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ANTIPROLIFERATIVE ACTIVITY OF METHANOLIC FRUIT EXTRACT FROM CUCURBITA DIGITATA

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ABSTRACT

To investigate the antiproliferative / cytotoxicity effect of methanolic fruit extract of *Cucurbita digitata* on human hepatocellular carcinoma HepG2 cell line. Cytotoxicity tests on HepG2 liver cancer cell lines were performed by Caspase assay, Flow cytometry and DNA fragmentation assays. Cell proliferations measured by Caspase assay exhibited significant inhibition of cell growth with an increase in concentration of the Methanolic active fraction. The Flow cytometry and DNA fragmentation analysis also proved similar effects against HepG2 liver cancer cell lines. Thus MeOH fruit extract of *Cucurbita digitata* exhibited a highly significant inhibitory effect on HepG2 cell proliferation which was evidenced by a reduction in viable cell count. The results were confirmed by microscopical examination of cell morphology. The antiproliferative activity of *Cucurbita digitata* thus contributes the underlying data for the isolation of active compound for antiproliferation and the primary intracellular mechanism.

KEY WORDS: ***Cucurbita digitata,*** **Caspase assay, antiproliferative and cytotoxicity**

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INTRODUCTION

According to WHO estimates, more than 80% of people in developing countries depend on traditional medicine for their primary health needs and 60% of cancer patients use vitamins or herbs as therapy[1]. However, only a few medicinal plants have attracted the interest of scientists to investigate the remedy for cancer. There is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from natural product sources[2]. The potential of using natural products as anticancer agents was recognized in the 1950s by the U.S. National Cancer Institute (NCI), and has major contributions to the discovery of new naturally occurring anticancer agents. Hence, an attempt has been made to explore the potential of newly discovered anticancer compounds, from medicinal plants, as a lead for anticancer drug development[3]. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity[4]. Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, are of 60% natural origin[5]. Several reports describe that the anticancer activity of medicinal plants is due to the presence of antioxidants present in them. So, it is anticipated that plants can provide potential bioactive compounds for the development of new 'leads' to combat cancer diseases. Reports confirmed that the members of same genus cucurbitaceae possess medicinal property for example *Curcumin longa Lin*. treats stomach ulcers and prevents liver cancer[6]**.** The fruits and leaves of *Momordica charantia Lin*. cure liver diseases[7]. Curcumin extracted from *C. longa* has anti-inflammatory and antioxidant properties and shows antiviral activity for HBV and hepatitis C virus[8,9]. *Momordica charantia* has antidiabetic, anti-opacity, anticancer, hypoglycemic, antifertility activities. However, very less evidence and articles are available to show the pharmacological property of *Cucurbita digitata*. Hence through this study, we investigated the anticancer activity by testing antiproliferative effect of MEOH extracts of *Cucurbita digitata* on human hepatocellular carcinoma HepG2 cell line which is a well-differentiated transformed cell line closely related to Hepatocellular carcinoma (HCC).

MATERIALS AND METHODS

Extract preparation

Fresh fruits of *C. digitata*, were collected directly from Tirunelveli district and were immediately used for extract preparation. The fruits were washed well using tap water and twice with distilled water. Then the seeds from *Cucurbita digitata* were removed manually to avoid its intrusion. The fruits were then shade dried for a period of 20 days, at an ambient temperature of 35˚C. The dried samples were ground properly using a mortar and pestle and later using a grinder, to obtain the fibrous form. The extracts of the fruit sample was prepared by soaking 100 g of dried powder in 900 ml of methanol 48 hours. The extract was then filtered using Whatman filter paper. The filtrate was concentrated under reduced pressure in vacuum at 40°C for 25 min using a rotary evaporator (Superfit-ROTAVAP, India).

Methanolic active fraction

The crude methanol extracts of *Cucurbita digitata* was subjected to column chromatography and the active fraction identification was based on separated fractions tested in MTT assay against HepG2 cell lines as indicated through a previous study by L. Helen Mary and Jerrine Joseph[10].

Cell lines

HepG2 cell lines were purchased from the National Centre for Cell Sciences (Pune, India). The cells were cultured in media containing L-Glutamine and 25mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES), Penicillin (100 µg/mL), Streptomycin (50µg/mL) and 10% foetal bovine serum. Cells were maintained in DMEM at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

ANTICANCER ASSAYS

Flow Cytometry

Flow cytometry analysis was performed to sort cells after treatment with the Methanolic active fraction of the fruit extract[11]. Approximately 5x10³cells/well (MCF 7) were seeded into micro titre plate, 300µl of medium was added and incubated at 37ºC. After 24 hours, the medium was discarded and fresh medium was added with 2.5 µl, 5µl and 10µl of (20mg/ml). The plates were incubated for 48h at 37ºC in a CO_2 incubator. After incubation, the cells were harvested and washed in PBS and stored in -20^{o}C for 24h. Further, the cells were washed with cold PBS and stained with 0.4 ml Propidium iodide (500µg/ml). Data was acquired using a flow cytometer after 30 mins of incubation at 37^{o}C.

DNA Fragmentation assay

Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 base pairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis[12]. Cells were seeded in 24 well plate and kept in CO_2 incubator. Cells were treated by the Methanolic active fraction of the fruit extract in three different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml) for 48 hours. At the end of incubation period, the cells were centrifuged for 1000 rpm for 3mins at 14^{o}C. The pellet was resuspended in a lysis buffer (10 mMTris-HCl, pH 8.0, 10 mMNaCl, I0 mM EDTA, 20mg/ml Proteinase K, 10% SDS), and incubated at 37^{o}C. The DNA was extracted by phenol-chloroform method, precipitated overnight in -20^{o}C ethanol containing 0.3 M final concentration sodium acetate. The pellet was dissolved in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). DNA samples were electrophoretically separated on 1.8 % agarose gel containing ethidium bromide (0.4µg/mL). DNA was visualized by a UV (302 nm) transilluminator[13,14]. Quercitin treatment was used as positive control, while untreated cells were used as control.

CASPASE assay

Caspases[15] activities were determined by chromogenic assays using caspase-3 and caspase-9 activation kits according to the manufacturer's protocol (Calbiochem, Merck). After treating with the Methanolic active fraction of fruit extract, the cells were lysed using Lysis buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM DTT, 100mM EDTA). Lysates were centrifuged at 10,000×g for 1 min. The supernatants (cytosolic extract) were collected and protein concentration was determined by the Lowry's method using BSA as a standard. 100–200 µg protein (cellular extracts) was diluted in 50 µl cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtitre plates with 5 µl of the 4mM p-nitroanilide (pNA) substrates, DEVD--pNA (caspase-3 activity) for 2 hours at 37°C. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405nm in a microtiter plate reader. Relative caspase-3 activity was calculated as a ratio of the absorbance of treated cells to untreated cells.

RESULTS

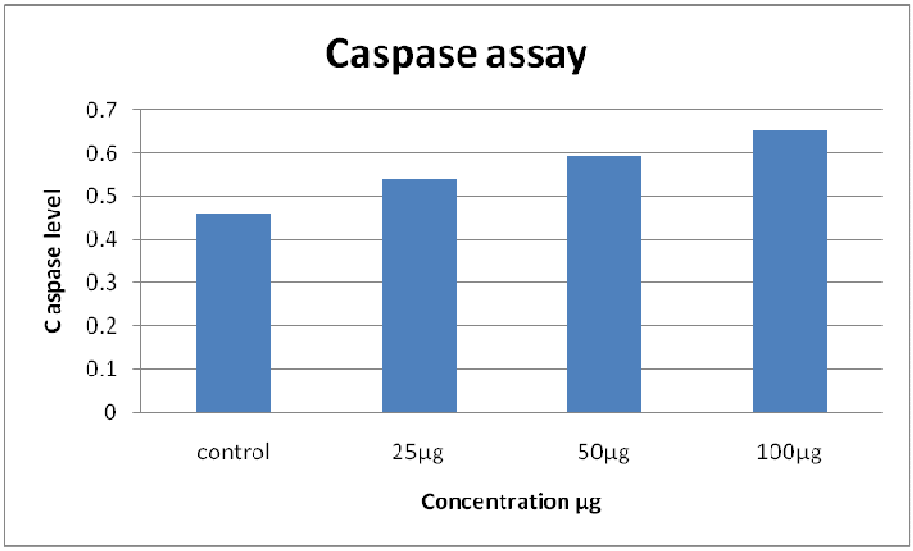
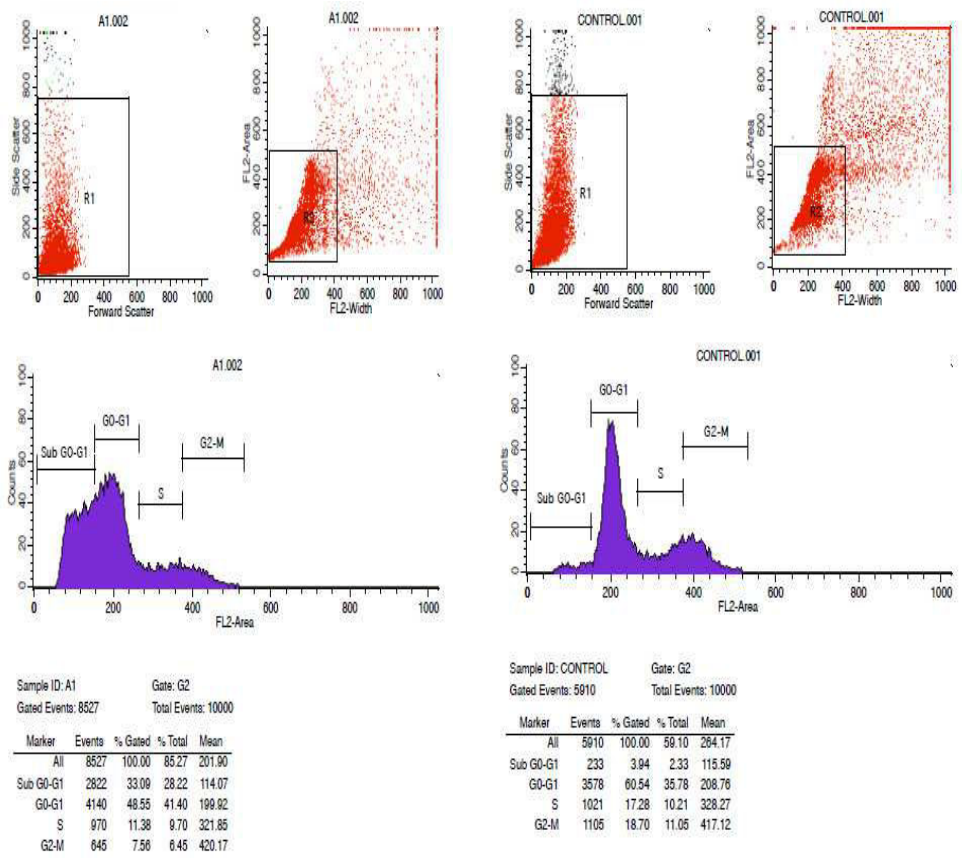


Figure 1
Caspase levels in treated HepG2 cells in comparison to control cells

Lane 1	Reference DNA Ladder
Lane 2	DNA from untreated cells
Lane 3	DNA from cells treated with 25µg compound
Lane 4	DNA from cells treated with 50µg compound
Lane 5	DNA from cells treated with 100µg compound

Figure 2
DNA laddering pattern in cells treated with fruit extract of C.digitata.



Sample ID: A1 Gate: G2
Gated Events: 8527 Total Events: 10000

Marker	Events	% Gated	% Total	Mean
All	8527	100.00	85.27	201.90
Sub G0-G1	2822	33.09	28.22	114.07
G0-G1	4140	48.55	41.40	199.92
S	970	11.38	9.70	321.85
G2-M	645	7.56	6.45	420.17

Sample ID: CONTROL Gate: G2
Gated Events: 5910 Total Events: 10000

Marker	Events	% Gated	% Total	Mean
All	5910	100.00	59.10	264.17
Sub G0-G1	233	3.94	2.33	115.59
G0-G1	3578	60.54	35.78	208.76
S	1021	17.28	10.21	328.27
G2-M	1105	18.70	11.05	417.12

Figure 3
Flow cytometry of active compound treated HepG2 cells (3a) and control (3b)

DISCUSSION

Previous phytochemical study in *C.digitata* showed the presence of important phytochemicals such as flavanoids, quinones, coumarins, carbohydrates, phytosteroids and cardiac glycosides. The DPPH, NO, SO and FRAP assays conducted earlier have shown important antioxidant property. The earlier invitro MTT Assay results also confirmed the induced cytotoxicity and decreased cell viability in the presence of the methanolic fruit extract[10]. This contributively instigated towards this research investigation on antiproliferation/ cytotoxicity effects in column fractionated Methanolic active fraction of fruit extract. In the caspase assay, Caspase 3 activation was used as a positive marker for apoptosis and the activity in extract at 25µg, 50µg and 100µg of Methanolic active fraction was measured to be 0.541, 0.594 and 0.652 as shown in figure1. It showed the activity of Caspase 3 to increase with increase in concentration of the fruit extract . *Cucurbita digitata* thus shown the enhanced apoptotic activity in which Caspase activity cascade is an integral event. In the DNA fragmentation assay for antiproliferative study, the fluorescence of fragmented DNA was taken as the indication for apoptosis taken place by the Methanolic active fraction of *Cucurbita digitata*. The lane 2 of untreated cells was used as a control and the cells treated with 25µg, 50µg and 100µg of Methanolic active fraction in lane 3, lane 4 and lane 5 is compared as shown in fig 2. The fluorescence of lane 3, 4 and 5 increases, which signifies the formation of DNA fragments constantly at increasing concentration of the sample. The Flow cytometry was useful to detect and measure apoptosis by analyzing cells with a DNA content less than 2n ("sub-G_1 cells"). In flow cytometry the fluorescent dyes allowed accurate cell cycle analysis in the cell

populations. In the illustration, the absence of a sub-G_0/G_1peak in control signifies that the DNA replication for cell growth has significantly reduced and cell death has occured. Thus the results of the Cell viability assays showed apoptotic effect in HepG2 cell growth proving *Cucurbita digitata* to inhibit the growth of cancer cell lines without affecting the growth of normal cells. The investigation thus forms the basis for further research in the isolation of active compound responsible for cytotoxicity and intracellular cancer pathway to disclose the significant mechanism involved in the cancerous cell inhibition.

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