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In vitro investigation of anti-cancer potential of *Spilanthes acmella*

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Abstract

Aim: The present research work was carried out to identify the phytoconstituents and *in-vitro* anticancer activity of *Spilanthes acmella* extract.

Methods: The whole plant was used for crude extraction. The extraction was carried out by cold percolation as well as hot percolation method (Soxhlet method) both and 70% ethanol was used as extraction solvent. The existence of phytoconstituents was determined by using typical protocols. The anticancer potential activities were determined by sulforhodomine B dye (SRB) assay method. In this experiment, Mitomycin-C (anticancer drug) was used as a positive control.

Results: The qualitative test result of plant extract showed the presence of Protein, Alkaloids, Phenolics and Flavonoids. The plant extract was tested for *in-vitro* anticancer activity against liver cancer (HEP-2) cell line which showed 77±1.90% growth inhibition and colon cancer (HT-29) cell lines which showed 74±1.03% growth inhibition by Sulforhodamine B (SRB) assay. The statistical analysis showed significant results against selected cancer cell lines.

Conclusion: Plant contains bioactive organic compounds viz Protein, Alkaloids, Phenolics and Flavonoids that may possess anti-cancer activities. Finally, it is conclude that the phytoconstituents of this plant compounds can be further used herbal formulations in pharmaceuticals.

Key Words: Anti-cancer, Bioactive, Flavonoids, Mitomycin-C, Phytoconstituents.

INTRODUCTION

Cancer is marked by abnormal growth of cells which leads to the development of tumor and spread by metastasis. According to WHO, cancer is the second leading cause of death worldwide. Treatments for cancer may involve surgery, radiotherapy and chemotherapy and often a combination of two or all three may employed but these type of treatment causes more adverse effect on normal cells [1]. The Study of review/articles before last few decades clearly indicated that herbal products exhibit a variety of therapeutic properties and provide more health security to the patients [2-3]. Plants are rich source of therapeutic bioactive agents, hence extensive research is going on to investigate plant derived non-toxic phytochemical compounds [4]. The medicinal plants have a diverse group of highly valuable and readily available source of bioactive organic compounds, e.g. alkaloids, tannins, essential oils, proteins and flavonoids [5-7]. The traditional system of medicine and knowledge of Ayurveda help in the discovery of new herbal drug leads with high activity and low toxicity for cancer therapy. Initial research focuses on the isolation and purification of bioactive herbal compounds, chemical modification and improving other pharmacological profiles [8]. The evaluation of medicinal plants as a source of anticancer was started in the 1950s, with the discovery of vinblastine and vincristine alkaloids from vinca (*Catharanthus roseus*) plant [9]. In recent time there is a great emphasis has been given towards herbal research on complementary and alternative medicine that deals with the treatment and cure of cancer [10]. Mainly the two plant products like vinblastine and vincristine both are used in combination for the treatment of a variety of cancers, including leukaemias, lymphomas, testicular cancer, breast, lung cancers, and sarcoma [11]. The anticancer activity of the plant *Tectaria cicutaria* extract was tested *in-vitro* using Sulforhodamine B (SRB) assay against some cancer cell lines namely Human Leukemia Cell Line (K-562), Human Nasopharyngeal Cell Line (KB), Human Colon Cell Line (HT-29) and Human Colon Cell Line (Colo-205) which showed significant result [12]. This plant is also known as toothache plant. Its extract contains an active compound know as Spilanthol that used against blood parasites including malaria. The extract have also insecticidal and anti-bacterial activities [13]. The cytotoxic property was also determined by MTT assay, which indicated a potential cytotoxic effect on cancer cell lines such as HeLa and V79 cell lines [14]. The ethanolic extract of Argemon mexicana showed cytotoxic activity using SRB dye assay against lung cancer cell line (A-549), colon cancer cell line (502713), liver cancer cell line (HT-29) and neuroblastoma cancer cell line (IMR-32) [15]. So, Keeping above review literature in mind, our research work was therefore aimed to investigate and evaluate the presence of bioactive herbal compounds and anticancer

potential of *Spilanthes acmella* extract against two selected liver (HEP-2) & colon (HT-29) cancer cell lines.

MATERIALS AND METHODS

Plant Materials and Extractions

Spilanthes acmella plants were collected in the months of February to March in the year 2012 from the area of forest research institute, Dehradun, India and authenticated from Botanical survey of India (BSI) of Accession No – 114534, Family- Asteraceae, Botanical Name- *Spilanthes acmella* L. The whole plants were washed in the laboratory and dried at room temperature then it was mild grinded. The plant extract was extracted by cold and hot percolation (Soxhlet) method using 70% ethanol as a solvent. The extract was then filtered, concentrated and freezed at 6-8 ^{0}C. At the time of experiment, the extract was redissolved in Dimethyl sulphoxide (DMSO) and again filtered.

Protocol for Qualitative Identification of Phytoconstituents

The qualitative test for phytoconstituents were performed using standard protocols. The concentration of each extract was 100 µg/ml which were used in this experiment [16].

Test for protein:

1 ml extract solution was taken in a clean test tube and then 5-6 drops of concentrated nitric acid solution (HNO_3) was added. The appearance of yellow color showed the presence of proteins/ amino acids in the sample.

Test for Steroids:

1 ml solution of plant extract was mixed with 2 ml chloroform. Then very carefully H_2SO_4 solution was added. The reddish brown colour was produced which indicated the presence of steroid molecules in the extract.

Test for Alkaloids

For the test of alkaloids, extract was redissolved in dilute HCl and then filtered. The filtrate was then mixed with saturated picric acid solution resulting the formation of light brown precipitate showed the presence of alkaloids.

Test for Phenolics

100 µl plant extract solution was gently mixed with 2 ml of 2% $FeCl_3$ solution. The formation of Bluish color showed the presence of phenolics.

Test for Flavonoids:

The presence of flavonoids was confirmed by two unique tests-

(a) Lead Acetate Test: 2-5 drops of freshly prepared lead acetate solution was added in to 2 ml of extract solution. After mixing, the formation of yellow color showed the presence of flavonoids.

(b) NaOH Test: 2-5 drops of sodium hydroxide solution was added in to 0.1 ml of extract solution. The formation of yellow color showed the presence of flavonoids. The yellow color was disappeared on addition of dilute HCl.

Test for Terpenoids

0.2 ml extract solution was taken in a test tube and then 2-3 ml of pure chloroform solution was added. Then 2 ml of con H_2SO_4 solution was added and gentle mixed. The formation of dark red/brown color showed the presence of terpenoids.

Test for Tannins:

0.1 ml extract solution was mixed with 2 ml of 2% $FeCl_3$ solution in a test tube. The formation of black color showed the presence of tannins.

Separation of Phytoconstituents by TLC Method

The TLC was performed by using standard protocol which is described below-[17 -18]

The slurry was prepared by using silica gel-G and distilled water, then it was applied on glass plate as a thin and uniform film layer. The thickness of layer was maintained between 2-4 mm and the plates were activated by putting it in to oven at 65^{0}C for 2 hours. After removing, the plant extract of concentration 100µg/ml was loaded with the help of capillary tube. The glass plates were air dried and further put upright inside the glass tank contained mobile phase for up to 2-3 hours (ascending technique). After removing from the mobile solution, plates were air dried and different spots were visualized in sun light and the distance of solute front as well as distance of solvent front were measured. The *Rf* values of each spots were calculated. The spots were scrubbed and collected in separate vials containing methanol as solvent and each vials sample were further used for phytochemical tests by standard qualitative protocol.

Solvent System and Calculation of *Rf* Values

The solvent system for TLC, shown in the given table 1.

Table 1: Mobile System for TLC

Name of Plant	*Spilanthes acmella*
Composition of solvent system	$CHCl_3$:Methanol (35:65)

The *Rf* values were calculated by the given formulae-

$$Rf = \frac{\text{Distance of solute front (in cm)}}{\text{Distance of solvent front (in cm)}}$$

Study of Anticancer Potential Assay

Source of Cell lines

The cancer cell lines were obtained from National Centre for Cell Sciences, Ganeshkhind, Pune, India, 411007.

Culture of Human Cancer Cell Lines

The two human cancer cell lines like liver cancer (Hep-2) and colon cancer (HT-29) were selected for the experiment. These cancer cell lines were grown in RPMI-1640 media with 2 mM L-glutamine of pH 7.2. The antibiotics penicillin was dissolved in phosphate buffer saline (PBS) and sterilized by filtering through 0.2µ cellulose filter paper under laminar. The growth media was freezed at 4^{o}C. The growth medium used for cryopreservation contained 20% FCS (foetal calf serum) and 10% DMSO (Dimethyl sulfoxide). The entire cancer cell lines (Hep-2, HT-29) of the experiment were adjusted at 37^{0}C, 5% CO_2 and 90% humidity [19].

Preparation of Cell Suspension for Assay:

Human cancer cell lines (HEP-2 & HT-29) were grown in multiple tissue culture flasks (TCFs) at 37C^0 in the atmosphere of 5% CO_2 and 90% relative humidity in the presence of complete growth medium to obtain enough number of cancerous cells. The tissue culture flasks

(TCFs) with cells at compatible stage were selected for the experiment. The whole cells were harvested by treatment with Trypsin-EDTA buffer. All cells were separated to single cell suspension using micro pipette and the viable cells were counted through haemocytometer after treatment with trypan blue solution. The cell viability at this stage was 98%. Viable cancer cell density was maintained and adjusted to 5,000-40,000 cells/100µl. The fully grown cancer cell suspension (100µl) with 100µl of complete growth medium was added in to each 96 well plate. The 96 well plates were placed in CO_2 incubator at 37^0C for 24 hours in an atmosphere of 5% CO_2 and 90% relative humidity for incubation. After 24 hours of incubation, the test material (plant extract), DMSO (vehicle control) and positive control(mitomycin-C) were added [20].

Sulforhodamine- B Dye (SRB) Assay

The anticancer activity was assessed by the cytotoxic potential of test material using cancer cell lines. These cancer cell lines were grown on tissue culture plates in the presence of test material as well as control solution. The inhibition of cell growth was calculated using ELISA reader after staining with Sulforhodamine B (SRB) dye. The SRB dye strongly bound with basic amino acid residues (Arg, Lys) by trichloroacetic acid (TCA) fixed cells [21].

The antiproliferative SRB assay was performed to assess growth inhibition. It is a colorimetric method assay which estimated the total cell number indirectly by staining cellular active proteins by SRB dye which descrived below- [22].

We followed the given protocol -

1. 100 µl of cancer cell suspension was taken in to each well of 96-well plates.
2. Adjusted the concentration of the substances to be tested in the culture medium when diluted to 2X. The concentration of extract was 100 µg/ml and the concentration of Mitomycin-C was 10^{-6} M. The Mitomycin-C was used as a positive control.
3. Then 100 µl of test sample was added in each wells of culture medium except positive control and mixed by pipetting method.
4. The 96-wells culture plate was kept inside the incubator for 24 hours. After completion of 24 hours, Culture plate was removed from incubator and then add ice cold 100 µl TCA to fixed the cells and then kept inside the incubator at 4^0C for an hour.
5. Culture plate was then removed and washed 5-6 times with double distilled water and allowed to air dry.
6. SRB dye solution was added in to each well for staining at room temperature.
7. After 30 Minutes of staining, all wells were washed with 1% acetic acid to remove the unbound dye and then air dried.
8. 100 µl of 10mM Tris base (pH 10.5) was added in to each wells and shaken on mechanical shaker for 5 minutes.
9. Culture plate was removed from shaker and then optical density (absorbancy) was measured through ELISA reader at 515 nm.
10. It was found that the optical density ((absorbancy)) of SRB dye in each well is directly proportional to the presence of number of cells.

Positive Control

The positive control in this experiment was Mitomycin-C (Anti-cancer drug).

Calculation

Cell growth in the presence of test material (extract) was calculated by the given formula-

Percent growth in presence of test material (extract):

= (Cell growth in presence of test material / Cell growth in absence of test material) X 100

Percent cell growth inhibition in presence of test material was determined as:

100 ─ Cell growth in presence of test material

Criteria to the Calculation of Test Sample Activity:

If, the test sample shows growth inhibition of ≥ 70% at 100 µg/ml then it was considered as active.

Statistical analysis: Results showed in Mean ± SEM. Statistical calculations were carried out by using Microsoft excel 2010.

RESULTS

The 70% ethanolic extract of *Spilanthes acmella* showed the presence of many organic compounds. The qualitative biochemical test results showed the presence of proteins, Alkaloids, Phenolics and Flavonoids (Table-2).

Table 2: Qualitative Biochemical tests

Compounds	Present
Protein	+
Alkaloids	+
Phenolics	+
Flavonoids	+

+ sign indicated the presence of compounds

TLC Result of Plant Extract

The glass plate was removed from mobile phase and air dried keeping upright position and chromatogram was developed. All spots were visualised very carefully and then distance of solvent front and solute front were measured and *Rf* Values were calculated (table 3).

Figure 1: Chromatogram of Extract

Calculation of *Rf* Values

Table 3: *Rf* Values of each compound
This is the ratio of solute front to solvent front in cm.

Name of the Plant *- Spilanthes acmella*	
Rf_1	0.20
Rf_2	0.16
Rf_3	0.10
Rf_4	0.06

(a) Rf: Retardation factor, (b) cm: Centimetre
(b)

TLC Analysis of Extract

The phytoconstituents of TLC Plates were separated in methanol containing vials and sample of each vials were tested by standard protocol [15]. The each plant extract sample showed identical result as shown in table No 2.

Result of *In -Vitro* Anticancer Activity

The result of anticancer activity of extract was given in table 4.

Table 4: Percentage inhibition of cancer cell lines

	Plant- *Spilanthes acmella*	**Drug-** Mitomycin-C
Solvent	70% ethanol	water
Concentration	100µg/ml	$1X10^{-6}M$
% Cell Growth inhibition		
Hep-2	77±1.90	79±1.59
HT-29	74±1.03	77±1.34

Results are expressed as mean values ± SEM (n = 6), µg/ml: Microgram per millilitre, M: Molar solution

According to previous research, if test result shows growth inhibition of more than 70% at 100 µg/ml concentration, ultimately it will be accepted and considered as significant. The experimental findings were summarized in tabular form (table 4). The anticancer activity was determined using human liver (HEP-2) and colon (HT-29) cancer cell lines. The HEP-2 showed significant anticancer activity of 77±1.90 % while Mitomycin-C (positive control) showed 79±1.59 % cell growth inhibition. In case of HT-29, which showed significant anticancer activity of 74±1.03 % and standard drug Mitomycin-C showed 77±1.34 % cell growth inhibition.

DISCUSSION

The qualitative tests for phytoconstituents showed that *Spilanthes acmella* extract contained primary and secondary metabolites viz: proteins, alkaloids, phenolics and flavonoids. The TLC result also justified the qualitative result and showed the presence of proteins, alkaloids, phenolics and flavonoids [5-7]. The SRB assay determined the anticancer potential activity against HEP-2 and HT-29 cell lines. The cell growth of both cell lines were measured on ELISA reader after staining with SRB dye which binds with basic amino acids in presence of trichloroacetic acid fixed cells [21].

Therefore, *Spilanthes acmella* extract serve as a source of bioactive compounds for the treatment of HEP-2 and HT-29 cancer cell lines and may used as anticancer drug [15].

The previous reviews showed that the possible cause of cancer are free radicals which are generated during catabolism. Some active agents are also reported that act as antioxidant and the activity of these compounds more likely depends on the concentration and type of cell lines [11]. Recently, there are many challenges to design new herbal drug that will be highly effective regarding cure and treatment of cancer. It was also found that many plant extracts has ability to inhibit the cancer growth by activating growth regulators and apoptosis [19].

CONCLUSION

The present research work was authenticated that *Spilanthes acmella* belongs to family Asteraceae and contained many bioactive compounds like proteins, phenolics, alkaloids, and flavonoids. These bioactive compounds have inhibitory effect on human cancer cell lines like HEP-2 and HT-29. The possible mechanism may be to reduce the free radical formation and induce the apoptosis including growth regulators. Many research articles revealed that plant metabolites played important roles for curing and treating the diseases including cancer. The recent research work was highly interesting for qualitative identification, isolation and evaluation of anticancer potential activity from the plant bioactive compounds. Finally, we concluded that the bioactive compounds of selected plant can be further used for pharmacological formulation to develop herbal anticancer drug that impose least side effect.

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Conflict of Interest

We have no any conflict of interest regarding this manuscript.

Authors Contributions

This manuscript was prepared by the collaboration of three authors. The first Author Shivsharan Singh, searched the review literature, design the protocol and first draft the manuscript. The second Author Satish Kumar Verma managed the analysis of the study and editing. The third Author, Santosh Kumar Singh design the study and performed the statistical analysis.

Note- In this research work, ethical approval, consent of cancer patient and consent for research publication are not applicable.

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