

## **ANTIOXIDANT AND ANTICANCER ACTIVITY OF METHANOLIC EXTRACT FROM *STEPHANIA ELEGANS***

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### **ABSTRACT**

**Objective:** The present work was aimed to investigate the *in vitro* antioxidant and anti-cancer activities of methanolic extract of *Stephania elegans*, an unexplored species from *Menispermaceae* family.

**Methods:** The methanolic extract of *S. elegans* tubers was prepared and phytochemical screening and total phenolic content were analyzed by using standard methods. *In vitro*, antioxidant potential of methanolic extract was determined by 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays. Cytotoxicity against human breast cancer cell line, Michigan Cancer Foundation-7 type (MCF-7) was evaluated by 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay.

**Results:** Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, carbohydrates, tannins, terpenoids, steroids, and saponins in the methanolic extract of *S. elegans* tubers. The total phenolic content in the methanolic extract was 23.0±0.06 mg GAE/g (dry mass). As revealed by ABTS assay, the methanolic extract of plant tubers showed significant radical scavenging activity (IC<sub>50</sub> 41.66±0.015 µg/ml). The reducing power activity of the extract increased with the concentration of the extract. MTT assay indicated that *S. elegans* has potent cytotoxic activity towards MCF-7 cells (IC<sub>50</sub> 158.7±0.13 µg/ml).

**Conclusion:** This is the first study demonstrating the antioxidant and anticancer capabilities of the methanolic extract of *S. elegans*. This study also provides a significant basis for further isolation and characterization of bioactive compounds from *S. elegans*.

**Keywords:** *S. elegans*, Anti-cancer, Antioxidant, FRAP, MCF-7

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### **INTRODUCTION**

Cancer is a hyperproliferative condition where cellular homeostasis of a healthy cell fail, leading to activation of numerous genes that are actively involved in survival, cell cycle, metastasis, and angiogenesis. It continues to be a global threat, the greatest cause of mortality and morbidity worldwide [1-3]. A number of novel concepts have been developed for therapeutic intervention in malignant diseases with the aim of recognising specific targets and overcoming the resistance against existing cytotoxic treatments. The numerous cancer cure modalities include surgery, anti-tumor medications and some targeted therapies including the use of radiation and Photodynamic therapy [4, 5]. Advanced therapies designed to stimulate the effector pathways involved in cell death, including apoptosis could be utilised as an effective strategy for cancer management [6]. Deploying plant-derived compounds or phytochemicals to the work is one such promising approach. The use of plant-derived bioactive compounds for anticipation, mitigation and therapeutics for cancer have been applied extensively. Various researchers have recognised species of plants that have established anticancer activities, especially those that have been used in herbal medicine in developing countries [7-9]. The natural compounds isolated from medicinal plants are believed to be major leads in the designing of anticancer medications. Screening of medicinal plants and their bioactive constituents for several biological activities, such as anticancer activity, has been a key research area since last few decades [10].

Free radicals and related species have attracted a great deal of attention in recent years. Reactive oxygen species (ROS)/Reactive nitrogen species (RNS) and other free radicals are responsible for countless diseases, such as arteriosclerosis, heart diseases, diabetes, aging process and cancer [11, 12]. ROS/RNS can activate the initiation, promotion and advancement phases of cancer by damaging DNA bases and the deoxyribose backbone leading to severe mutations in fundamental genes [13, 14]. A lot of studies have reported that cancers

are associated with ROS production [15]. Antioxidant defences encompass a variety of distinct compounds and enzymes that are linked together through their capacity to neutralise and scavenge ROS. Phenolic extracts or isolated polyphenols from different plants have been studied for their anti-cancerous effects on various cell lines representing different stages of cancer [16, 17].

Over the last few decades, there has been increased interest by pharmaceutical industries to discover the new drugs from the ethnobotanicals to provide new and alternative drugs to synthetic drugs for the treatment of dreadful diseases. *Stephania elegans* belong to *Menispermaceae* family, including 65 genera and 350 species [18]. The members of this family are generally herbs or shrubs and are small climbers with peltate and membranous leaves and are generally seen in temperate and tropical regions of lower Himalayas in India, Bhutan, Burma, China and Nepal at altitudes of 2000-2200 m. These plants (especially tubers) have recognised medicinal values and traditionally have been used by locals for the treatment of asthma, tuberculosis, dysentery, hyperglycemia, cancer, fever, intestinal complaints, sleep disturbances and inflammation [19-21]. *Stephania* is the principal genus comprising 43 species and a lot of studies have been carried to investigate anticancer properties of diverse *Stephania species* [22-26]. Till date, no scientific work has been conducted on bioactivities mediated by *S. elegans* phytochemicals, however *S. elegans* was found to be an alkaloid rich plant, and identification of nine alkaloids have been reported [27]. These days, pharmaceutical agencies significantly relies on natural products obtained from traditional medicinal plants and also an emphasis is given on unexplored ones. The current report demonstrates novel antioxidant and anticancer activities (against MCF-7 cells) of the methanolic extract of *S. elegans* tubers.

### **MATERIALS AND METHODS**

#### **Materials**

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT), 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS),

ascorbic acid and Gallic acid were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 2,4,6-tripyridyl-s-triazine (TPTZ), ferrous sulfate ( $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$ ), trypsin, vincristine, penicillin and streptomycin were purchased from HiMedia Laboratories Pvt. Ltd. Dimethyl sulphoxide, potassium persulfate, sodium bicarbonate, Mayer's reagent, Benedict's solution, acetic anhydride lead acetate, ferric chloride, glacial acetic acid, hydrochloric acid, sulfuric acid and sodium hydroxide were purchased from Loba Chemie. All organic solvents like methanol, chloroform were of HPLC grade.

#### Preparation of extract from *S. elegans*

Plant tubers were collected from the Koti village of Solan, Himachal Pradesh, India. Botanical identification of species was done by renowned taxonomist of Y. S Parmar University, Nauni (H. P., India), identified as *S. elegans* and linked to UHF-Herbarium with Field book No. 13530. Dried tubers were powdered and macerated by methanol at a ratio of 1:5 (w/v) for 72 h. The extract was filtered using Whatman filter paper and then concentrated in vacuo at 40 °C using a rotary evaporator and then stored as a methanolic extract for experiments.

#### Preliminary phytochemical screening

The methanolic extract of *S. elegans* was screened for the presence of various families of phytoconstituents viz. Alkaloids, carbohydrates, glycosides, saponins, tannins, quinones, coumarin, amino acids, terpenoids and steroids by respective reagents using standard procedures [28-30].

#### Quantitative determination of total phenolics

Folin Ciocalteu reagent was used for the analysis of the total phenolic content of the methanolic extract [31]. A volume of 0.2 ml of the extract was introduced into test tubes followed by 0.5 ml Folin-Ciocalteu's reagent (diluted 10 times with water).

The solution was then kept in the dark for 5 min and then 1 ml sodium carbonate (7.5% w/v) was added. After 30 min, the absorbance of the mixture was measured by UV-vis spectrophotometer at 765 nm and compared to a gallic acid calibration curve. Gallic acid served as the standard for preparing the calibration curve ranging from 20 to 80 µg/ml assay solution. The determinations were carried out in triplicate and the total phenolic content was expressed as Gallic acid equivalents (mg of GAE/g of sample).

#### *In vitro* antioxidant assay (ABTS assay)

ABTS assay was conducted to analyse antioxidant attributes of *S. elegans*. The ABTS<sup>•+</sup> is generated by reacting an oxidizing agent (e. g. Potassium persulfate) with the ABTS salt. The reduction of the blue-green ABTS<sup>•+</sup> by hydrogen-donating antioxidant is calculated by the suppression of its characteristic long wave absorption spectrum and by the effect of this reaction blue ABTS radical cation is converted back to its colourless neutral form. ABTS solution was prepared by dissolving light green coloured ABTS in distilled water to get a final concentration of 7.4 mmol. 2.46 mmol potassium persulfate solution was also prepared. Then both solutions were blended rigorously in ratio 1:1 and the solution was kept in the dark for 24 h. Further, the working solution was prepared by diluting the solution 1:25 in methanol [32].

Sample extract was prepared in DMSO (1 mg/ml). Ascorbic acid, a notable antioxidant was considered as a positive control and methanol was used as a blank whereas DMSO was used as negative control. To 3 ml of ABTS<sup>•+</sup> solution, sample extracts containing antioxidant were added at the concentrations 20, 40, 60 and 80 µg/ml. Absorption was taken using light absorption spectrophotometer at a wavelength of 734 nm. The assay was performed three times, and percentage inhibition (IC<sub>50</sub> value) was calculated by Graphpad Prism 5.0.2.

#### FRAP assay

The FRAP assay was carried out by Benzie and Strain method [33] with certain modifications. The stock solutions for the assay included 300 mmol acetate buffer (3.1 g  $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$  and 16 ml  $\text{C}_2\text{H}_4\text{O}_2$ ), pH 3.6, 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution.

The fresh working solution was prepared by mixing 16 ml acetate buffer, 2 ml TPTZ solution, and 2 ml  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution and then warmed at 37 °C before carrying out the experiment. Sample (150 µl) was allowed to react with 2.850 ml of the FRAP solution for 30 min in the dark condition. FRAP values were calculated by measuring the absorbance of coloured product [ferrous tripyridyl triazine complex] at 593 nm and then relating it to a ferrous ion standard solution. Results were expressed in µM TE/g fresh mass.

#### Cell culturing and maintenance

To evaluate anticancer properties of *S. elegans*, MCF-7 (breast carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Following standard procedures, stock cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and amphotericin B (5 µg/ml) in a humidified atmosphere of 5%  $\text{CO}_2$  at 37 °C until confluent. The stock cultures were grown in 25 cm<sup>2</sup> tissue culture flasks, and all experiments were carried out in 96 well Microtiter plates.

#### Subculturing

Used media were disposed of followed by a PBS wash in order to take out the dead cells and debris. Trypsinization of cells was carried out using 0.25% of trypsin. Further centrifugation was done at 2000 rpm for 10 min; the pellet was then cultivated.

Fresh aliquots were made and transferred to new culture dishes for sub-culturing ( $1 \times 10^4$  viable cells/well were seeded). Cell counting was conducted using a hemocytometer, 2000 cells/well in 200 µl media were seeded in a 96 well plate and further incubated for 24 h for optimal growth in nourished media and environment.

#### *In vitro* cytotoxicity assay

The methanolic extract of *S. elegans* was tested for *in vitro* cytotoxicity by using MTT reagent (5 mg/ml) [34]. Cells (2000 cells/well) were seeded in media (DMEM) in a 96 well plate followed by overnight incubation.

*S. elegans* extract in different concentrations (25, 50, 100, 200 µg/ml) were added followed by a 24 h incubation. Vincristine (10 µg/ml) and DMSO were used as positive and negative controls, respectively. The optical density was noted at 595 nm using a microplate reader. IC<sub>50</sub> values were calculated by Graphpad Prism 5.0.2.

#### Statistical analysis

Results were expressed as mean ± standard error. The one-way ANOVA and multiple comparisons of 2-tails of Student's t-test (2 groups) were used to evaluate the difference between the control and test samples by the Graphpad Prism software for windows. A p value ≤ 0.05 was considered to be significant.

## RESULTS

#### Phytochemical analysis

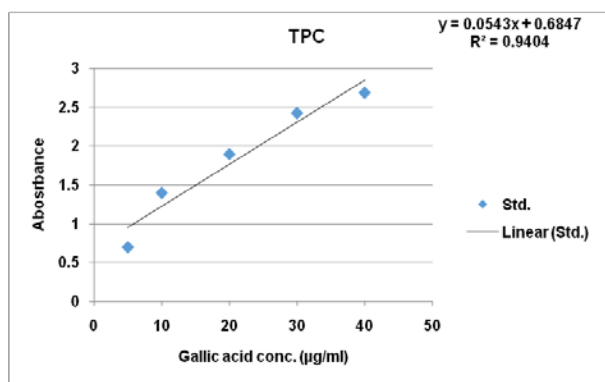
40 g of plant tuber powdered material yielded 4.1 g of methanolic extract (10.25%). The phytochemical analysis revealed that the methanolic extract of *S. elegans* contains alkaloids, carbohydrates, flavonoids, saponins, terpenoids/steroids and tannins (table 1). The quinones, coumarin, amino acids and glycosides were absent in the plant extract.

**Table 1: Phytochemical assessment in methanolic extract of *S. elegans* (+indicates the presence and -indicates the absence of a phytochemical)**

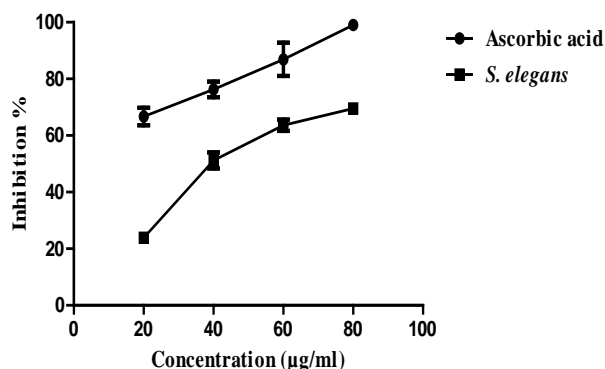
Phytochemicals	Methanolic extract of <i>S. elegans</i>
Alkaloids	+
Carbohydrates/reducing sugars	+/+
Flavonoids	+
Tannins	+
Terpenoids/steroids	+
Saponins	+
Amino acids	-
Quinone	-
Coumarin	-
Glycosides	-

**Total phenolic content (TPC)**

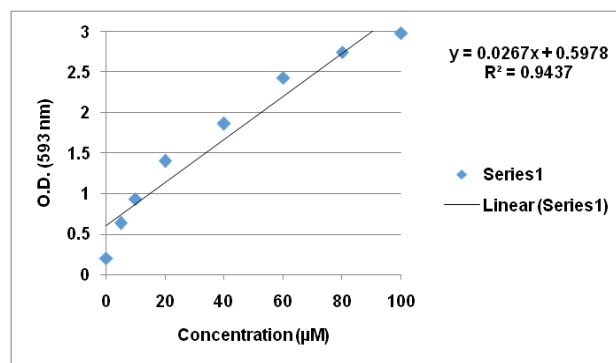
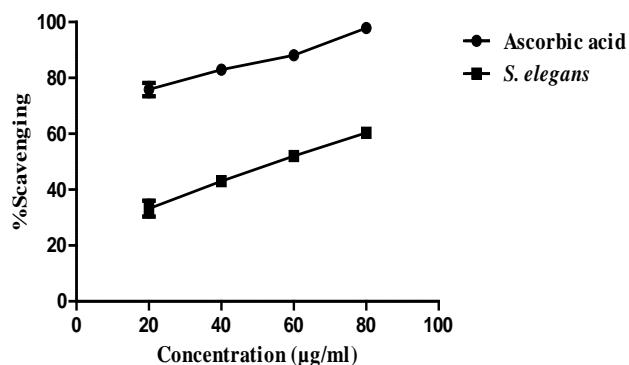
The total phenolic content was reported as Gallic acid equivalents (fig. 1) with reference to a standard curve (standard curve equation:  $y = 0.0543x + 0.06847$ ,  $R^2 = 0.9404$ ). The total phenolic content measured in methanolic extract was  $23.0 \pm 0.06$  mg GAE/g of dry sample.

**Fig. 1: Standard curve of Gallic acid for determination of total phenolic content****In vitro antioxidant assay**

*In vitro* ABTS assay was conducted to study the antioxidant activity of *S. elegans* at a concentration range of 20-80 µg/ml. Ascorbic acid, a well-known antioxidant compound was taken as positive control (fig. 2). As compared to ascorbic acid ( $IC_{50} 12.56 \pm 0.1$  µg/ml), *S. elegans* extract also showed significant antioxidant activity with an increase in concentration ( $IC_{50} 41.66 \pm 0.015$  µg/ml) (table 2, fig. 2). Thus the selected plant possesses high antioxidant potential.

**Fig. 2: Antioxidant activity of standard ascorbic acid and methanolic extract of *S. elegans*. The experiment was done twice (in triplicate) using two different samples****FRAP assay**

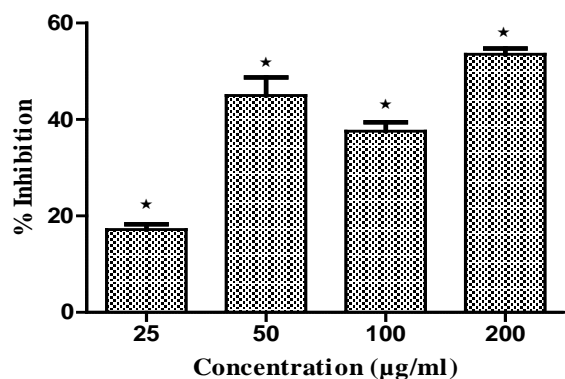
FRAP assay was carried out, and results were compared with a standard curve of ferrous sulphate ( $y = 0.0267x + 0.5978$ ,  $R^2 = 0.9437$ ) (fig. 3). The antioxidant activity of methanolic extract of *S. elegans* (20-80 µg/ml) as determined by FRAP assay varied from 33.33-58.76 µM TE/g. The methanolic extract of *S. elegans* revealed significant reducing potential ( $IC_{50} 51.62 \pm 0.023$  µg/ml) which increased with the increase in concentration from 20-80 µg/ml (table 2, fig. 4).

**Fig. 3: Standard curve of ferrous sulphate****Fig. 4: Ferric reducing antioxidant power of standard ascorbic acid and methanolic extract of *S. elegans*. The experiment was done twice (in triplicate) using two different samples and data was significant as \* $p < 0.05$** **In vitro cytotoxicity assay for MCF-7**

*In vitro* cytotoxicity was analysed on MCF-7 cells by MTT assay at four different drug concentrations (25, 50, 100, 200 µg/ml). *S. elegans* showed significant cytotoxicity from 50 µg/ml to 200 µg/ml, and  $IC_{50}$  value was calculated as  $158.7 \pm 0.13$  µg/ml (fig. 5). The highest activity was recorded at 200 µg/ml, i.e. 54%.

**Table 2: % Inhibitory concentration (IC<sub>50</sub>) of methanolic extracts of *S. elegans* as determined by the ABTS and FRAP assays. Each value represents mean±SEM (n = 3)**

IC <sub>50</sub> value (µg/ml)	ABTS	FRAP
Ascorbic acid	12.56±0.1	7.24±0.14
<i>S. elegans</i>	41.66±0.015	52.96±0.024

**Fig. 5: Cytotoxic effect of *S. elegans* methanolic extract on MCF-7 cells. Experiment was done twice in triplicates at different concentrations**

## DISCUSSION

Cancer is one of the prominent threats to humankind. Hence, an interdisciplinary mitigating approach involving both chemical and natural sources of medications is the need of the hour. Plant-derived phytochemicals have been widely used for their immense potential to counter cancer. Herein, qualitative phytochemical screening of *S. elegans* methanolic extract indicated the presence of alkaloids, carbohydrates, tannins, saponins, terpenoids, steroids and flavonoids, which advocated this plant to be an important source for providing therapeutic agents especially for antioxidant and anticancer activity. *S. elegans* was elucidated for its antioxidant potential by ABTS and FRAP assays which revealed excellent radical scavenging and reducing abilities. The total phenolic content measured in the methanolic extract was 23.0±0.06 mg GAE/g. Polyphenolic compounds are known to have antioxidant activity, and this activity is believed to be mainly due to their redox properties, which help in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [35, 36]. Additionally, the alkaloids and saponins have been shown to possess immense antioxidant potential [37-39]. Plant-derived antioxidants are vitally used these days for prevention and cure of numerous disorders [40]. Earlier, ethanolic extract of *S. glabra* and *S. hermandifolia* have been reported to exhibit free radical scavenging activity [41, 42].

Cytotoxicity assay displayed promising anticancer activity of *S. elegans* methanolic extract against MCF-7 cells at a concentration range of 50 to 200 µg/ml. Earlier, in a study of 14 plants, *S. tetrandra* demonstrated antiproliferative and proapoptotic effects in hepatic stellate cells, HSC-T6 [43]. The ethanolic extract from the tubers of *S. venosa* showed potent cytotoxicity against small human cell lung cancer (NCIH187) and human breast cancer (MCF-7), while the same extract also showed high antioxidant potential [44]. Also, cepharanthine, a biscochlorine amphipathic alkaloid isolated from the plant *S. cepharantha Hayata* reduced mitochondrial membrane potential and upregulated the level of cleaved caspases [45]. Tetrandrine, a bis benzylisoquinoline alkaloid isolated from the roots of *S. tetrandra* S. Moore induces G1 arrest in human colon carcinomas, HT29 [46]. The phytochemical constituents such as flavonoids and terpenoids are the major components that are responsible for the potential cytotoxic activities [47-49]. Various plants possessing antioxidants, such as flavonoids, phenolic acids, carotenoids, and ascorbic acid, have been projected to be active anticancer constituents [50-52].

## CONCLUSION

To the best of our knowledge, this is the first report describing the antioxidant and anticancer potential of *S. elegans* methanolic extract. The methanolic extract of *S. elegans* showed significant antioxidant activity and hence can be used as a source of natural antioxidants for curing diseases caused by oxidative stress. The study also determined the promising anticancer potential of the plant which could be used as a source of novel drug to fight cancer. Much interest is given these days towards naturally-derived photocomponents as they are considered to have less side effects compared to current treatments such as chemotherapy. Also, there is a growing demand for alternative treatments with naturally-derived anticancer agents with plants being the desired source. Medicinal plants like *S. elegans* can be of significant use in this context. However, further investigations need to be carried out to isolate and characterise the specific bioactive compounds responsible for such activities. Moreover, *in vivo* studies are also necessary to confirm the anticancer potential of this plant in more detail.

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## CONFLICT OF INTERESTS

The authors declare no conflict of interest

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