenols and flavonoids are used for the prevention and cure of various diseases which are mainly associated with free radicals [7]. Phenolic compounds have gained much attention, due to their antioxidant activities and free radical-scavenging abilities, which potentially have beneficial implications for human health [8]. Flavonoids are regarded as one of the most widespread groups of natural constituents found in plants [9], which are been reported to have radical scavenging effects through scavenging or chelating processes [10]. Several plant species rich in flavonoids are reported having disease preventive and therapeutic properties [11]. Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer [12]. Therefore, in recent years, considerable attention has been directed towards the identification of plants with antioxidant ability.

The evaluation and the discovery of new anticancer agents is long-term process that encompasses many steps. The goal of screening medicinal plant is to search for excellent anticancer agent avertable to human malignancies. In defiance of astonishing advances in modern medicine, such as surgery, radiotherapy, chemotherapy, and hormone therapy, cancer remains a worldwide health problem. Newman and Cragg [13] reported in their analysis that sources of many new drugs in recent years are derived directly from natural products. Alkaloids, the naturally occurring pharmacologically active organic compounds have made major impact on plant medicine because of its vast application. In addition to possessing various medicinal properties [14, 15, 16], alkaloids have been shown to be potent anti-cancer agents [17]. These facts are in favor with the new call for medicinal plant identification namely local plants, in conjunction with anticancer properties.

Several members of the genus Ficus (Family: Moraceae) are being used traditionally in a wide variety of ethnomedical remedies. One of them is *Ficus racemosa* syn. *Ficus glomerata* (Gular; Udumbara) [18], a moderate sized avenue tree widely distributed throughout India, northern Australia, and other parts of Asia. *Ficus racemosa* Linn. Commonly known as ‘cluster fig’, is used widely in Indian folk medicine for the treatment of various diseases, including jaundice, dysentery, diabetes, diarrhea and inflammatory conditions [19]. Apart from the usage in traditional medicine, scientific studies indicate that *F. racemosa* possesses various biological effects such as hepatoprotective [20], chemopreventive [21], anti-diabetic [22], anti-inflammatory [23], antipyretic [24], and antidiuretic [25]. The bark has also been evaluated for cytotoxic effects using 18R3, Hep
G2, HL-60 cell lines and found to be safe and less toxic than aspirin, a commonly consumed anti-inflammatory drug [27]. The present study aims to explore the phytochemical properties, antibacterial, antioxidant and cytotoxic activities of cold and hot (Soxhlet) ethanol extracts of Ficus racemosa. The phytochemical and antioxidant screening of these plants is a prerequisite for verifying their potential for utilization as new sources of herbal drugs. Although several studies are available on various Ficus spp., less work has been done on F. racemosa. In this study, we have performed a detailed analysis of all the phytochemical properties of F. racemosa which determine its significance as a rich source of molecules with high pharmaceutical value. In addition, this study has also explored the anticancer properties of these extracts against lung anaplastic carcinoma cell line Calu6.

**MATERIALS AND METHODS**

**Processing of plant materials and extraction**

The different parts of Ficus racemosa viz. leaves, fruits and bark were collected from their natural habitat. They were washed with distilled water and were shade dried and powdered. Cold and hot (Soxhlet) ethanolic extracts of the powdered plant material were prepared. For cold extraction, twenty-five grams of powdered sample was soaked in 250 mL of ethanol, agitated manually, and allowed to extract for 48 hours. Extracts were then filtered using Whatmann No 1 Filter paper and the filtrates were evaporated. The extracts were stored at 4°C until further processing. For hot (Soxhlet) extraction, twenty-five grams of dried powder of plant material was extracted with ethanol. The ethanol extracts were then distilled, evaporated and vacuum dried. The crude extracts thus obtained were used directly for phytochemical screening and also for assay of antibacterial, antioxidant and cytotoxic potential.

**Phytochemical screening**

**Determination of total phenolic contents**

The plant extracts were analysed for total phenolic, flavonoid and alkaloid contents. The total phenolic content of plant extracts were determined using Folin-Ciocalteau reagent [28]. Plant extracts (100 µL) were mixed with 0.5 mL of Folin-Ciocalteau reagent and 1.5 mL of 2% NaCO₃ solution. The mixture was shaken well and made up to 5 mL using distilled water. The reaction was allowed to stand for 2 hour and then the absorbance was measured at 765 nm against a reagent blank. The data obtained were used to estimate the phenolic contents using a standard graph obtained from various concentrations of gallic acid. The total phenolic content is expressed as µg of gallic acid equivalents (GAE) per mL of the plant extracts.

Total flavonoid content was determined as described by Jia et al. [28]. Five hundred micro liter of extract was diluted with 2 mL of distilled water and subsequently with 1.5 mL of 5% NaNO₂ solution. After 6 min 200 µL of 10% AlCl₃·H₂O was added and mixed. Immediately the volume is brought to 5 mL using distilled water and allowed to stand at RT for 15 min. The absorbance of the mixture was determined at 510 nm. Quercetin was used as standard and the results were expressed as µg of quercetin equivalents (QE) per mL of the extract.

Total alkaloid content was measured by Dragendorff’s method [29]. It is based on the formation of yellow bismuth complex in nitric acid medium with thiourea. 5 mL of extract was dissolved in dilute HCL and 2 drops of Dragon drop’s was added, a crystalline precipitate indicates presence of alkaloid. After centrifugation, the supernatant was decanted completely and the precipitate was further washed with alcohol. After centrifugation, filtrate was discarded and the residue is then treated with 2 mL of 1% disodium sulfide solution. The brownish black precipitate formed was then centrifuged. Completion of precipitation was checked by adding 2 drops of disodium sulfide. The residue was dissolved in 2 mL concentrated nitric acid and diluted to 10 mL in a standard flask with distilled water. 1 mL was then pipetted out and 5 mL of 3% thiourea solution was added to it and absorbance was measured at 435 nm against the blank containing nitric acid and thiourea. The amount of bismuth present in the solution was calculated by multiplying the absorbance values with the factor, taking suitable dilution factor in to consideration. The factor is obtained from the standard curve, which is a constant for different concentrations.

**Factor = Concentration / Absorbance**

The calibration curve was obtained with Bismuth nitrate pentahydrate stock solution. Series dilutions of the stock solution were made by pipetting out 1, 2, 3, 4, 5, 6, 7, 8, and 9 mL stock solution into separate 10 mL standard flasks and diluting to volume with distilled water. 1 mL of this solution was taken, and 5 mL thiourea solution was added to it. The absorbance value of the yellow solution was measured at 435 nm against colorless reagent blanks.

**Assay of antibacterial activity of plant extracts**

Antibacterial activity was assayed with the standard agar well diffusion method [30]. Different concentrations of the extracts (100, 200, 300 µg/ml) were prepared by using 2% DMSO (dimethyl sulphoxide). 10µl of 24h test cultures such as Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis were seeded onto respective Mueller-Hinton agar medium by spread plate method. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. Antibacterial activity was also assayed with the standard disc diffusion method. The filter paper discs (5mm in diameter) impregnated with the extracts is placed on test organisms. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the disc. The DMSO was used as a negative control.

**Evaluation of total Antioxidant Capacity (TAC) by Phosphomolybdenum method**

The total antioxidant capacity of the plant extracts was determined by phosphomolybdate method using ascorbic acid as a standard [31]. The assay is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green phosphate / Mo(V) complex at acid pH. An aliquot of 0.1 mL of sample solution was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. A typical blank contained 1 mL of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. The antioxidant activity is obtained by the number of gram equivalent of ascorbic acid. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicates and values are expressed as equivalents of ascorbic acid in µg / mg of extract [32].

**Assay of antioxidant activity by DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity**

The free radical scavenging capacity of the extracts was determined using DPPH [33]. The DPPH solution (0.006% w/v) was prepared in 95% ethanol. Different concentrations of the test sample which is to be examined for antioxidant activity is prepared (10-50µg/mL). 3 mL of different concentration of test sample of F. racemosa extracts were mixed with 1 mL of DPPH solution in dark. Ascorbic acid which is a strong antioxidizing agent is taken as standard. 3 mL of different concentration of standard solution of ascorbic acid was mixed with 1 mL of DPPH solution in dark.

The prepared solution of ascorbic acid and plant extracts samples was incubated for half an hour and then absorbance is taken with the help of U.V. Spectrophotometer at 517 nm. Ethanol serves as a blank and the experiment was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

\[
\text{DPPH radical scavenging activity (}%) = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]
Assay of antioxidant activity by ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

ABTS assay was performed according to the protocol Re et al. [34]. The stock solution was prepared by mixing equal volumes of 7 mM ABTS solution and 2.45 mM potassium persulfate solution followed by incubation at 12 h at room temperature in the dark to yield a dark-colored solution containing ABTS radicals. Working solution was prepared freshly before each assay by diluting the stock solution by mixing of stock solution to 50% methanol for an initial absorbance of about 0.700 (± 0.02) at 745 nm, with temperature control set at 30°C. Varying concentrations (10-50μg/ml) of the plant extracts were allowed to react with 3 ml of the ABTS solution and the absorbance readings were recorded at 734 nm [34]. Ascorbic acid was used as positive controls. The scavenging activity was estimated based on the percentage of ABTS radicals scavenged by the following formula:

\[
\text{ABTS radical scavenging activity (％)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

Determination of Cytotoxicity of the extracts

Cell lines and culture medium

The Lung anaplastic carcinoma Calu6 (ATCC NO. HTB-56) were obtained from the American Tissue Culture Collection (ATCC, USA). The Calu6 cells were grown and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 50 μg/ml of amphotericin B. The cells were cultured in a 5% CO₂ incubator at 37°C in a humidified atmosphere. The culture was sub-cultured and maintained by changing media every two to three days and routinely checked under an inverted microscope for any contamination.

XTT assay for cell viability

Assay of antitumor activity of the extracts

Cells were grown in T25 tissue culture flasks to the 60-70 % confluency. The cells are trypsinized, counted in haemocytometer and diluted with DMEM media. 50,000 cells were seeded in 96 well microtitre plates in 100μl of DMEM media and incubated for 24 hrs at 37°C in 5% CO₂ for cell adherence. The diluted ranges of extracts were added to each well and the final concentrations of the test extracts were 10, 20, 40, 80, and 160 μg/ml. Untreated cells were used as reference. After treatment, the plates are incubated for 24 hrs at 37°C in 5% CO₂. After 24 hrs of incubation, 50μl of XTT-PMs solute was added to each well and incubated in a humidified atmosphere for another 2-4 hrs for color development. The absorbance of the color was measured on 96-well microplate reader Tecan micro plate reader (Magellan™data analysis software) at 450nm wave length. Percentage viability of the cells was calculated at corresponding concentrations of the sample with reference to untreated cells.

Experiments were carried out in triplicate wells, repeated at least three times. Values are presented as the mean ± SD cell viability ± SD.

\[
\% \text{ Anticancer Activity} = \frac{(A_{c} - A_{a}/A_{c}) \times 100}{}
\]

Where Ac and As referred to the absorbance of control and the sample, respectively.

Statistical analysis

All tests were conducted in triplicate. The results are expressed as means ± SD. Analysis of variance and significant differences among the means were tested by the one-way ANOVA, using SPSS (Version 16.0).

RESULTS AND DISCUSSION

Qualitative and Quantitative and Estimations of Phytochemicals

The results of quantitative screening of phytochemicals are presented in Table 1. The ethanolic extracts of F. racemosa fruits, leaves and bark were evaluated for the presence of total phenolics. The total phenolic content was found to be higher in the cold extracts of bark (1042±15.04 μg mL⁻¹) followed by cold extract of leaf (1012±26.4 μg mL⁻¹). A positive correlation was observed between phenolic content and free-radical scavenging activity of the extracts and these observations corroborate with results from other studies [35, 36, 37, 38 and 39]. Among the studied samples, the flavonoid content was ranging from 21.3±4.04 μg mL⁻¹ (hot extraction of leaves) to 43.7±6.65 μg mL⁻¹ (hot extraction of fruits) (Table 2). Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals [40] implicated in several diseases. The results of total alkaloids extracted from different parts of Ficus racemosa are presented in Table 1.

The results show that the bark contains relatively high percentage of alkaloids compared to leaves and fruits. According to the studies conducted by Garba and Okenyi [41], alkaloids extracted from C. papaya, C. procera, M. indica and P. guajava were effective against most of the test microbes indicating a broad spectrum of activity.

<table>
<thead>
<tr>
<th>Phytochemicals (µg mL⁻¹)</th>
<th>Bark cold</th>
<th>Leaves cold</th>
<th>Fruits cold</th>
<th>Bark hot</th>
<th>Leaves hot</th>
<th>Fruits hot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>1042±15.04</td>
<td>1012±26.4</td>
<td>802±30.6</td>
<td>199±13.5</td>
<td>174±7.93</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>39.03±3.6</td>
<td>23.4±3.64</td>
<td>21.3±4.04</td>
<td>29.5±4.33</td>
<td>43.7±6.65</td>
<td></td>
</tr>
<tr>
<td>Alkaloids</td>
<td>355±22.9</td>
<td>51.3±6.02</td>
<td>29.7±4.5</td>
<td>80.6±8.96</td>
<td>90.3±8.43</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± SD of data from three independent experiments.

<table>
<thead>
<tr>
<th>Test Microorganisms</th>
<th>Diameter of zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bark cold</td>
</tr>
<tr>
<td>E. coli</td>
<td>8.8±0.76</td>
</tr>
<tr>
<td>S. aureus</td>
<td>8.3±0.64</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>16.6±1.52</td>
</tr>
<tr>
<td>P. aeroginosa</td>
<td>11.13±1.02</td>
</tr>
</tbody>
</table>

Values are the means ± SD of data from three independent experiments.
Total Antioxidant Capacity Phosphomolybdenum assay

The result of total antioxidant activity (TAC) is shown in Figure 1. Among all the extracts tested hot ethanolic extracts of bark (78.8 µg mL⁻¹) was found to be more effective in reduction of Mo (VI) to Mo (V) while the lowest effects was shown by cold ethanolic extracts of fruits (20.3 µg mL⁻¹).

**DPPH and ABTS radical scavenging activity**

Table 3 shows the significant decrease in the concentration of DPPH radical due to scavenging ability of the *Ficus racemosa*. The results of the present study showed that cold extract of bark had high (IC₅₀ 52.04 µg/mL) DPPH radical scavenging activity than leaves and fruit extracts. The lowest activity (IC₅₀ 91.23 µg/mL) was seen in hot extracts of leaves (the concentration of the plant extract used for assay was 50µg/mL).

This indicates that bark of *Ficus racemosa* can be good source of natural antioxidants. The suppressive effect on ABTS cation radical was assayed at 50 µg/mL for all the extracts (Table 3). The maximum scavenging activity of 35.73% was observed in soxhlet extraction of bark followed by leaves extract (34.15%). This finding demonstrates that bark, leaf and fruit extracts of *Ficus racemosa* are capable of non-enzymatically inhibiting the free radical, produced in biological systems, which is a precursor of many ROS and is shown to be harmful for various cellular components [42].

Oyedemi *et al.*, [43] attributed the antioxidant activity observed in the aqueous stem bark extract of *S. henningsii* to the presence of flavonoids, flavonols, phenols and proanthocyanidins. Hence scavenging activity of ABTS and DPPH radicals by the *Ficus racemosa* plant extracts was found to be considerable; which implies that it may be useful for treating radical related pathological damage especially at higher concentrations.

**Table 3: Antioxidant activity (% of scavenging) and IC₅₀ value of cold and soxhlet [hot] ethanolic extract of bark, leaves and fruits of *Ficus racemosa* by Phosphomolybdenum method.**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>DPPH % of scavenging</th>
<th>ABTS % of scavenging</th>
<th>DPPH IC₅₀ (µg mL⁻¹)</th>
<th>ABTS IC₅₀ (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bark</td>
<td>74.86±1.4</td>
<td>84.86±0.1</td>
<td>42.17</td>
<td>32.14</td>
</tr>
<tr>
<td>Cold</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit</td>
<td>52.86±0.37</td>
<td>30.94±0.39</td>
<td>52.04</td>
<td>81.03</td>
</tr>
<tr>
<td>Leaves</td>
<td>41.47±0.1</td>
<td>26.33±0.62</td>
<td>59.41</td>
<td>114.41</td>
</tr>
<tr>
<td>Bark</td>
<td>32.44±0.53</td>
<td>26.11±0.31</td>
<td>74.87</td>
<td>116.86</td>
</tr>
<tr>
<td>Leaves</td>
<td>45.14±0.14</td>
<td>35.73±0.25</td>
<td>59.91</td>
<td>69.31</td>
</tr>
<tr>
<td>Soxhlet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit</td>
<td>39.39±0.2</td>
<td>19.35±0.36</td>
<td>60.81</td>
<td>15.408</td>
</tr>
<tr>
<td>Leaves</td>
<td>30.22±0.05</td>
<td>34.15±0.26</td>
<td>91.23</td>
<td>95.62</td>
</tr>
</tbody>
</table>

Values are the means ± SD of data from three independent experiments.

**Determination of Cytotoxic Activity by XTT assay**

The crude hot ethanolic extracts of bark, fruits and leaves of *Ficus racemosa* were evaluated in vitro for their inhibitory ability against the growth of Calu-6 cell lines using the XTT colorimetric assay (Figure 2).

It was found that all the extracts showed a significant inhibition at the higher concentrations of 160µg/mL. The highest percentage of inhibition of 58.8 and 39.6% was exhibited by hot ethanolic extracts of bark and fruits, respectively. The cytotoxic effect of plants is principally contributed by the presence of secondary metabolites like alkaloid, glycoside, steroid, tannin, terpenoid and flavonoid in their extract [44]. This is also consistent with our observation because the phytochemical group analysis of the extract showed the presence of alkaloid, phenols and flavonoids in significant higher concentration.

**CONCLUSION**

The present research work concludes that *Ficus racemosa* is important medicinal plant with varied pharmacological spectrum. The phytochemical screening revealed chemical constituents that form the foundation of their pharmacological activity. The cold and soxhlet (hot) extracts of Ficus racemosa has good efficacy against gram positive and gram negative bacteria in higher concentration. The ABTS and DPPH in vitro assays indicate that these plant extracts are a significant source of natural antioxidant, which might be helpful in preventing the various diseases associated with oxidative stresses. The cytotoxicity exerted against cancer cell lines suggests bioactive principles in the plant. This shows that the plant could be useful as antitumor, anticancer and as antimicrobial agent.

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