

***In vitro* cytotoxic activity of *Aesculus indica* against breast adenocarcinoma cell line (MCF-7) and phytochemical analysis**

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Abstract: *Aesculus indica* (Linn.) (Sapindaceae) is an ethanobotanically important plant specie traditionally used against rheumatism, skin and vein complaints. Cytotoxic potential of *Aesculus indica* crude leaf extract and its fractions was investigated against MCF-7 cell line. Crude extract of *Aesculus indica* was prepared in methanol by maceration technique. Crude extract was fractionated into four organic and one aqueous fraction on polarity basis. MTT assay was used to evaluate the reduction of viability of MCF-7 breast cancer cell line. Cell viability was inhibited by *Aesculus indica* crude extract in a dose dependent manner ranging from 34.2% at 10 µg/ml to 94% at 500µg/ml. Activity was found in an ascending order from hexane showing 29.8% inhibition to aqueous fraction indicating maximum inhibition, 60%. Phytochemical analysis of crude and fractionated extracts revealed presence of flavonoids, saponins, coumarins and tannins upto varying degrees. Methanol and aqueous fraction of methanol extract of *Aesculus indica* can be good source of cytotoxic compounds.

Keywords: *Aesculus indica*, cytotoxic, MCF-7 cell line, MTT assay

INTRODUCTION

Cancer is the most common and fatal disease responsible for 2-3% of deaths recoded worldwide annually. While in women; breast cancer is most widespread (Parkin and Fernandez, 2006) and its incidence in Pakistan is reported highest among South-Central Asian countries (Nisa *et al.*, 2011). About 60% of anticancer drugs used nowadays are obtained from natural sources (Sakpakdeejaroen and Itharat, 2009).

Medicinal plants can be a promising source of novel chemotherapeutic agents including cancer. Isolation of vincristine and vinblastine from *Catharanthus roseus*, have provided a clue for it. In addition, synthesis of topotecan and irinotecan derived from camptothecin (*Camptotheca*) further provided evidence that plant derived compounds if not effective as a drug can be converted to an effective agent (Daniels *et al.*, 2006).

Out of total 250,000 plant species existing on earth approximately one thousand have anticancer potential. A large number of plant species have been screened through bioassays for search of novel plant based anticancer drugs (Abu-Dahab and Afifi, 2007). Bioactivity guided isolation is an important strategy for discovery of potent anticancer agents (Bibi *et al.*, 2010).

Aesculus indica (Linn.) is a large deciduous tree belonging to family Sapindaceae. Commonly it is known

as bankhor by local people. Plant is widely distributed in northern western himalayas. *Aesculus indica* is well known for its medicinal importance. Seeds, fruits and roots are traditionally used against rheumatism, skin diseases and vein complaint. The plant contains saponins, flavonoids, glycosides and fatty oils (Zhang *et al.*, 2010). Antioxidant activity of its leaf extract was found significant (Chakraborty, 2009a). Methanol extract *Aesculus indica* leaves stimulated cell mediated immune response (Chakraborty, 2009b). Crude and fractionated extracts of its leaves also proved active against different pathogenic bacteria (Bibi *et al.*, 2011). Present investigation was aimed to assess the cytotoxic potential of methanol extract of leaves of *Aesculus indica*.

MATERIALS AND METHODS

Collection, identification, drying and extraction

Fresh *Aesculus indica* (Linn.) leaves were collected in May from Northern area of Pakistan (Taien Valley Lower Barian) and identified by Dr. Mir Ajab Khan, Department of Plant Sciences Quaid-i-Azam University Pakistan. Plant material was thoroughly washed and dried under shade. Dried material was ground to fine powder.

Cold maceration technique was used for extraction. Powdered plant material (1.5kg) was dipped in methanol (2000 ml) and kept at room temperature. After seven days, the extract was filtered under vacuum through Whatman filter paper No. 1. The residue was again dipped in methanol for additional seven days and filtered thereafter. The filtrates were combined and methanol was

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evaporated under vacuum using rotary evaporator (Buchi Rotavapor R-200) at 45°C. The dried extract (250g) was stored at 4°C until further analysis.

Fractionation

Fractionation of crude extract was carried out by suspending 250g of extract in 150 ml water and then partitioning with different organic solvents (hexane, chloroform, ethyl acetate and methanol) in order of increasing polarity by using separating funnel. All the six fractions were dried by evaporating respective solvent using rotary evaporator. Quantities obtained were 22, 45, 98, 50 and 25g of hexane, chloroform, ethyl acetate, methanol and aqueous fractions, respectively. Summary of scheme used for fractionation of crude leaf extract of *Aesculus indica* is shown in fig. 1.

Cytotoxic activity against MCF-7 cell line

Cell culture

Human breast adenocarcinoma MCF-7 cell line was maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with FBS (Fetal bovine serum), penicillin (10000 units), streptomycin (10 mg/ml), and l-glutamine (200 mM) at 37°C under 5% CO₂ and relative humidity 95%.

Dilution of extracts

Crude extract and fractions were separately dissolved in DMSO (Dimethylsulfoxide) at concentration of 2 mg/ml. Required dilutions in µg/ml were made under sterile conditions by adding calculated amounts of DMEM.

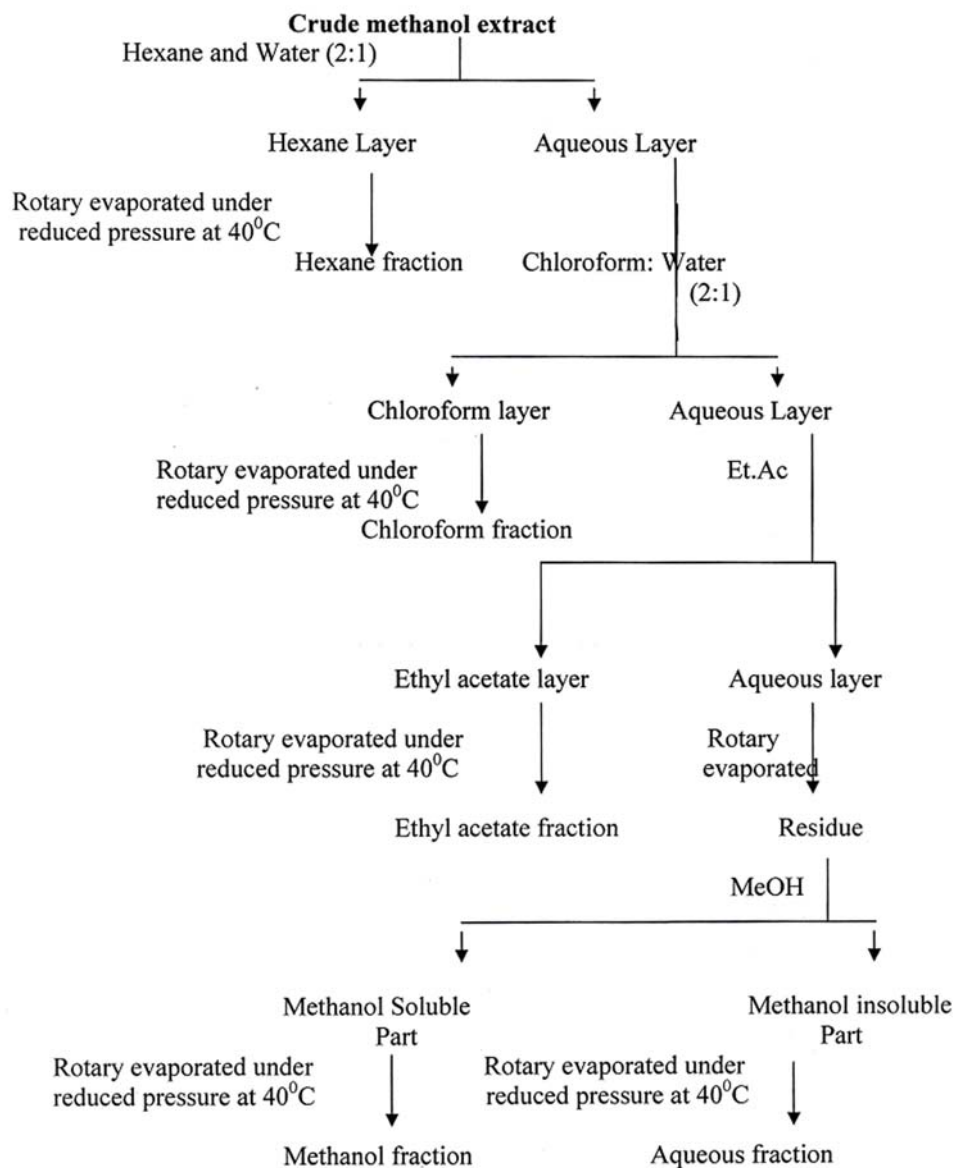


Fig. 1: Scheme used for fractionation of crude extract of *Aesculus indica* leaf.

MTT assay

Standard MTT assay was used to evaluate cell line viability in the presence and absence of extracts (Son *et al.*, 2003). In 96 well plate, 100 µl medium (RPMI 1640) was poured in each well and seeded with 5000 MCF-7 cells/well. Cells were allowed to attach overnight and then various concentrations of the crude extract and fractions were added to respective wells. After 24 h incubation at 37°C, 5% CO₂ and relative humidity 95%, 10 µl of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well. After further 4 h incubation, 100 µl of DMSO solution was added to each well to solubilize MTT crystals. The plates were again incubated overnight at conditions mentioned above. The plates were read for optical density at 570 nm, using a plate reader. Percentage inhibition was calculated by the following formula:

$$\text{Percentage inhibition} = \frac{(B - A) - (C - A)}{(B - A)} \times 100$$

A= Average absorbance of media

B= Average absorbance of media +cells

C= Average absorbance of extract sample

The test was performed in triplicate and data obtained from fractions assay was statistically analyzed by ANOVA and LSD using MSTATC.

Phytochemical analysis of crude extract and fractions

Different chemical tests were conducted for preliminary qualitative analysis to determine presence or absence of different phytochemicals in crude extract and fractions (Bibi *et al.*, 2010). The results were evaluated by visual inspection as change in color or precipitation.

Test for alkaloids**Mayer's reagent**

Mercuric chloride (0.3555 g) was dissolved in 60 ml of water and 5 g of potassium iodide was dissolved in 20 ml of water. Two solutions were mixed and volume was made up to 1000 ml with distilled water.

Dragendorff's reagent

Solution A: Basic bismuth nitrate (1.7 g) and 20 g of tartaric acid was dissolved in 80 ml of distilled water.

Solution B: Potassium iodide (16 g) was dissolved in 40 ml of distilled water

Solution A and B were mixed in ratio of 1:1

Plant extract (0.5-0.6 g) was mixed with about 8 ml of 1% HCl, warmed and filtered. 2 ml of filtrate were treated separately with Mayer's Reagent and Dragendorff's reagent. Turbidity or precipitation was observed to indicate the presence of alkaloids.

Test for anthraquinones

Plant extract (1 g) was boiled with 6 ml of 1% HCl and filtered. The filtrate was shaken with 5 ml of benzene.

The benzene layer was removed and then 10% NH₄OH was added. Formation of pink, violet or red color in alkaline phase was observed for the presence of anthraquinones.

Test for coumarins

Moistened plant extract (0.5 g) was taken in a small test tube and covered it with filter paper moistened with 1 N NaOH. The test tube was placed for few minutes in boiling water. Filter paper was removed and examined under UV light for yellow fluorescence to indicate the presence of coumarins.

Test for flavonoids

Prepared extract (0.5 g) was shaken with pet ether to remove the fatty materials. The defatted residue was dissolved in 20 ml of 80% of ethanol and filtered. The filtrate was used for the following test

- Filtrate (3 ml) was mixed with 4 ml of 1% AlCl₃ in MeOH in a test tube. Formation of yellow color was observed to indicate the presence of flavonols, flavones and/or chalcones.
- Filtrate (3 ml) was mixed with 4 ml of 1% KOH. A dark yellow color was observed to indicate the presence of flavonoids.

Test for saponins

Plant extract (0.5 g) was dissolved in boiling water in a test tube and allowed to cool. The tubes were shaken well. Froth appears indicate the presence of saponins.

Test for tannins

Plant extract (0.5 g) was boiled in 20 ml of distilled water in a test tube and then filtered. 0.1% FeCl₃ was added to filtrate. Appearance of brownish green or blue black coloration indicate the presence of tannins.

Test for sterols

Concentrated H₂SO₄ (2 ml) was added to a small quantity of extract. Formation of purple ring at upper surface indicate presence of sterols.

RESULTS

Crude extract of *Aesculus indica* proved active showing inhibition ranging from 34.2% at 10µg/ml to 94% at 500µg/ml. Average absorbance value was found to be 0.2 at 10µg/ml and 0.0843 at 500µg/ml. The extract showed activity in a dose dependent manner from lowest to highest concentrations. A well marked increase in percentage inhibition was seen between 10-25µg/ml and 25-50µg/ml in comparison with higher concentrations where very minor increase was noticed (fig. 2).

Among fractions *Aesculus indica* aqueous fraction showed maximum inhibition (59%). Remaining all fractions showed moderate activity with 29.8% inhibition

by hexane fraction while 30.6% inhibition was found in chloroform fraction. Ethyl acetate fraction showed 45% inhibition while 53% inhibition was found in methanol fraction (fig. 3).

Preliminary qualitative phytochemical analysis of *Aesculus indica* crude extract showed presence of flavonoids, coumarins, saponins and tannins. Sterols, anthraquinones and alkaloids were absent in crude extract as well as fractions. Flavonoids were present in all fractions except in ethyl acetate fraction. Coumarins were present in ethyl acetate and methanol fraction in low quantities. However, were absent in other fractions. Saponins were present in low quantity in hexane fraction while high in other fractions. Tannins were moderately found in chloroform and ethyl acetate fractions (table 1).

DISCUSSION

Extracts proved active in crude form however a dose dependent efficacy was observed. Same results were reported by Ogunlana *et al.* (2008) that dose dependent activity was found in crude extract of *Morinda Lucida*. Likewise similar findings were also shown by several other researchers that activity increased with increase in concentration (Jagetia and Rao, 2006; Aisha *et al.*, 2009; Rahman *et al.*, 2010).

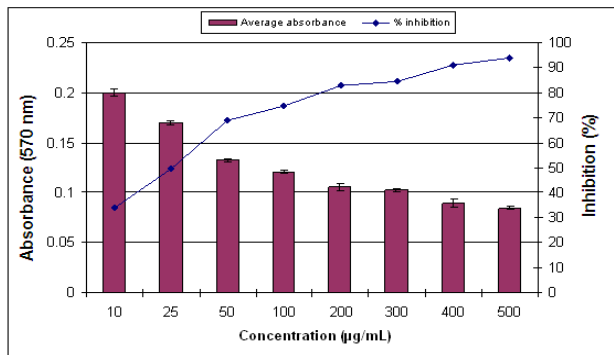


Fig. 2: MTT assay of crude *Aesculus indica* methanol extract

Among fractions a trend of increase in activity was observed by fractions of *Aesculus indica* from non polar (hexane) fraction to polar (aqueous) fraction. Maximum activity in aqueous fraction proved that compounds responsible for activity are polar in nature. It is also evident by traditional use of water decoctions to treat ailments (Kamuhabwa *et al.*, 2000). Increase in activity while moving towards polar solvent indicate presence of compounds in different fractions but their quantity might be increased in aqueous fraction due to ability of water to dissolve maximum of compounds than other solvents. Moderately polar and polar fractions of *Aspidosperma tomentosum* Mart extract proved active than non polar fractions when tested for antiproliferative activity (Kohn *et al.*, 2006).

Phytochemical screening revealed presence and distribution of various phytochemicals among fractions. This preliminary knowledge of phytochemicals serves as guideline about overall chemical components present in plants material which can be helpful for further investigation as well as crude herbal drugs development (Gurumurthy *et al.*, 2008).

CONCLUSIONS

Methanol and aqueous fraction can be suitable candidates

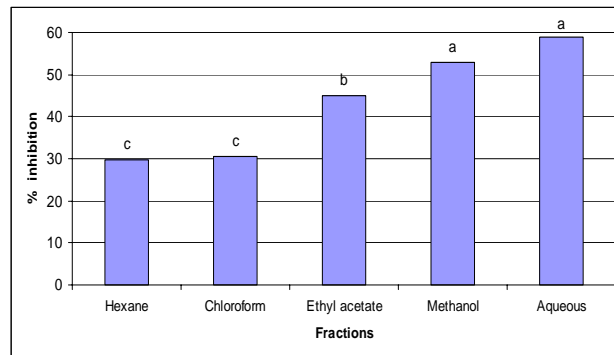


Fig. 3: Activity of *Aesculus indica* extract fractions against MCF-7 cell line. The alphabets on the bars present statistical difference in values obtained after LSD.

Table 1: Qualitative phytochemical analysis of crude extract and fractions of *Aesculus indica*

Chemical Test	Crude extract	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Methanol fraction	Aqueous fraction
Alkaloid	Absent	Absent	Absent	Absent	Absent	Absent
Flavonoid	Low	Moderate	Moderate	Absent	Moderate	Moderate
Coumarins	Low	Absent	Absent	Low	Low	Absent
Anthraquinones	Absent	Absent	Absent	Absent	Absent	Absent
Saponins	Low	High	High	High	High	High
Tannins	Low	Absent	Moderate	Moderate	Absent	Absent
Sterols	Absent	Absent	Absent	Absent	Absent	Absent

for isolation of active components against MCF-7 cell line. Furthermore maximum activity in aqueous fraction might be due to the presence of polar flavonoids and saponins either synergistically or individually. These extracts should be tried on normal cells in order to measure the potential difference in cytotoxicity which could make these findings even more valuable.

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