

PHYTOCHEMICAL ANALYSIS, LIQUID CHROMATOGRAPHY, AND MASS SPECTROSCOPY AND *IN VITRO* ANTICANCER ACTIVITY OF *ANNONA SQUAMOSA* SEEDS LINN.

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ABSTRACT

Objective: The objective of the present study is to evaluate *in vitro* anticancer property and phytochemical analysis using liquid chromatography and mass spectroscopy (LCMS) method of hydroalcoholic extract of seeds of *Annona squamosa* (AS) Linn. Seeds of AS Linn. are traditional medicine treating various diseases and have shown anticancer activity. Due to lack of survival benefit, cancer is a deadly global disease.

Method: The anticancer activity was evaluated using the sulforhodamine B assay method on five cancer cell lines: Breast cancer cell line, cervix cancer cell line (SiHa), colon cancer cell line (HT)-29, liver cancer cell line, and ovary cancer cell line (Ovcar). The phytochemical analysis was performed using LCMS method.

Result: The phytochemical characterization was done using LCMS method which showed 15 different molecular weight compounds. The extract showed an average *in vitro* anticancer activity at a concentration of 100 µg/ml against all cancer cell lines. The best activity was observed against Ovcar-5 cell line (69.72) and was also significant against HT and SiHa cell lines.

Conclusion: The phytochemical analysis showed the wide range of phenols and flavonoid which are showing potent anticancer activity of AS seeds.

Keywords: *Annona squamosa*, Phytochemicals, *In vitro* anticancer, Liquid chromatography and mass spectroscopy.

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INTRODUCTION

Cancer is one of the leading causes of death worldwide. According to the WHO (2004), 12.5% of the population dies due to cancer. The disease is characterized by the uncontrolled and abnormal growth of cells in the human body, forming tumors of malignant cells with the potential to be metastatic [1,2]. Major causes of cancer may be physical inactivity, heredity, unbalanced diet, and various environmental factors [3].

Currently, chemotherapy, radiotherapy, and immunotherapy treatments and surgery cause several toxic effects on non-targeted cells/tissues. This arouses the need of using alternative treatments and therapies against cancer [1,4]. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with the present chemotherapeutic agents [5]. Over the past decades, herbal medicines have made an impact on both global health, and they have been well accepted worldwide [6].

Medicinal plants play a critical role in the healthcare system of a majority of the world's population. Among several medicinal plants worldwide, including India, only a few medicinal plants have attracted the interest of scientists to investigate the remedy for the prevention and treatment of cancer [5]. Natural bioactive compounds such as phenol and flavonoids occurring in the medicinal plants protect the biological systems against harmful effect. They have been studied for their antitumor, proapoptotic, and antiangiogenic effects [7,8].

In history, plant secondary metabolite derives anticancer constituents such as vincristine, vinblastine, camptothecin, podophyllotoxin, flavopiridol, and silvestrol have been used worldwide [9]. Medicinal plants possess good immunomodulatory and antioxidant properties, leading to anticancer activities. The antioxidant phytochemicals protect the cells from oxidative damage. Further, it will be of great significance

to develop new drugs from these medicinal plants. Taking into consideration the above facts, an attempt has been made to evaluate the *in vitro* anticancer activity particularly against totally unexplored ovarian cancer cell lines i.e. SiHa cervix and ovcar-5 ovary cancer cell lines and phytochemical study of the seeds of *Annona squamosa* more over LCMS analysis will be the part of study.

METHOD**Chemicals and drugs**

The following drugs and chemicals were used: Ethanol, Roswell Park Memorial Institute (RPMI) media, trypsin-EDTA (Sigma Chemical Co.), fetal bovine serum, penicillin, streptomycin, phosphate-buffered saline, sulforhodamine b, and dimethyl sulfoxide (DMSO). All chemicals used were of analytical grade.

Plant material

The seeds of AS were obtained from Botanical Garden, Dr. H. S. Gour University, Sagar, M.P, specimen No. BOT/H/12/14/20.

Extract preparation

The seeds of AS were washed with running water to remove impurities. It was dried to avoid the growth of microorganisms and reduced to coarse powder and subjected to hydroalcoholic extraction with the help of a Soxhlet apparatus. The hydroalcoholic extract was filtered, concentrated under reduced pressure, and lyophilized for drying. The dried extract was kept in the airtight container and stored at 4°C till further studies. The air-dried seeds (150 g) were powdered and then extracted with 1 L of 50% ethanol using Soxhlet apparatus at 68°C [10]. Percentage yield was calculated (Table 1).

Phytochemical tests

Phytochemical analysis was performed to detect various compounds such as tannins, flavonoids, alkaloids, and steroids [11,12].

Table 1: Percentage yield of hydroalcoholic extract of the plant

Solvent	Plant	Part used	Dry weight (g)	Yield (g)	Time (h)	Temperature (°C)	% yield
50% ethanol	AS	Seeds	150	25.25	96	68	16.83

AS: *Annona squamosa***Liquid chromatography and mass spectroscopy (LCMS) analysis**

Seeds of AS were subjected to LCMS analysis using the instrument, Mass Hunter SG11251102 (Agilent Technologies), which was conducted in both polarities (positive and negative) of electron spray ionization (ESI) mode. Better response was found in positive ESI. Two solvents system, i.e. Solvent A (water+0.1% formic acid) and Solvent B (acetonitrile) with column Chromolith - 50 × 4.6 mm were used for analysis. The fragmentor voltage 135 V, run time 30 min, and flow rate 0.5 ml/min were maintained [13,14]. In a total run of 30 min in the LCMS, approximately 16 compounds were detected. Their retention time and mass have been confirmed (Table 2).

Anticancer activity

The *in vitro* anticancer activity of hydroalcoholic extract of seeds of AS was performed on five human cancer cell lines, i.e., human breast adenocarcinoma breast cancer cell line, human cervix cancer cell line (SiHa), colon cancer cell line (HT-29), liver cancer cell line, and ovary cancer cell line (Ovar-5) which were procured from Regional Research Laboratory, Council of Scientific and Industrial Research, Jammu (J & K). The cell lines were cultured in RPMI containing 20% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin. All cell lines were maintained at 37°C in 5% CO₂ atmosphere with 95% humidity. Maintained cultures were passaged weekly, and the culture medium was changed twice a week.

In vitro anticancer activity by sulforhodamine B (SRB) assay method

The protocol was established by the National Cancer Institute [15,16] for human tumor cytotoxicity for the assessment of growth inhibition. The colorimetric assay estimated cell number indirectly by staining total cellular protein with the dye SRB. Single-cell suspensions were prepared by the treatment of cells with 0.51 ml of 0.1% trypsin-EDTA (Sigma Chemical Co.). The viable cells were counted using a Coulter counter and diluted with RPMI medium, and final densities of 100 × 10⁴ cells/ml were obtained. Cell suspensions (100 µl/well) were seeded in 96-well microtiter plate containing 1 ml of media and incubated for cell attachment. After 24 h, the cells were treated with the extracts. Extract was initially dissolved in 100% DMSO (1 mg/ml for extract) and further diluted in RPMI medium to produce a concentration of 100 µg/ml. 100 µl of the medium was added to the control wells. The plates were incubated for 48 h. After 48 h, adherent cell cultures were fixed by adding 50 µl of cold 50% (w/v) trichloroacetic acid and incubating for 60 min at 4°C. The supernatant was then discarded, and the plates were washed 5 times with de-ionized water and then dried. 100 µl of SRB solution (0.4% w/v in 1% acetic acid) was added to each microtiter well, and the culture was incubated for 30 min at room temperature for the complete staining reaction. Unbound SRB was removed by washing five times with 1% acetic acid and then the plates were air-dried. Bounded stain was solubilized with Tris buffer (10 mM) and the optical densities were read on an automated spectrophotometric plate reader (Molecular Devices, USA) at a wavelength of 492 nm, and optical density (OD) of SRB in each well is directly proportional to the cell number. The experiment was performed in triplicate, and the mean SRB absorbance was taken for the calculation of inhibition. Percentage inhibition was calculated by comparing the OD of control well (Table 3) with that of test sample [16,17].

Statistical analysis

The experiment was performed in triplicate and the data are given as mean SRB absorbance for the calculation of inhibition ± standard error of the mean. Percentage inhibition was calculated by comparing the OD of control well with that of test sample Fig.1:

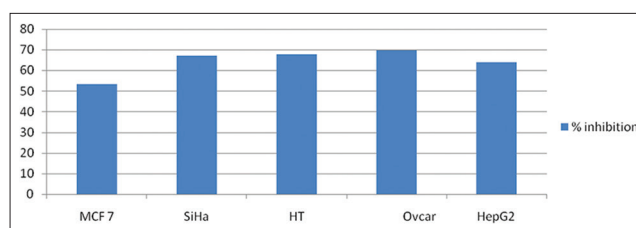
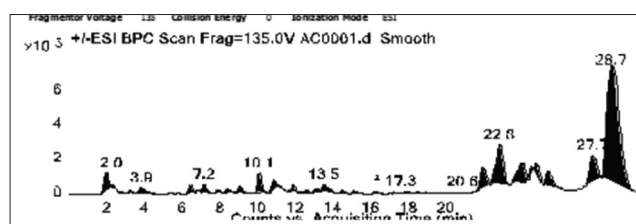
Table 2: LCMS analysis of AS seed extract

Retention time range (min.)	Mass-to-charge (m/z)
2.3–2.5	181.10
6.3–6.6	313
7.0–7.3	327
8.2–8.7	652
9.9–10.3	848
12.9–13.2	837.30
15.0–15.4	549.10
16.1–16.5	269.10
18.3–18.7	635.40
18.9–19.2	656.40
19.9–20.1	654.40
11.7–12.1	832.40
21.7–22.2	638.40
24.4–24.6	638.40
25.2–25.7	638.40

LCMS: Liquid chromatography and mass spectroscopy, AS: *Annona squamosa*

Table 3: Anticancer activity of hydroalcoholic extract of AS

Type of cell line	Concentration of Extract (µg/ml)	% inhibition
MCF 7	100	53.34
SiHa	100	67.15
HT	100	67.66
Ovarc	100	69.72
HepG2	100	63.82

AS: *Annona squamosa*, MCF 7: Breast cancer cell line, SiHa: Cervix cancer cell line, HT: Colon cancer cell line, Ovarc: Ovary cancer cell line, HepG2: Liver cancer cell lineFig. 1: Anticancer activity of hydroalcoholic extract of *Annona squamosa*Fig. 2: Liquid chromatography and mass spectroscopy chromatogram of hydroalcoholic extract of *Annona Squamosa*

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Optical density of the treated cells}}{\text{Optical density of the control}} \right) \times 100$$

RESULTS AND DISCUSSIONS

The percentage yield of the extract of seeds of AS was 16.83 (Table 1). It showed the presence of flavonoids, tannins, alkaloids, and other phytochemicals. The LCMS analysis revealed the presence of 15 different molecular weight compounds at different retention time (Table 2 and Fig.2). The mass spectrum of extract by LC-MS showed various peak of different peak of 15 compounds of which molecular ion peak at m/z 316 which resembles the molecular weight of the Isorhamnetin. However isorhamnetin is methylated metabolite of quercetin which may acting as flavonoid in hydroalcoholic extract of extract of seeds of AS. The extract showed an average *in vitro* anticancer activity at a concentration of 100 µg/ml against all cancer cell lines. The best activity was observed against Ovar cell line (69.72) and was also significant against HT and SiHa cell lines (Table 3). The anticancer activity of the plant may be due to the presence of phytochemicals, such as flavonols and flavonoids, which were investigated to determine chemoprevention activity against cancer [18]. Phenols, flavonoids, quercetin, genistein, and baicalein obtained from plant extracts are also effective against tumor [19]. Similarly, alkaloids such as schischkinnin and montamine have been isolated from the seeds of *Centaurea schischkinnii* and *Centaurea montana* showed anticancer property [20]. Further, the active compounds of the plant can be isolated and their individual activity can be analyzed for several pharmacological activities.

CONCLUSION

On the basis of phytochemical and anticancer activity, it has been found that the *Annona Squamosa* seeds indicates potential against ovarian cancer cell lines. Phytochemical analysis indicates the presence of flavonoids and its derivatives are present in the *Annona Squamosa* seeds and can be concluded that anticancer potential may be due to presence of isorhamnetin.

AUTHOR CONTRIBUTIONS

Sarvesh paliwal planned, design and supervised the research and manuscript. Shuchi Dave Mehta involved the design of the research, interpretation of data, analysis of the result and to the writing of the manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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