PHARMACOGNOSTICAL, PHYSICO-CHEMICAL, PHYTOCHEMICAL ANALYSIS AND ANTICANCER SCREENING OF CROTALARIA VERRUCOSA L.

Ramesh Kannan N.¹*, Agnel Arul John N.¹ and Natarajan E.²

¹Department of Biochemistry (Centre for Research), Srimad Andavan Arts and Science College (Autonomous), Tiruchirappalli, Tamil Nadu, India.
²PG & Research Department of Botany, National College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

ABSTRACT

Objective: To establish the standardization parameters for pharmacognostic, physico-chemical, phytochemical and evaluation of anticancer activity of Crotalaria verrucosa L, belonging to family Fabaceae, an important plant in the Indian system of medicine.

Methods: Macroscopical, microscopical, physic-chemical evaluations, florescence analysis, preliminary phytochemical analysis, quantification of phytoconstituents of C. verrucosa were investigated and aqueous and ethanolic extracts of C. verrucosa were subjected for screening of anticancer potentials against Ehrlich Ascites Carcinoma using in-vitro methods. Results: Chemo-microscopy revealed the presence of lignin, starch grains and calcium oxalate crystals. The different fluorescent light shades were obtained under short and long UV light for both powder as well as the extracts of the leaves. Physico-chemical evaluation used to determine numerical standards showed a result with total ash (6.30±0.27) % w/w, water soluble ash (4.4±0.37) % w/w, acid insoluble ash (1.80±0.42) % w/w, loss on drying (1.08±0.11) % w/w, ethanol soluble extractive (08.7±0.46) % w/w and water soluble extractive (24.6±1.12) % w/w. Behavior characteristics of the leaves powder and various extracts of C. verrucosa showed presence of Terpenoids, Flavones, Steroids, Alkaloids, Phenol, Tannin, Saponin, and Glycosides. The quantification of the few secondary metabolites in aqueous and ethanol extracts was estimated. In vitro cytotoxic effect was
examined using MTT Assay, the aqueous extract showed 77.61 ± 0.89% while ethanolic extracts inhibits 84.09 ± 1.57% against EAC cell lines. **Conclusions:** The results from pharmacognostic, physic-chemical, phytochemical analysis and anticancer investigations of *C. verrucosa* are suggested that ethanol extracts of *C. verrucosa* is very useful towards discovering a new drug against cancer cells.

**KEYWORDS:** *Crotalaria verrucosa* L; Pharmacognostical study; Phytochemical analysis; MTT; Cytotoxicity.

**INTRODUCTION**

Preemption is decisively the sensible tactic towards the definitive goal of cancer control. Numerous approaches are being for the healing of cancer in modern medicine. Chemotherapy, radiotherapy, and surgery are plays a major roles in treatment of cancer. Among them, chemotherapy is now considered as the most applicable method of cancer treatment. Intercession with chemo-preventive drugs at the initial stage in carcinogenesis is theoretically more balanced than attempting to eliminate completely matured cancer cells. Nevertheless, chemotherapeutic drugs severely affect the normal cells in human body. Hence the usage of natural products has been expected as of incomparable value in the control of cancer and its eradication program.[1] Plant-based secondary metabolites such as alkaloids, phenols, flavonoids, tannins, saponins, terpenes, etc., have received significant consideration in recent years because of their various pharmacological screening including cytotoxic and cancer chemo-preventive effects.[2]

*Crotalaria verrucosa* L. (Fabaceae) commonly called, blue rattlesnake; distributed within India, in the tropical regions, from Himalayas to Ceylon.[3] Much Branched herbaceous, usually annual plant with blue, sometimes white, flowers.[4] Leaves extract is used in impetigo and scabies both externally and internally, also considered effective in weakening salivation.[5] The leaf extract is given orally to cure jaundice. Aqueous and ethanolic extracts of aerial parts of *C. verrucosa* were effective for fertility and estrogenic implantation in Albino rats and also has exhibited very substantial hepato protective property against paracetamol induced hepatotoxicity study models in Wistar rats.[6] Hence, we put an attempt to inspect pharmacognostic, physicochemical, phytochemical characteristics and anticancer potential of leaves extract of *C. verrucosa* against EAC using *in vitro* methods.
MATERIALS AND METHODS

Plant Material

The plant, *Crotalaria verrucosa* was collected in Kolli Hills, Namakkal District (Tamil Nadu). The plant was identified with the help of Flora of Karnataka and authenticated by Rabinet Herbarium, St. Joseph’s College, Tiruchirappalli, Tamil Nadu. The leaves of the plant were dried, powdered and passed through 40 mesh sieve and stored in an airtight container for further use.

Macroscopic and microscopic analysis

Macroscopic studies were done by using simple microscope. The color, odour, taste, size and shape of leaves were determined. Microscopic studies were done by preparing thin hand section of leaves of *C. verrucosa*. The sections were cleared with chloral hydrate solution, stained with phloroglucinol- hydrochloric acid (1:1) and toluidine blue. Powder of the dried leaves was used for the observation of powder microscopical characters. The powdered drug was separately treated with phloroglucinol-hydrochloric acid (1:1) solution, acetic acid and iodine solution to determine the presence of lignified fibres, calcium oxalate crystals and starch grains respectively.[7]

Physico-chemical Analysis

Physico-chemical studies are evaluated to determine the quality and purity of the leaf powder of *C. verrucosa*. Physico-chemical parameters of the powdered drug such as total ash, water-soluble ash, acid-insoluble ash were determined. Alcohol and water-soluble extractive values were determined to find out the amount of water and alcohol soluble components. The moisture content was detected by loss on drying method.[8,9]

Fluorescence analysis

Fluorescence of the powder was observed under day and UV light (254nm) treating with acids and alkaline solutions of the drug.[10]

Preliminary Phytochemical screening

The leaf powder of *C. verrucosa* and extraction from different solvents (Hexane, Chloroform, Ethyl acetate, Ethanol and Water) were studied for the presence and absence of secondary metabolites like alkaloids, glycosides, saponins, phenolics, terpenoids, flavonoids, coumarins, steroids, sugars, quinines, lignin and tannins by standardized qualitative chemical tests.[11-17]
Quantification of Secondary Metabolites

Estimation of Total Alkaloids
The extracts of plant sample were treated with 0.1N HCl and aqueous acidified layer thus obtained was partitioned with Chloroform in a separating funnel. The Chloroform layer was discarded and the aqueous layer was basified with Ammonium hydroxide to alkaline pH and partitioned with Chloroform in a separating funnel. The aqueous layer was discarded and the Chloroform layer was evaporated, the resultant content was treated as total alkaloid and confirmed for alkaloid with dragendroff’s reagent.[18]

Following formula is used to calculate the total alkaloid percentage:

$$\text{Alkaloid \%} = \frac{\text{Weight of Residue}}{\text{Total Weight}} \times 100$$

Estimation of Total Flavonoids
The plant extract was extracted with ethyl acetate. The extracts were dried over anhydrous sodium sulphate, filtered and concentrated under vacuum up to a concentration of 1g/ml of extract. They are further diluted with ethyl acetate to obtain 0.01g/ml solution and used in the experiments. About 10ml of the solution was transferred into a 25ml volumetric flask, 1ml of 2% aluminium chloride was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30m, the absorbance was measured at 390nm.[19] A blank was also maintained.

Estimation of Total Tannins
Content of tannins in plant extract was determined by Follin-Denis method. Colorimetric estimation of tannins is based on the measurement of blue colour formed by the reduction of phosphotungstomolybdic acid by tannin like compound in alkaline medium. 1ml of plant extract and standard solution of tannic acid (10 – 50µg/ml) was made upto 7.5ml with distilled water. Then 0.5ml of Folin-Denis reagent and 1ml Na₂CO₃ solution were added. The volume was made up to 10ml with distilled water and absorbance was measured at 700nm.[20] The total tannic acid content was expressed as mg of tannic acid equivalent per gram of extract.

Estimation of Phenol
0.5 to 1gm of the sample was weighed and ground well with 10 times volume of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20m. The supernatant was
collected and the residue was re-extracted with 5 times the volume of 80% Ethanol. Centrifuged and the supernatant was collected. It was then evaporated to dryness. The residue obtained was dissolved in 5ml of distilled water. Different aliquots (0.2 – 2.0ml) were pipetted out in test tubes. The volume was made up to 3ml with water. 0.5ml of Folin’s reagent was added to all the tubes after 3m and 2ml of 20% Sodium bicarbonate solution was added. The content was mixed thoroughly and the test tubes were placed in boiling water bath for 1m, cooled and the colour developed was measured at 650nm. \([21]\) The amount of total phenol was calculated using the standard graph.

**Estimation of Ascorbic Acid**

500mg of the plant sample was weighed exactly and it was grinded with a mortar pestle with 10ml of 4% oxalic acid which was centrifuged for 10m. The supernatant was used for the estimation. 5ml of the working standard pipetted out into 100ml of conical flask. Then add 10ml of 4% oxalic acid and titrated against the dye (\(V_1\)ml). End point is the appearance of pink colour which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. 5ml of the supernatant was pipetted out, 5ml of 4% oxalic acid was added and titrated against the dye (\(V_2\)ml). The ascorbic acid in the plant was expressed in the mg/100g of the plant sample. \([22]\)

**MTT/cytotoxicity assay against cancer cell lines**

MTT assay is based on the ability of viable cells with active mitochondrial to produce succinate dehydrogenate enzyme which cleave the tetrazolium rings of MTT where the optical density (OD) obtained was proportional to the number of healthy viable cells.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1X10^6 cells/ml using PBS. To each well of the 96 well microtitre plate, 0.5ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 μl of different test concentrations of test drugs were added and made up with PBS on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO\(_2\) atmosphere, and microscopic examination was carried out and observations were noted every 24h interval. After 24h, the drug solutions in the wells were discarded and 20 μl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 4h at 37°C in 5% CO\(_2\) atmosphere. The supernatant was removed and 100 μl of DMSO was added and the
plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage of growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line.

\[
\% \text{ of Growth Inhibition} = \frac{\text{Mean OD of Individual test group}}{\text{Mean OD of Control group}} \times 100
\]

**Statistical Analysis**

All experiments were repeated at least three times. Results are reported as Mean ± Standard deviation.

**RESULTS AND DISCUSSION**

**Macroscopic characteristics**

Blue Rattle pod is a branched annual herb, 50-100 cm high and branches are acute and angled. Flowers occur in 10-12 flowered racemes at the end of branches or laterally. The racemes are typically 15-20 cm long. Flower tube is about 2 cm, bluish purple and white. "Standard" petal 1.5 cm, obovate, veined with dark purple lines. Pods are densely silky hairy when young, 3-4 cm long and flowering happens during November month. Leaves have characteristic odour, bitter in taste and the leaves of *C. verrucosa* are ovate to triangular in shape and 5-15 cm long and leaf stalk is 2-4 mm.

**Microscopic characteristics**

Microscopical evaluation is indispensable in the initial identification of herbs as well as in detection of adulterants and identifying the plant by characteristic tissue features. The microscopical study of leaves Midrib (Figure 1) showed the presence of upper and lower epidermis, collenchyma, parenchyma and vascular bundles. Upper epidermis is made up of single layered thick walled large round to oval shaped cells. In adaxial side the upper epidermis is followed by1-2 layers of large round and oval shaped parenchyma cells. But smaller and round shaped parenchyma cells in center. Lower epidermis is made up of small round cells. Abaxial side 1-2 layers of collenchyma polygonal angular collenchyma cells are seen. In abaxial side many layers of polygonal parenchyma cells are seen. Aadjacent to the parenchyma cells a 3-4 layers of selerenchyma cells with thin lignified walls were also observed. Collateral vascular bundle with distinct phloem and xylem was also noticed. Phloem located in abaxial side of the midrib which is made up of many layers of smaller polygonal cells. Some of the phloem cells are filled with simple starch grains and prismatic
Calcium oxalate crystals. Xylem region is appeared in adaxial side and which is made up of xylem vessels and xylem fibers. Xylem vessels are spiral and reticulate thickening.

T S of lamina (Figure 2) showed the presence of upper and lower epidermis. Upper epidermal cells are wide and large rectangular and elongated with thick layer of striated cuticle. Lower epidermis is made up of small narrowly elongated and oval shaped cells with cuticle. Unicellular covering trichomes with smooth surface with wide and narrow lumen were also seen. Upper epidermis is followed by palisade parenchyma of 2 layers of elongated polygonal cells. Vascular bundles were also observed in some places of the lamina and the xylem vessels showed spiral and reticulate thickening. Spongy parenchyma is made up of 2-3 layers of round, oval and irregular shaped cells with small intercellular space were also seen. Epidermal cells are wavy and stomata anomocytic also noticed.

Figure 1: T.S of Leaf Midrib region of *Crotalaria verrucosa* L.
Fluorescence Analysis

Fluorescence is an important phenomenon exhibited by various phytochemical constituents present in plant material, when suitably illuminated. The fluorescence colour is specific for each compound. A nonfluorescent compound may fluoresce if mixed with impurities that are fluorescent. Some constituents show fluorescence in the visible range in day light. The ultra violet light produces fluorescence in many natural products, which is not visible in day light. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives after reacting with different reagents, hence some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation. The results of fluorescence analysis of leaves powders showed their characteristic fluorescent colour.

The dry powder of *C. verrucosa* treated with various reagents and produced different shades of color such as green, black, orange, Red, brown and yellow under day light and Black, brown, orange, green Red, orange and pink under UV light. The formation of different colour under fluorescence analysis indicates the presence steroids, alkaloids, flavonoids, phenols and Tannins (Table 1).

After treating with some chemical reagents, fluorescence Character of *Crotalaria verrucosa L* powder and extracts from various solvents were observed at 24h and 48h under normal and ultraviolet light at 254 nm and recorded in Table 1.
Table 1: Fluorescence analysis of *Crotalaria verrucosa* L. powder.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>DAY light</th>
<th></th>
<th></th>
<th>UV light</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 Hour</td>
<td>24 Hour</td>
<td>48 Hour</td>
<td>0 Hour</td>
<td>24 Hour</td>
<td>48 Hour</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Dry Powder</td>
<td>Green</td>
<td>Bluish green</td>
<td>Dark green</td>
<td>Black</td>
<td>Black</td>
<td>Black</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Dry Powder+ Aqueous 1N NaOH</td>
<td>Dark green</td>
<td>Black</td>
<td>Black</td>
<td>Dark Brown</td>
<td>Dark Brown</td>
<td>Dark Brown</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Dry Powder+Alcoholic 1N NaOH</td>
<td>Light green</td>
<td>Dark green</td>
<td>Dark green</td>
<td>Orange</td>
<td>Yellowish Green</td>
<td>Green</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Dry Powder+ 1N HCl</td>
<td>Light green</td>
<td>Orange</td>
<td>Orange</td>
<td>Light Green</td>
<td>Green</td>
<td>Dark Green</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Dry Powder+ 50% H$_2$SO$_4$</td>
<td>Light green</td>
<td>Red</td>
<td>Red</td>
<td>Dark Green</td>
<td>Black</td>
<td>Black</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Dry Powder+Chloroform</td>
<td>Green</td>
<td>Orange</td>
<td>Orange</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Dry Powder+Hexane</td>
<td>Green</td>
<td>Red</td>
<td>Red</td>
<td>Orange</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Dry Powder+Ethyl Acetate</td>
<td>Light brown</td>
<td>Red</td>
<td>Red</td>
<td>Light Orange</td>
<td>Reddish Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Dry Powder+Acetone</td>
<td>Light brown</td>
<td>Green</td>
<td>Dark Green</td>
<td>Orange</td>
<td>Reddish Pink</td>
<td>Pink</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Dry Powder+Benzene</td>
<td>Yellowish green</td>
<td>Pale yellow</td>
<td>Yellow</td>
<td>Light Orange</td>
<td>Reddish Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Dry Powder+Alcohol</td>
<td>Light green</td>
<td>Reddish orange</td>
<td>Red</td>
<td>Light Pink</td>
<td>Light Pink</td>
<td>Pink</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Dry Powder+Water</td>
<td>Light green</td>
<td>Green</td>
<td>Dark Green</td>
<td>Light Green</td>
<td>Light Green</td>
<td>Green</td>
<td></td>
</tr>
</tbody>
</table>

Physico-chemical Analysis

Table 2: Physicochemical Constants

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parameters</th>
<th>Value % W/W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Foreign Matter</td>
<td>0.76±0.16</td>
</tr>
<tr>
<td>2.</td>
<td>Loss on drying</td>
<td>1.08±0.11</td>
</tr>
<tr>
<td>3.</td>
<td>Total Ash Content</td>
<td>6.30±0.27</td>
</tr>
<tr>
<td>4.</td>
<td>Water soluble Ash</td>
<td>4.4±0.37</td>
</tr>
<tr>
<td>5.</td>
<td>Acid insoluble Ash</td>
<td>1.80±0.42</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE (n=3)

Extractive Values analysis

Table 3: Successive Extractive Values.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parameters</th>
<th>Value % W/W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>0.72±0.21</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform</td>
<td>2.28±0.31</td>
</tr>
<tr>
<td>3.</td>
<td>Ethyl acetate</td>
<td>3.04±0.19</td>
</tr>
<tr>
<td>4.</td>
<td>Alcohol</td>
<td>8.7±0.46</td>
</tr>
<tr>
<td>5.</td>
<td>Water</td>
<td>24.6±1.12</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE (n=3)
**Physico-chemical constants analysis**

The Physicochemical parameters have been evaluated to determine the percentage of foreign matter, moisture content; total ash, water soluble, acid insoluble ash and extractive value as per Indian Pharmacopoeia.

Foreign organic matter involves removal of matter other than source plant to get the drug in pure form. Herbals should be entirely free from moulds or insect, including excreta and visible contaminant such as sand and stones, poisonous and harmful foreign matter and chemical residues.

In Table 2, the physicochemical contents of *C. verrucosa* were recorded. The percentage of foreign matter was determined as 0.76%, which means the plant material *C. verrucosa* has less contaminant. The moisture content of leaves of *C. verrucosa* is 1.08% which clearly indicates that the plant extracts will not allow microbial contamination during storage. Total ash value is 6.3% which indicate the amount of minerals and earthy material attached to the plant material. The amount of the water soluble ash is 4.4% and acid insoluble siliceous matter present in the plant 1.8%. Less acid insoluble ash value clearly indicates the presence of less inorganic content and the purity of the plant sample.

The Table 3 showed the extractive values of leaves of *C. verrucosa* soaked in different solvent which are useful to evaluate the chemical constituents present in the crude drug and also help in the estimation of specific constituents soluble in a particular solvent. These are indicative of weights of the extractable chemical constituents of crude drug under different solvents. The Hexane (0.72%) and chloroform (2.28%) extractive values are less when compared to ethyl acetate (3.04%). The solubility of leaf powder of *C. verrucosa* in water (24.6%) is found to be higher compared to ethanol solubility (8.7%). Water soluble extractive value (24.6%) is higher than any other solvent, which indicates the presence of highly polar chemical constituents such as Phenol, flavonoids and alkaloids.
Phytochemical Analysis.

Table 4: Behaviour of leaf powder and different extracts of *C. verrucosa* with Various Chemical Reagents

<table>
<thead>
<tr>
<th>S. No</th>
<th>Test for</th>
<th>Powder</th>
<th>Hexane</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Ethanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Terpenoids</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavones</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Steroids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Phenol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Tannin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Saponin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Coumarin</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Lignin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Quinone</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates Presence  
- indicates Absence

Behaviour of leaf powder and different extracts of *C. verrucosa* with Various Chemical Reagents

The dry powder and various extracts of leaves of *C. verrucosa* are subjected to qualitative chemical examination for the presence or absence of secondary metabolites. From the table 4, it is observed that the *C. verrucosa* powder showed the presence of Terpenoids, Flavones, Steroids, Alkaloids, Phenol, Tannin, Saponin and Glycosides.

The results of the preliminary phytochemical screening of various extracts are shown in Table 4. Phenol, Flavones, alkaloids and Glycosides are present in all the extracts. Steroids are present in hexane, chloroform, ethylacetate and water extracts where as, Coumarine is present in hexane, ethyl acetate and ethanol extracts. Terpenoid is found to be present in ethanol and water extracts; Tannin, Saponin, Lignin and Quinone are absent in all the extracts of *C. verrucosa*. Based on the results from extractive value and preliminary phytochemical screening tests, the aqueous extracts of *C. verrucosa* (AECV) and ethanolic extracts of *C. verrucosa* (EECV) were selected for further investigations.
Quantitative Analysis of Phyto Constituents.

Table 5: Estimation of Important Phyto Constituents.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Organic constituents (mg/kg)</th>
<th>Ethanol Extract</th>
<th>Aqueous Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total Alkaloids</td>
<td>15.23±0.26</td>
<td>12.45±0.39</td>
</tr>
<tr>
<td>2.</td>
<td>Total Flavonoids</td>
<td>5.18±0.29</td>
<td>4.23±0.12</td>
</tr>
<tr>
<td>3.</td>
<td>Phenols</td>
<td>8.13±0.25</td>
<td>7.15±0.16</td>
</tr>
<tr>
<td>4.</td>
<td>Tannins</td>
<td>0.25±0.08</td>
<td>0.23±0.07</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE (n=3)

The secondary metabolites present in the plants play an essential role in the pharmacological potential of the crude drug. Phytochemical quantification is very important in identifying therapeutically and industrially important compounds like alkaloids, flavonoids, phenolic compounds and tannins present in the crude drug. The quantitative analysis of important secondary metabolites were carried out for AECV and EECV and recorded in Table 5.

The alkaloids are more diverse group of secondary metabolites found in plants and have a range of structure, types, biosynthetic pathways and pharmacological activities. From the results, alkaloids are found to be significantly rich in ethanol extract (15.23±0.26mg/g) and followed by water extract (12.45±0.39mg/g). These results indicated that the ethanol extract has high content of alkaloids and might have free radical scavenging activity and anticancer property.

Flavonoids are a collection of poly phenolic molecules with accepted properties which include free radical scavenging, inhibition of hydrolytic & oxidative enzymes and anti-inflammatory action. Flavonoids have ability of scavenging hydroxyl radicals, superoxide anion radicals and lipid peroxyradicals which are important for prevention of diseases associated with oxidative damage of membrane, proteins and DNA. The quantity of total flavonoids content of ethanol extract and water extract were found to be 5.18±0.29 and 4.23±0.12 mg/g dry weight respectively. The flavonoids play crucial role in fighting the free radicals which harm the human cells. Frequent epidemiological studies validate major relationship between the high dietary intake of flavonoids and the reduction of carcinogenic risk. There have been an increasing number of reports that directly challenge the reputed role of flavonoids as antioxidant and anticancer agents.

Phenols are ubiquitous secondary metabolites in plants and comprise a large group of biologically active ingredients which has biochemical activities such as antioxidant,
antimutagenic, anticarcinogenic activities and the ability to modify the gene expression. Total phenolic content of the leaf extracts are found to be 8.13±0.25mg/g and 7.15±0.16mg/g for ethanol and water respectively. Studies have shown that phenolics play an important preventive role in the development of cancer, heart diseases and ageing related diseases. The high content of phenolic compounds contributes to the anticancer activity.

Tannins are widely distributed in almost all plants. The tannin are used as antihelminthic, antioxidant, antimicrobial and antiviral drugs. The highest total tannins content is found in water extract (0.23±0.07mg/g) followed by ethanol extract (0.25±0.08mg/g). Herbs that have tannins as their constituent are astringent in nature and are used for treating intestinal disorders, revealing antimicrobial activity. Li et al. (2003) assessed the biological activities of tannins and observed that they have notable activity in cancer prevention.[24]

**MTT Assay**

In this study, EAC cell lines were treated with AECV and EECV at different concentrations for 24h. A mitochondrial enzyme in living cells, succinate dehydrogenase cleaves the tetrazolium ring of MTT and converting it to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. Viability of the EAC cancer cells was decreased with increasing concentration of AECV and of EECV (250 to 1000µg/ml). EECV exhibited a high cell inhibition rate against EAC as 24.15 ± 1.36, 55.46 ± 0.77, 77.61 ± 0.89 and 93.79± 0.70% and AECV also showed remarkable range of inhibitory activity against EAC as21.42 ± 0.81, 44.60 ± 1.16, 70.08 ± 1.55 and 84.09 ± 1.57%at250, 500, 750 and 1000 (µg/ml) respectively. IC50 value of AECV for EAC was found to be 565.87µg/ml and EECV for was 487.19µg/ml.

![Figure 3: Effect of AECV and EECV on EAC cell lines (MTT Assay).](image-url)
Many studies carried out on cell cultures and animal models indicated that polyphenols were the main phytochemicals with antioxidant and anti-proliferative properties from higher plants. These molecules may act as cancer-blocking agents, preventing the initiation of the carcinogenic process as cancer-suppressing agents and inhibiting cancer promotion and progression. In our preliminary study, EECV contained a higher phenolic content than other extracts, which coincides with its cytotoxic activity. This finding showed that the leaves ethanolic extract of *C. verrucosa* has are responsible for cytotoxic properties.

**CONCLUSION**

In conclusion, *C. verrucosa* has shown specific diagnostic characters during the observation of the powder. It mainly constitutes stone cells and pigment cells. From the results of physic-chemical and phytochemical analysis, it was observed that the existence of bioactive components in *C. verrucosa*. The AECV and EECV possess a promising antitumor activity, which was confirmed by the MTT assay against Ehrlich's ascites carcinoma cell lines. Henceforward, the study determines the value of medicinal plant used in pharmacy and considerable interest to the development of new drugs from the medicinal plants. Further investigations have to be carried out in isolation and the characterization of active constituents from *C. verrucosa*.

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