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Antioxidant and *in vitro* cytotoxic activity of extracts of aerial parts of *Cocculus hirsutus* (L) using cell line cultures (breast cell line)

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Abstract

The aim of the present study was to evaluate the *in vitro* cytotoxic activity of methanolic and aqueous extract of aerial parts of *Cocculus hirsutus* on MCF-7 breast cell lines. Qualitative phytochemical screening tests were performed to detect phytochemicals in the extracts. Antioxidant activity of the plant extracts were characterized by using DPPH free radical scavenging method. The cytotoxic activity of the extracts of *Cocculus hirsutus* on MCF-7 cells was investigated *in vitro* through MTT assay. The results showed Antioxidant activity using DPPH were found to be increased in a concentration dependent manner and decreased cell viability and cell growth inhibition in a dose dependent manner. The findings from this study indicated that methanolic and aqueous extracts of *Cocculus hirsutus* leaf possessed vast potential as a medicinal drug especially in breast cancer treatment.

Keywords: *Cocculus hirsutus*, DPPH, MTT assay, Antioxidant, Cytotoxicity.

Introduction

Plants have been the traditional source of raw materials for medicines. Ancient scholastic works like Atharva Veda, Charaka, Sushruta are a rich heritage of knowledge on preventive and curative medicines. As per an estimate, around 13,000 plant species worldwide are known to have been used as drugs. These plant species contain biologically active compounds that protect human health. Compounds from plants could act as protective agents with respect to human carcinogenesis, acting against initiation, promotion or progression stages, or destroying/blocking the DNA damaging mutagens, thus avoiding cell mutations. The plant sources of India are likely to provide effective anti-cancer agents. These activities have been co-related to the presence of certain phytochemical substances. Herbs have a vital role in the prevention and treatment of cancer.

The screening of plant extracts has been of great interest to scientists in the search for new drugs for effective treatment of several diseases. [1] Cancer is one of the most dangerous diseases in humans and presently there is considerable scientific discovery of new anticancer agents from natural products. Also, it is the second leading cause of death in America. Drug discovery from medicinal plants has played an important role in the treatment of cancer and indeed, over the last half century most of the plant secondary metabolites and their derivation have been used towards combating cancer.[2, 3] The major cause of cancer is: smoking, dietary imbalances, hormones, chronic infections/inflammations.

Many of the discovered phytochemicals seem to fight diseases such as cancer, heart attack and stroke. At the same time other scientists are conducting epidemiological studies to determine the relationship between the consumption of phytochemicals and human health. Most studies showed that diets rich in plants gave lower rates of cancer and heart disease. The principles underlying herbal medicines are relatively simple, although they are quite distinct from conventional and herbal medicine.

Plants have been a major source of highly effective conventional drugs for the treatment of many forms of cancer and while the actual compounds isolated from the plant frequently may not serve as the drug, they provide leads for the development of potential novel agents like

paclitaxel, vinblastine and vincristine. Plant derived natural products have received considerable attention in recent years, due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects.

The major alkaloidal phytoconstituents present in Cocculus hirsutus are Cohirsine, Cohirsinine, Cohirstinine, Hirsutine, Shaheenine, Haiderine, Syringasesinol, Isotriboline, Triboline and Jamtinine. The other phytoconstituents like triterpenoid alcohol Hirsudiol, essential oil, beta-sitosterol and gineol have also been reported. [4-8] The aqueous extract of aerial parts showed significant diuretic activity, antihyperglycemic activity and laxative effect in rats. [9, 10] The juice of leaves used externally as cooling and smoothing agent in prurigo, eczema and impetigo. [11] The roots of *Cocculus hirsutus* possess anti-inflammatory and analgesic properties. [12]

Hence, the present work is an attempt to study the anti-cancer activity of one of the plants of the same family; Menispermaceae named *Cocculus hirsutus* (L) using cell line cultures.

Materials and Methods

Collection of plant material, chemical and cell line

Healthy, disease free leaves of *Cocculus hirsutus* were collected from the local area near Baroda (India). The plant was authenticated by Botanist and Pharmacognocist. Washed and shade dried, fresh leaves were cut into small pieces in pulverized blender. The required chemicals for extraction were collected from departmental store. Essential chemicals from sigma labs and Himedia. Breast cancer cell lines from NCCS, Pune.

Preparation of Extract

The leaves of *Cocculus hirsutus* were washed, shade dried, pulverized in a blender. Coarsely powdered aerial parts (leaves) were extracted with petroleum ether followed by benzene, chloroform, methanol, by the process of successive soxhlet extraction.

10 gm of pulverized leaf material was mixed with 100 ml of methanol and kept in a rotary shaker at 100 rpm overnight and filtered with Whatman no. 1 paper and concentrated to dryness at 40ºC, lyophilized and stored at 4ºC until further use. Different concentrations of the methanolic extracts (500, 250, 125 µg/ml) were prepared in 5% DMSO for determining cytotoxicity.

Antioxidant activity

The evaluation of the antioxidant activity of methanolic extracts of *Cocculus hirsutus* was done through *in vitro* assays by *1, 1*-Dipheny-2-Picrylhydrazyl (DPPH) radical scavenging activity. [13] The free radical scavenging activity of different extracts of *Cocculus hirsutus* and L-ascorbic acid (Vitamin C as standard) was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. About 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in water at different concentrations (20-100 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. The lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scavanged} = \frac{\text{Acont} - \text{Atest}}{\text{Acont}} \times 100$$

Where, Acont is the absorbance of the control reaction, Atest is the absorbance in the presence of the extracts.

The antioxidant activity of the extracts ware expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50 %. The results of anti-oxidant activity of extracts of *Cocculus hirsutus*, using DPPH free radical scavenging method is tabulated in table 1 and figure 1.

In vitro Cytotoxic Activity by MTT assay

The cell lines were prepared and cryopreserved using reagents such as DMSO which preserve the cell during freezing. DMSO is toxic at room temperature. The freezed ampoule is brought to room temperature by slow agitation (thawing). The freezed cryovials plunged into the water bath and is rapidly thawed until it gets liquified. Solution, centrifuged with saline for 10 mins to remove the DMSO. The saline is discarded and aliquot is taken for cell counting, cell viability and for sub-culturing. MTT assay is a quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the ability of living cells. The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl) - 2, 5-diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity.[14-17] The result of *In vitro* cytotoxic activity of methanol and aqueous extracts of *Cocculus hirsutus* on Breast cancer cell lines (MCF 7 cell line) is tabulated in table 2 and figure 2.

Preparation of Herbal extract for the assay

- 0.5 ml of stock (100 mg/ ml) herbal extract was dissolved in 4.5 ml of DMSO for a concentration of 10 mg/ ml.
- The fresh working suspension was filtered through 0.45 µm membrane filter prior to the assay.
- Using the 1 mg/ ml concentration herbal extract, nine serial doubling dilutions of the extract of 500µl each was prepared in DMSO to get the concentration of the extract as indicated and the diluted extracts will be transferred to 10 wells of a 12 well culture plate.
- 500 µl of 48h culture of MCF 7 cell lines at a concentration of 105 cells/ ml was added to each well.
- Two control wells received only cell suspensions without plant extract. The plate was incubated in a humidified CO_2 incubator at 37°C for 4 - 6 h.
- The plate was microscopically examined for confluent monolayer of cells, turbidity and toxicity.

Assay Process

- After incubation, the medium from the well was aspirated carefully and then discarded.
- Each well was washed with Eagle's Minimum Essential Medium (EMEM) without Fetal Calf Serum (FCS).

- 200 µl of MTT solution (5mg MTT/ ml of PBS, pH 7.2) will be added to each well.
- The plate was incubated for 6-7 h at 37°C in a CO_2 incubator with 5% CO_2. After incubation 1 ml of DMSO was added to each well and mixed with pipette and left for 45s at room temperature.
- Purple formazan was formed in the wells.
- Cell control and solvent controls were included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments.
- The suspension was transferred to a spectrophotometer cuvette and the optical density (OD) was measured at 540nm using DMSO as blank.
- The % cell viability was calculated with the following formula: Cell viability % = Mean OD of wells receiving each plant extract dilution / Mean OD of control wells x 100.

Determination of IC_{50}

IC_{50}, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of Extract) vs % cell inhibition. A line drawn from the 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of the compound). The antilog of that value gives the IC_{50} value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where, At = Absorbance of Test, Ab= Absorbance of Blank (Media), Ac= Absorbance of control (cells) % cell inhibition = 100 − % cell survival

Data interpretation

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

% cell survival= {(At-Ab) / (Ac-Ab)} x100

Where, At= Absorbance value of test compound Ab= Absorbance value of blank Ac=Absorbance value of control

% cell inhibition= 100-cell survival

Results and Discussion

The aim of the present study was to evaluate the *in vitro* cytotoxic activity of methanol extracts of the leaves of *Cocculus hirsutus* on MCF-7 cells from human breast cancer. Qualitative phytochemical screening tests were performed to detect phytochemicals in the extracts. The antioxidant activity of the plant extract was then characterized using the DPPH radical scavenging method. Antioxidant activity using DPPH was found to increase in concentration dependent manner. All the extracts of aqueous and methanol exhibited potential antioxidant activity with an IC_{50} value of 25 µg/ml when compared to the standard Ascorbic Acid with an IC_{50} value of the 7.5 µg/ml.

The cytotoxic activity of the extracts of *Cocculus hirsutus* on MCF-7 cells from human breast cancer was investigated *in vitro* 3-(4) 5-Dimethyl-thiazol-Zyl) - 2,5 biphenyl tetrazolium bromide (MTT). The results showed decreased cell viability and cell growth inhibition in a dose dependent manner. The IC_{50} value of standard Tamoxifen, methanol extract were 9.3, 39.06 µg/ml respectively. Methanol extracts of *Cocculus hirsutus* demonstrated strong antioxidant and anti-proliferative activities. Accumulating evidence clearly indicates that apoptosis is a critical molecular target by dietary bioactive agents, in the prevention of cancer. Since the phytochemical analysis has shown the presence of potent phytochemicals like alkaloids, phenols, flavonoids, terpenoids, glycosides, saponin, steroids, tannin and sugars, etc. Several authors reported that phenolic, acids, flavonoids, steroids, terpenoids are known to be bioactive principles.

Table 1: Antioxidant activity of extracts of *Cocculus hirsutus*, using DPPH free radical scavenging method

Conc in µg/ml	DPPH Scavenging Activity		
	MECH	AECH	Standard
20	48.42 ± 1.03	45.4 ± 1.03	43.19 ± 1.112
40	51.78 ± 0.9	50.18 ± 0.9	46.63 ± 1.10
60	56.66 ± 0.72	53.54 ± 0.72	50.4 ± 0.83
80	59.9 ± 1.12	55.8 ± 1.12	52.29 ± 0.53
100	64.71 ± 0.69	59.35 ± 0.69	56.32 ± 0.44
IC_{50} Value	29.065	36.2789	61.46

Where, MECH: methanolic extract of *Cocculus hirsutus*; AECH: Aqueous extract of *Cocculus hirsutus*; Standard: Ascorbic acid

Table 2: *In vitro* Cytotoxic Activity of methanol and aqueous extracts of *Cocculus hirsutus* on Breast cancer cell lines (MCF 7 cell line)

S. No	Concentration (µg/ml)	Dilutions	*In vitro* cytotoxic activity		Tamoxifen
			MECH	AECH	
1	1000	Neat	12.32 ±0.25	32.56 ±0.59	12.33 ± 0.51
2	500	1:1	15.96 ±0.89	41.23 ±0.32	17.18 ±1.58
3	250	1:2	18.96 ±1.23	48.92 ±0.57	20.98 ±1.47
4	125	1:4	25.36 ±0.89	52.35 ±0.44	24.13 ±0.55
5	62.5	1:8	38.59 ±1.05	59.87 ±0.14	28.78 ±0.87
6	31.25	1:16	52.55 ±1.47	61.23 ±0.57	37.17 ±0.69
7	15.625	1:32	58.79 ±1.69	68.77 ±0.22	43.46 ±0.54
8	10	1:64	65.32 ±0.58	72.02 ±1.06	48.19 ±1.25
9	7.8125	1:128	78.19 ±0.47	75.12 ±1.08	54.33 ±1.06
10	3.125	1:256	90.23 ±0.55	85.64 ±1.89	61.22 ±0.88
11	Cell control	-	100	100	100

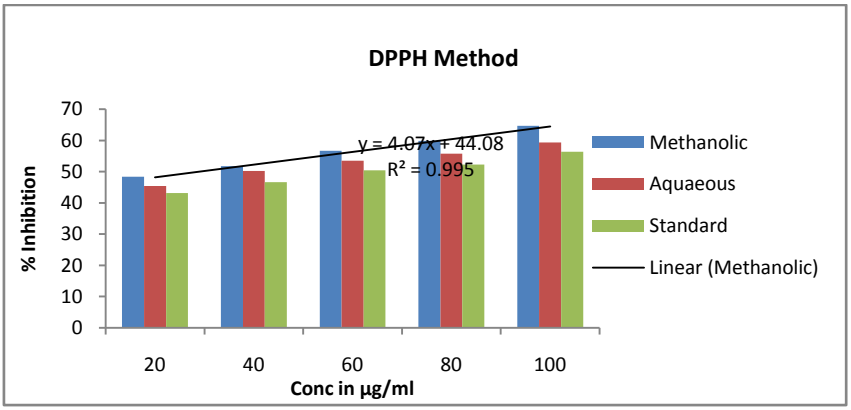

Figure 1: Antioxidant activity of extracts of *Cocculus hirsutus*, using DPPH free radical scavenging method

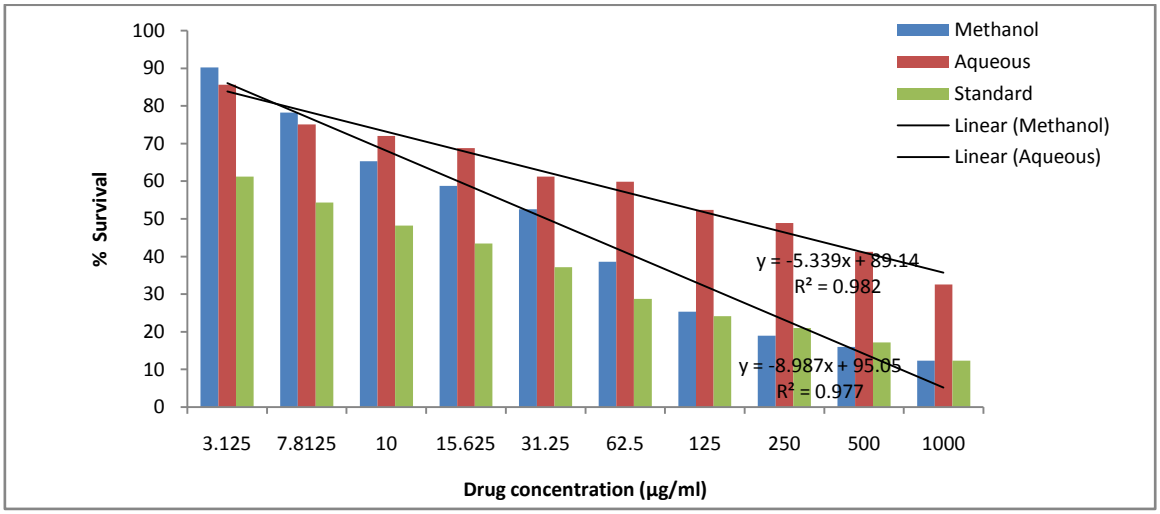

Figure 2: *In vitro* Cytotoxic activity of methanol and aqueous extracts of *Cocculus hirsutus* on breast cancer cell lines (MCF 7 cell line)

Conclusion

Therefore the crude extracts of *Cocculus hirsutus* leaf could be new sources of development of new plant based therapy for management of diseases. Further research is to be carried out to fractionate and purify the extract, in order to find out the molecules responsible for the anti proliferation activity observed. The findings from this study indicated that methanol extracts of *Cocculus hirsutus* leaf possessed vast potential as a medicinal drug especially in breast cancer treatment.

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