Antiproliferative, antioxidant and antimicrobial screening of *Strychnos colubrina* Linn extracts

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**Abstract:** Medicinal plants are the most precious gift to mankind offered by nature. For most of the known health issues there is a natural plant remedy. But most of these plants are not properly exploited. The present study deals with the scientific study of the therapeutic use of a medicinal plant *Strychnos colubrina* Linn. The plant is traditionally used by the tribe for skin cancer and infections. The present study consists of invitro screening of antiproliferative, antioxidant and antimicrobial activity of *Strychnos colubrina* Linn extract. The chloroform extract was prepared using ariel plant parts and the antimicrobial activity as assessed by Cup plate method using gram –ve and +ve strains. The antioxidant activity of extract was screened by six different methods that include H₂O₂, superoxide, hydroxyl radical and NO scavenging assays, Reducing power assay and DPPH assay. Also the invitro antiproliferative activity was studied by MTT assay on HaCaT skin cell line using the extract. These studies were shown that the extract is having antimicrobial, antioxidant and antiproliferative efficacy.

**Key words:** *Strychnos colubrina*, chloroform extract antimicrobial, antioxidant, MTT assay.

**Introduction**

Medicinal plants are the valuable wealth of man-kind. Because of their natural origin, the deleterious effects exert is much less compared to known synthetic compounds. So for a healthy wellbeing, the man should explore and utilize the priceless medicinal wealth offered by nature.

The genus *Strychnos*, is the largest genus of the family Loganiaceae. About 44 species of Strychnos are endemic to Asia. *Strychnos colubrina* Linn (*S.colubrina* Linn) is one of the rare species of Strychnos genus which is traditionally used by the tribe for tumors, mania, arthralgia, diarrhoea, pain, chicken pox etc.,. But these beneficial uses are not scientifically studied.

Most of the antimicrobial or anticancer agents available in the market are under the threat of microbial resistance. So it is the need of decade to have new medicinal agents to subside the diseases with minimum side effects. The present study aims to reveal the antioxidant, antimicrobial and antiproliferative activities of *Strychnos colubrina* Linn extract.

**Materials and Methods**

**Plant Material and Extraction**

The ariel parts of *Strychnos colubrina* Linn was collected from village of Idukki in the month of October 2014. The plant material was identified and authenticated by the Botany department at Nirmala college of Arts &
Science, Muvattupuzha, Kerala, India. The ariel parts of the plant was shade dried at ambient temperature and the dried materials were crushed into fine powder using an electric blender. About 200g of powdered plant material was weighed and defatted with petroleum ether (1L) using soxhlet apparatus at 40°C for 48hrs. Then the residue was extracted with 750mL of chloroform and methanol successively. The extracts were concentrated in a rotary evaporator to yield a dark brown mass (5 g).

**Antimicrobial activity**

**Micro organisms:** Bacteria including *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) were used for the experiment. The clinical strains used in this work are our laboratory collection

**Cultivation of Bacteria:** The antibacterial activity of extract was determined by agar well diffusion method. The agar plates were seeded with 0.1ml of overnight culture and allowed to incubate for 24hrs. Cups were made in petri plates using sterile cork borer (0.85cm) and 50μL of extract was added to plate well. Then the plates were incubated at 37°C for 24Hrs.The extract treated plates were triplicated and the mean zone of inhibition was determined. The zone of inhibition was expressed in mm.

**Antioxidant Activity**

**DPPH Assay:** The radical scavenging activity of extract was determined by using DPPH assay The decrease in the absorbance of the DPPH solution after the addition of an antioxidant was measured at 517 nm. 0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol. Different volumes of plant extract were made up to 40μl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm.3ml of DPPH was taken as control.

\[
\text{% inhibition} = \left( \frac{\text{Control} - \text{Test}}{\text{Control}} \right) \times 100
\]

**2.3.2. \text{H}_2\text{O}_2\text{Scavenging activity}**: The extract was dissolved in 3.4ml of 0.1M phosphate buffer (pH 7.4) and mixed with 600μl of 43mM solution of hydrogen peroxide. The absorbance value (230nm) of the reaction mixture was recorded after 10 minutes.

\[
\text{% inhibition} = \left( \frac{1 - \frac{\text{Test}}{\text{Control}}}{} \right) \times 100
\]

**Hydroxyl radial scavenging activity:** One milliliter of the final reaction solution consisted of aliquots (500μl) of various concentration of the extract 1μM FeCl₃, 1μM EDTA, 20μM H₂O₂, 1μM ascorbic acid and 30μM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 hour at 37°C and further heated in a boiling water bath for 15 minutes after addition of 1ml of 2-8% (w/v) trichloro acetic acid and 1ml of 1% 2-thio-barbituric acid. The colour development was measured of 532nm against a blank containing phosphate buffer.

\[
\text{% inhibition} = \left( \frac{\text{Control} - \text{Test}}{\text{Control}} \right) \times 100
\]

**Nitric Oxide Scavenging Activity:** Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmol/L) in phosphate buffered saline (pH-7.4), was mixed with different concentration of the extract prepared in methanol and incubated at 25°C for 30minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with
N-1 naphthyl ethylene diamine dihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard.\(^{14}\)

\[
\% \text{ inhibition} = \frac{(\text{Control} - \text{Test}) \times 100}{\text{Control}}
\]

**Reducing power activity**: Different concentrations of extract were mixed with 2.5ml of phosphate buffer(200mM)(pH 6.6), and 2.5ml of 1% potassium ferric cyanide was added and boiled for 20 minutes at 50°C. After incubation, 2.5 ml of 10% TCA were added to the mixtures followed by centrifugation at 650xg it for 10 minutes. The upper layer (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride was added and the absorbance was read at 700nm.\(^ {15}\)

\[
\% \text{ inhibition} = \frac{(\text{Control} - \text{Test}) \times 100}{\text{Control}}
\]

**Super oxide free radical scavenging activity**: Super oxide anion are generated in riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product. 0.02ml of extracts, 0.05ml of Riboflavin solution(0.12mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The absorbance of solution was measured at 560nm using DMSO as blank after illumination for 5 min and difference in OD was determined after 30 minutes incubation in fluorescent light. Absorbance was measured after illumination for 30 min. at 560nm on UV visible spectrophotometer.\(^ {16}\)

\[
\% \text{ inhibition} = \frac{(\text{Control} - \text{Test}) \times 100}{\text{Control}}
\]

**Invitro anticancer activity by MTT assay**:

HaCaT (keratinocyte) were purchased from NCCS Pune were maintained in Dulbecco’s modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°C in 5 % CO2 in a humidified atmosphere in a CO2 incubator(NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (Himedia)) for 2 minutes and passaged to T flasks in complete aseptic conditions.6.25,12.5,25,50,100µg/ml of sample was added from 1mg/ml stock to grown cells and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

The cells was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT - 5mg/ml dissolved in PBS). It was then incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 200µl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA).\(^ {17,18,19,20,21,22}\)

\[
\% \text{ viability} = \frac{(\text{OD of Test/ OD of Control}) \times 100}{100}
\]

**Results**:

**Antimicrobial activity**: The extract showed asignificant activity against E.coli and S.aureus. The antimicrobial activity against P.aureuginosa and B.subtilis, is not as significant as the other two. The zone of inhibition is shown in figure-1. The zone of inhibition by the extract is depicted in table.1. From the table it is interpreted that the extract showed maximum activity against E.coli.
Figure 1: The zone of inhibition shown by extract against different bacterial strains

Table 1: The zone of inhibition for Antimicrobial effect of Strychnos colubrina Linn extract by cup-plate method

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Bacterial Strain</th>
<th>Standard</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>B. subtilis</td>
<td>27.2±1.32</td>
<td>10±1.10</td>
</tr>
<tr>
<td>2.</td>
<td>P. aeruginosa</td>
<td>30.35±2.13</td>
<td>16.12±1.45</td>
</tr>
<tr>
<td>3.</td>
<td>S. aureus</td>
<td>30±2.33</td>
<td>18±0.23</td>
</tr>
<tr>
<td>4.</td>
<td>E. coli</td>
<td>29.15±1.11</td>
<td>19.0±2.53</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SEM.

Antioxidant activity: The invitro antioxidant screening of Strychnos colubrina Linn extract showed significant protection against oxidizing agents/free radicals. The Table 2 and 3 shows the percentage inhibition by the extract by different invitro antioxidant screening methods. The maximum potency was shown at 2000 μg/ml concentration. In No scavenging assay the percentage inhibition of extract at 2000 μg/ml concentration is comparable to the standard.

Table 2: The percentage inhibition in different methods of antioxidant activity by Strychnos colubrina Linn extract

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (μg/ml)</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DPPH Assay</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>Extract</td>
</tr>
<tr>
<td>1.</td>
<td>125</td>
<td>10.24±3.0</td>
</tr>
<tr>
<td>2.</td>
<td>250</td>
<td>32.23±0.5</td>
</tr>
<tr>
<td>3.</td>
<td>500</td>
<td>38.48±1.3</td>
</tr>
<tr>
<td>4.</td>
<td>1000</td>
<td>66.12±2.1</td>
</tr>
<tr>
<td>5.</td>
<td>2000</td>
<td>69.14±0.7</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SEM.
Table 3: The percentage inhibition in H2O2 scavenging assay and Reducing power assay by *Strychnos colubrina* Linn extract

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Concentration (µg/ml)</th>
<th>H2O2 scavenging assay</th>
<th>Reducing power assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Percentage Inhibition</td>
<td>Percentage Inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>by Extract</td>
<td>by Extract</td>
</tr>
<tr>
<td>1.</td>
<td>125</td>
<td>6.03±1.12</td>
<td>20.23±1.5</td>
</tr>
<tr>
<td>2.</td>
<td>250</td>
<td>7.75±0.57</td>
<td>39.38±3.0</td>
</tr>
<tr>
<td>3.</td>
<td>500</td>
<td>8.64±4.1</td>
<td>42.03±2.8</td>
</tr>
<tr>
<td>4.</td>
<td>1000</td>
<td>9.5±1.22</td>
<td>43.44±1.56</td>
</tr>
<tr>
<td>5.</td>
<td>2000</td>
<td>10.05±4.23</td>
<td>45.22±0.50</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SEM

But the antioxidant activity by H2O2 scavenging assay showed a less effective response compared to other screening methods.

**In vitro anticancer activity by MTT Assay:** The result of MTT assay is shown in table 4 and figure 2. The extract is having a potent antiproliferative effect on HaCaT cell line.

![Figure 2](image1)

**Figure 2:** Viability of cell line in antiproliferative activity of *S. colubrina* Linn by MTT Assay: 2a-Control; 2b- 6.25 µg/mL sample treated; 2c- 12.5 µg/mL sample treated; 2d- 25 µg/mL sample treated; 2e- 50 µg/mL sample treated; 2f- 100 µg/mL sample treated.
Table:4 Percentage viability of cells in MTT Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Percentage Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td></td>
<td>95.69±1.30</td>
</tr>
<tr>
<td>12.5</td>
<td></td>
<td>88.29±0.04</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>78.32±2.30</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>56.38±1.01</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>39.17±0.77</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SEM

Discussion:

The antimicrobial study revealed that the extract is having broad spectrum of activity against both gram positive and gram negative microbes. Because of this wide antimicrobial activity the extract can be used in the treatment of various infections or inflammations. The mechanism of action should be identified by further works.

Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidant. DPPH is a stable free radical at room temperature. As the electron became paired in the presence of free radical scavenging the absorption vanishes and the resulting discoloration stochiometrically coincides with the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. The bleaching of DPPH molecules can be correlated with the number of available hydroxyl groups. We can infer that, the activity of the extract may be probably due to the presence of substance with an available hydroxyl group. The extracts are able to reduce the stable radical DPPH to the yellow coloured diphenyl picrylhydrazine. The discoloration increases as the radical scavenging activity increased. The results showed that as the increased concentration of extract of *Strychnos colubrina Linn* showed a proportional increase in activity. The saponins or flavonoids or other phenolic compounds in the extract may reduces the radicals to the corresponding hydrazine when it reacts with the hydrogen.

In H$_2$O$_2$Scavenging activity, the Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H$_2$O$_2$ can probably react with Fe$^{2+}$, and possibly Cu$^{2+}$ ions to form hydroxyl radical and this may be the origin of many of its toxic effects. The extract had shown only a mild H$_2$O$_2$ scavenging activity.

In Hydroxyl radial scavenging activity, the highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The effect of *Strychnos colubrina* on the inhibition of free radical mediated deoxyribose damage was assessed by means of the Iron (II)-dependent DNA damage assay. The Fenton reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe$^{2+}$ salts as an important catalytic component. Extent of hydroxyl radical scavenged was determined by the decrease in intensity of pink coloured chromophore was determined at 532 nm. The extract showed a competent hydroxyl radical scavenging activity with that of the standard.

The NO scavenging method is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The extract had shown potent NO scavenging activity at concentrations of 100, 200and 400µg/mL. The activity of extract at these concentrations is comparable with the standard.

As per the reducing power assay results, the extract showed a moderate antioxidant efficacy. The reducing power of extract might be due to their hydrogen donating ability. The reducing power of a compound is related to its electron transferability and may serve as a significant indicator of its potential antioxidant activity possibly, the extracts could react with radicals to stabilize and terminate radical chain reactions.

Superoxide has been observed to directly initiate lipid Peroxidation. Ferrous iron can initiate lipid peroxidation by the fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides.
into peroxyl and alkoxyl radicals. Superoxide anion radical plays an important role in the formation of other reactive oxygen-species such as hydroxyl radical, hydrogen peroxide, or singlet oxygen in living systems, so evaluating the scavenging effects of *S. colubrina* on superoxide radicals is one of the most important ways to clarify the mechanism of any antioxidant activity. At 200μg/mL concentration, it showed a maximum antioxidant activity

**Invitro anticancer activity by MTT assay:**

HaCaT is a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin. The agents that decrease the proliferation on these cells are an indication of their use in skin proliferation. The results are represented in the graph. It shows that the viability decreases with increase in concentration of extract. At concentrations of 100μg/mL it showed a better cytotoxic effect

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**References**


