OBJECTIVE: Clerodendrum infortunatum is a traditional Indian medicinal plant which has been used for the treatment of many diseases. The root bark juice is effective against indigestion and abdominal pain. Based on the preliminary screening study on the antioxidant activity of the plant, the present study was aimed to isolate tannins from the root bark of Clerodendrum infortunatum and it was qualitatively analysed and quantified. The in-vitro antioxidant activity and antiproliferative effect on HCT-15 cell lines was also evaluated.

METHODS: Tannins were isolated from the root bark of Clerodendrum infortunatum. It was qualitatively analysed by phytochemical screening, protein precipitation test and thin layer chromatography (TLC) and quantified by Folin-denis assay and protein precipitation assay. The modified method of protein precipitation assay was carried out to differentiate between condensed and hydrolysable tannins. Antioxidant and antiproliferative activity were also evaluated.

RESULTS: Phytochemical screening revealed the presence of tannins. TLC and protein precipitation ability of the extract confirmed the presence of tannins. The total tannin content by Folin-denis assay was found to be 166.6±5.607 mg tannic acid equivalents/g dry extract. This coincides with the radial diffusion method (163.75 ± 7.5 mg tannic acid equivalents/g dry extract). Isolated tannins exhibited significant antioxidant and radical scavenging activities compared to the standard antioxidant ascorbic acid. 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay demonstrated the antiproliferative effect of isolated tannins against HCT-15 cell lines and about 50% of the cells were found to be dead at a concentration of 100 µg/ml.

CONCLUSION: The antioxidant and antiproliferative properties exhibited by tannins isolated from Clerodendrum infortunatum suggest it as a new source of medicament.

KEYWORDS: Clerodendrum infortunatum, Verbenaceae family, Hydrolysable tannins, Antiproliferative effect, Colon cancer, Thin layer chromatography.
was added. The contents were mixed thoroughly and after 3 min, 2 ml of tannins. To 2 ml of the extract an equal volume of Folin-denis reagent based on the reducing power of the phenolic hydroxyl group of protein precipitation assay. The tannins were quantified by the modified method of Hangerman's concentration. To construct the standard curve, different concentrations (10, 25, 50, 100 mg/ml) of tannins. Tannin radial diffusion [19]. The protein precipitation test is performed with 1.1-diphenyl-2-picrylhydrazyl (DPPH) by the modified method [20]. 2 ml reaction mixtures containing 1 ml methanolic solution of DPPH (0.1 mM) and 1 ml of standard ascorbic acid and isolated tannins at various concentrations (1000-1.95 µg) were incubated in dark at 37 °C for 30 min. 1 ml of dimethyl sulfoxide (DMSO) served as the control. After incubation the absorbance was read at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and can be calculated using the formula % inhibition of DPPH radical = Abscontrol - Abssample x 100 Abscontrol IC50 value was calculated using the graph by plotting inhibition percentage against extract concentration. total antioxidant activity The total antioxidant activity of the tannins was determined by the ferric reducing power assay (FRAP) [21]. 0.1 ml of acetic acid (5-30 µg) serves as the standard. The antioxidant activity was expressed as mg ascorbic acid equivalents per gram of sample on a dry weight basis. ferric reducing antioxidant power (FRAP) assay Ferric reducing ability of the isolated tannins was estimated by the ferric reducing power assay [22]. 0.2 ml of the extract was added to 3 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer (pH 3.6), 1 part of 10 mM TPTZ solution and 1 part of 20 mM FeCl3·6H2O solution) and the reaction mixture was incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. FeSO4 (0.002-0.02 mmol/ml) was used as the standard. The antioxidant capacity of tannins based on the ability to reduce ferric ions was calculated from the linear calibration curve and expressed as mmol FeSO4 equivalents per gram dry extract. Ascorbic acid was used as the positive control.

antiproliferative effect of tannins The in-vitro antiproliferative effect of tannins was determined in colon cancer cell line HCT-15. HCT-15 cell lines were purchased from NCCLS Pune and were maintained in Dulbecco’s modified eagle's medium (Himedia) supplemented with 10% fetal bovine serum (Invitrogen) and grown to confluency at 37 °C in 5% CO2 (NBS, Eppendorf, Germany) in a humidified atmosphere in CO2 incubator. The cells were trypsinized (500 µl of 0.025% trypsin in PBS/0.5 mM
and it can be visualized as discrete bands. This confirmed tannins as 2). The reference compound tannic acid migrated from the origin (60/60/10) as mobile phase and fumigated with iodine vapour (fig. 2). The spot containing aqueous acetone extract remained at the origin.

**Thin layer chromatography**

The spot containing aqueous acetone extract remained at the origin when the TLC plates were developed in toluene acetone:formic acid (60/60/10) as mobile phase and fumigated with iodine vapour (fig. 2). The reference compound tannic acid migrated from the origin and it can be visualised as discrete bands. This confirmed tannins as the phytochemical component present in the aqueous acetone extract [20, 21].

**RESULTS AND DISCUSSION**

**Qualitative analysis**

**Percentage yield**

The percentage yield of the aqueous acetone extract of the root bark of *Clerodendrum infortunatum* was found to be 1.5%.

**Phytochemical analysis**

The phytochemical screening of aqueous acetone extract of the root bark showed the presence of tannins and phenols (Table 1). Plants have free radical scavenging molecules such as vitamins, terpenoids, phenolic acids, tannins, flavonoids, quinones, coumarins and alkaloids that have promising antioxidant properties [28]. These compounds make plants rich sources of drugs.

**Table 1: Phytochemical constituents of aqueous acetone extract of the root bark of Clerodendrum infortunatum Linn**

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Aqueous acetone extract of the root bark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
</tbody>
</table>

‘+’ indicates the presence and ‘−’ indicates the absence of phytochemicals.

**Protein precipitation test**

Opaque precipitate was observed in the form of a ring around the wells treated with aqueous acetone extract (fig. 1). The ability to precipitate proteins is the defining characteristics of tannins. They can cross-link with fibrous protein due to their polymeric nature. Opaque precipitate confirmed the presence of tannins in the root bark [19].

**Radial diffusion method**

The extracts at all concentrations precipitated proteins in the gel which can be viewed as ring around the wells. The diameter of the rings seemed to be increased with increase in concentration of the extract. From the standard curve of tannic acid, the amount of tannin in the extract was found to be 163.75 ±7.5 mg tannic acid equivalents/g dry extract.

**Selective determination of condensed or hydrolysable tannins**

Tannins possess immense structural variability and are classified into hydrolysable tannins and condensed tannins based on their hydrolytic reaction with mineral acids and enzymes and the nature of phenolic nuclei involved in the tannins structure. In the modified method of radial diffusion, ring of precipitation was not observed in the extract treated with hydroxylamine hydrochloride reagent whereas the untreated extract showed ring of precipitate around the well (fig. 3). Treatment of hydrolysable tannins with hydroxylamine hydrochloride resulted in the hydrolysis of ester bonds, which then decomposed into core polyols and hydroxamates of the phenolic acids (gallic acid and ellagic acid). These phenolics cannot precipitate proteins in the radial diffusion method. Condensed tannins are unaffected by hydroxylaminolysis [23]. This indicated the presence of hydrolysable tannins in the extract.

**Antioxidant assays**

The antioxidant and scavenging properties of tannins isolated from the root bark of *Clerodendrum infortunatum* were studied. A large number of diseases are caused due to the deleterious effects of free
radicals and reactive oxygen and nitrogen species. Antioxidants scavenge these molecules before they attack the body and thus protect from diseases. Currently the interest in natural antioxidants has increased due to the toxicity associated with the synthetic drugs [29].

E1-before hydroxylaminolysis, E2-after hydroxylaminolysis, T-tannic acid  

**Fig. 3:** Radial diffusion of isolated tannins before and after hydroxylaminolysis compared to tannic acid

Reducing power

Isolated tannins exhibited significant reducing power which increased with increasing concentration. Fig. 4 shows the dose-response curve for the reducing power of the tannins. The reducing power of tannins may be due to the presence of hydroxyl group of phenols, which possess hydrogen donating abilities [30]. It was previously reported that phenolic compounds scavenge free radicals by an electron-transfer mechanism [31].

Ascorbic acid is diluted 1:4. Values are the mean±SD, where n=4. A-Ascorbic acid, B-Tannins  

**Fig. 4:** Reducing power of isolated tannins and ascorbic acid

DPPH radical scavenging activity

The radical scavenging activity of tannins was determined by using DPPH as substrate. The decrease in the purple colour of DPPH indicated an increase in free radical scavenging activity. Thus isolated tannins exhibited concentration dependent radical scavenging activity (fig. 5). The percentage inhibition of DPPH radicals increases over the concentration range of 1.95 -31.25 µg/ml, equivalent to 1.95 -31.25 µg/ml of ascorbic acid. The IC₅₀ value of tannins also points to its antioxidant potential (table 2). The effect of antioxidant in DPPH can be attributed to its hydrogen donating ability [32].

**Table 2:** IC₅₀ value of isolated tannins and ascorbic acid

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>IC₅₀ Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>13.48±0.392</td>
</tr>
<tr>
<td>Tannins</td>
<td>20.70±1.497</td>
</tr>
</tbody>
</table>

Values are the mean±SD (n=4).

Ferric reducing ability

The ferric reducing ability of tannins was estimated by the redox linked reaction. Antioxidants act as the reductants and reduce the ferric tripyridyltriazine to the ferrous complex of intense blue colour and it was expressed as mmol equivalents of FeSO₄ and calculated from the standard plot of FeSO₄. This is a pH dependent reaction. The reducing ability of tannin was found to be 314.67±13.57 mmol FeSO₄ equivalents per gram dry extract, although it was less when compared to ascorbic acid (table 3). This indicated that they were capable of donating electrons to the free radicals to make them stable.

**Table 3:** Ferric reducing ability of tannins

<table>
<thead>
<tr>
<th>Extract</th>
<th>Antioxidant activity (mmol FeSO₄ equivalents per gram dry extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>610.83±13.57</td>
</tr>
<tr>
<td>Tannins</td>
<td>314.67±9.44</td>
</tr>
</tbody>
</table>

Values are the mean±SD (n=4).

Antiproliferative effect of tannins against colon cancer cell lines

Cancer remains one of the world's leading causes of death. Colon cancer, also known as colorectal or bowel cancer, which is characterized by uncontrolled cell growth in colon and rectum, is the third most common kind of cancer. Due to the lack of effective chemotherapeutic agents and the side effects associated with the existing ones, the search for alternative products from natural sources begins. The exposure of colon cancer cells to tannins exhibited significant reduction in the conversion of MTT as evident from the decrease in absorbance. The cleavage of tetrazolium ring in MTT involves mitochondrial succinate dehydrogenase and depends on the activity of respiratory chain and redox state of mitochondria [35]. The number of viable cells decreases with increase in concentration of the isolated tannins. At a concentration of 100 µg/ml, more than half of the cells were found to be dead by the cytotoxic effect of tannins (fig 6). The cells were examined under the microscope and found to possess structural features that characterize the loss of viability. All the treated
cells exhibited different levels of cytotoxicity. Cell shrinkage, aggregation, cell rounding and cell death was visible, depending upon the concentration (fig. 7). The cell was found to be detached from the surface. These morphological changes also points to the cytotoxic effects of tannins.

![a) Control (HCT-15 cell line)](image1)

![b) HCT-15 cells treated with 10µg/ml tannin](image2)

![c) HCT-15 cells treated with 50µg/ml tannin](image3)

![d) HCT-15 cells treated with 100µg/ml tannin](image4)

**Fig.7:** HCT-15 cell lines treated with tannins at different concentrations

![Graph: Effect of tannins on the viability of HCT 15 cancer cell lines](image5)

Values are the mean±SD (n=4).

**Fig. 6:** Effect of tannins on the viability of HCT 15 cancer cell lines

**CONCLUSION**

Tannins isolated from the root bark of *Clerodendrum infortunatum* possessed significant antioxidant activity. The results were compared with a known antioxidant, ascorbic acid. Reducing power of tannins increased with increasing concentration. They exhibited the ability to scavenge DPPH radical and significant total antioxidant activity. Free radicals are involved in the pathogenesis of many diseases and thus free radical scavengers can provide the protective effect from such conditions. In this view, the antiproliferative effect of tannins was evaluated and it exhibited significant cytotoxicity against HCT-15 cell lines. Thus tannins from the root bark of *Clerodendrum infortunatum* may act as a source of natural antioxidant and an anticancer agent.

**ACKNOWLEDGEMENT**

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**CONFLICT OF INTERESTS**

Declare None

**REFERENCES**