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Original Article

MANGO SEEDS: A POTENTIAL SOURCE FOR THE ISOLATION OF BIOACTIVE COMPOUNDS WITH ANTI-CANCER ACTIVITY

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ABSTRACT

Objective: The mango (*Mangifera indica* L.), is a fruit with high levels of phytochemicals and the seeds have antioxidant, anti-inflammatory and anti-obesity effects. This study aims at the extraction of metabolites from mango seeds and evaluation of the antiproliferative properties on cancer cell lines.

Methods: The antiproliferative effects of the ethanol extract of mango seeds were evaluated on the cancer cell line HeLa, CHO cell lines and also on normal human lymphocytes by MTT assay. The extract was purified by TLC and characterized by LC-MS methods.

Results: The ethanol extract had significant cytotoxicity to HeLa cells and the bioactive fraction from the crude extract had antiproliferative effects with an IC_{50} value of<10µg/ml. Fluorescence microscopy, caspase and LDH activity assays were confirming the anticancer potential of fraction 6. This fraction had arrested the HeLa cells in G2/M phase and decrease in the percentage of cells in S phase. By LC-MS analysis, it was found to have an m/z value of 701.8 indicating it to be a novel one.

Conclusions: Here mango seeds have shown promising potential as a novel source for the isolation of a bioactive compound with anticancer activity.

Keywords: Anti-cancer, Antiproliferative, Bioactive fraction, Cytotoxic, HeLa cells, Mango seeds.

INTRODUCTION

In the last 50 years diverse forms of cancer have become some of the world's leading causes of mortality. The increasing incidence of various cancers reported over the last few decades have led to the search and development of new anticancer drugs, drug combinations and chemotherapy strategies by systematic and methodical exploration of the synthetic and natural product [1]. Epidemiology suggests that cancers can be largely avoided [2]. There is a large amount of experimental evidence which indicates that fruits and vegetables lower the risk of cancer [3]. In light of the continuing need for effective anticancer agents, and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly being considered as sources of anticancer drugs [4].

Mango (*Mangifera indica* L.), the most important fruit in *Anacardiaceae* family, is a tropical fruit with high nutritional and medicinal value. Ripe mango fruit is considered to be invigorating and freshening. It is a phytochemically dense food with high levels of carotenoids and phenolic compounds [5-7]. It originated from the Asia Indo-Burmese region approximately 4000 years ago, and is now commercially grown in more than 87 countries [8]. Fully ripe mango is famous for its strong aroma, intense coloration, delicious taste, high amount of bioactive compounds such as phenolic compounds, β-carotene, vitamin C, and minerals [9-12]. Traditionally *Magnifera indica* have been reported to have many medicinal properties [13]. Bioactive compounds found in the mangos, among other plants and herbs have been shown to have possible health benefits with antioxidative, anticarcinogenic, antiatherosclerotic, antimutagenic, and angiogenesis inhibitory activitie [14]. Among the various polyphenolic compounds found in the mango, mangiferin (C-glucosyl xanthone) is a distinct one [15]. Mangiferin is a natural pharmacologically active phytochemical that was found to have anti-inflammation [16], anti-diabetic [17], immunomodulatory [18], anti-tumor [19, 20], and antioxidant activities [21, 22]. Lupeol, a triterpene present in mango and other fruits, has shown to possess anticancer properties *in vivo* and *in vitro* assays [23, 24].

Soong and Barlow [25] assayed the antioxidant activity of a variety of fruit seeds and reported that the antioxidant activity of the mango seed kernel was the highest, a fact attributed to its high polyphenolic content. Mango seed kernels are rich in polyphenols with potent antioxidative activity, but ironically the seeds are always discarded as waste during processing and consumption of the mango fruit. Ahmed *et al.* [26], identified and quantified various polyphenolic compounds in the mango seed kernel and reported the total polyphenolic content of the mango seed kernel extract as 112 mg (GAE)/100 g. Mango seed kernel extract with hot water (MSKE-W) influences anti-obesity effects, both *in vitro* and *in vivo* [27].

Based on the main components of Mango seed extract and its antioxidant properties, we hypothesized that Mango seed extract may have anticancer activities against cancer cell lines *in vitro*. However, there are no such reports. To confirm this hypothesis, the inhibitory effect of mango seed extract on the growth of human cervical cancer cells *in vitro* and partial purification and characterization of the active component responsible for this bioactivity were investigated in this experimental study.

MATERIALS AND METHODS

Sample collection, authentication and Preparation of extracts

The fruits of mango (*M. indica*) were collected from Bangalore, India on June–July 2011. The herbarium voucher specimens were identified and deposited in Jain University, Bangalore, India. The kernel was separated from the fruit and cut into small pieces, which were diced further and freeze-dried. The freeze dried samples were ground into a fine powder using a dry grinder, and then kept in an air-tight container and stored in a freezer (-20°C) before extraction. 30 grams of dried powder were used for serial extraction in a soxhlet apparatus using ethanol. The extracts were filtered and evaporated to dryness in a rotary evaporator. 1 mg/ml of the extract was prepared by dilution of the stock with sterile dimethyl sulphoxide (DMSO) [28].

Chemicals

DMEM medium, fetal bovine serum (FBS), penicillin, streptomycin and MTT were procured from HIMEDIA (India). Caspase Apoptosis

Assay Kit was purchased from G Biosciences (kit 786-205A), USA. All other chemicals and solvents used were of the highest purity grade available.

Cell lines and culture

HeLa and CHO cell lines were procured from National Center for Cell Sciences (NCCS), Pune. They were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum. Lymphocyte isolation was performed using the blood collected from few healthy male and female individuals, about 20 years of age, who were free from infection for the past six months and had not been under any medication. The ethical guidelines for research of the Indian Council of Medical Research (ICMR, 2006) were followed with regard to blood sampling. Hi Sep medium (HIMEDIA, India) was used for the isolation. Lymphocytes were used as control cells to assess the cytotoxicity of the extracts on humans. The cells were incubated at 37 °C with 95% air and 5% CO_2. All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide)] Assay

Hela and CHO cells growing exponentially were collected after trypsinization and plated in 96-well microtiter plates in 100 µl of culture medium and were allowed to adhere for 24 h before treatment. Increasing concentrations of ethanol extract of mango seeds, dissolved in DMSO, were added to different wells of the microtiter plates. Final concentration of DMSO in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. The cells were incubated for 24, 48 and 72 hours in the presence and absence of the extracts. Cytotoxicity was analyzed using MTT assay following the standard protocol [29]. Cytotoxicity was expressed as the concentration of the extract inhibiting cell growth by 50%, relative to cells incubated in the presence of 0.5% DMSO. The absorbance was read at 540 nm using the ELISA plate reader. Each experiment was performed in triplicates.

The following formula was used to calculate the percent of inhibition:

$$\text{Inhibition (\%)} = (1 - OD_s/OD) \times 100$$

(Where OD_s = Optical density of the sample and OD = Optical density of the control)

Chromatographic separation and identification of the bioactive compound (TLC)

The ethanol extract of mango seeds was subjected to thin layer chromatography (TLC) using pre-coated TLC plates (Silica gel 60 F 254 Merck) to fractionate the bioactive components from the crude extract. Chromatogram was performed with the following solvent systems (a) Toluene: ethylacetate: formic acid (2.5:1:1 v/v); (b) chloroform: acetone (6:4 & 8:4 v/v); (c) hexane: acetone (6:4 & 8:2 v/v); (d) dichloromethane: acetone (6:4 & 8:2v/v); (e) Toluene. The chromatograms were detected with the help of a UV transilluminator (254 and 366 nm). For the detection of active compound separated in TLC, bioactivity guided fractionation was followed. From the chromatogram developed as described above, each band was scraped, mixed with methanol and centrifuged at 3000 rpm for 15 min. Supernatant was collected in a pre-weighed vial and kept for evaporation. The partially purified fractions obtained from preparative TLC were tested for cytotoxicity against the HeLa cells, CHO cells and the lymphocytes by MTT assay as described earlier.

Phytochemical analysis

Preliminary qualitative phytochemical analysis was performed for the determination of the chemical groups of the bioactive fraction using standard protocols [30]. All the analyses were carried out using 0.5-1 mL of extract solutions. In the case of tests for carbohydrates, tests such as Fehling's and Benedict's tests were carried out. Tests for alkaloids (Wagner's and Mayer's tests), test for sterols (Salkowsky test), tests for the detection of phenolic compounds (test with neutral $FeCl_3$) and tannins, tests for proteins (Biuret test) and also tests for the detection of flavonoids and saponins, were performed.

Fluorescence microscopic analysis by Ethidium bromide/ Acridine orange (EB/AO) staining

HeLa and CHO cells growing exponentially were subcultured to 25 cm^2 culture flasks and were allowed to adhere for 24 h. After this period, the cells were treated with bioactive fraction 6 from the mango seed extract for 24 h. After removal of the incubation medium, cells were harvested by trypsinization and treated with EB/AO stain [31]. Stained cells were observed using a fluorescence microscope and the images were captured in a digital camera.

Caspase-9 apoptosis assay

Caspase-9 activity was assessed using the caspase-9 Colorimetric Assay Kit (G Biosciences, kit 786-205A). The assay is based on spectophotometric detection of the chromophore *p*-nitroaniline (*p*NA), after cleavage from the labeled substrate LEHD-*p*NA. The free *p*NA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an un induced control allows determination of the fold increase in caspase-9 activity. In brief, $2x10^6$ control or treated cells were lysed in 500 µl of lysis buffer and lysed by alternate freezing and thawing for 4-5 times. 50 µl of cell lysates added to 50 µl of 2X CasPASE™ assay buffer (containing 7.5 mM DTT). Blanks was also set up by adding 50 µl of lysis buffer into the wells instead of cell lysates. 5 µl of the substrate (1 mM AFC-conjugate) was added to the wells (50 mM final concentration) and the contents of the wells were mixed gently and the absorbance was detected in an Elisa plate reader at 405 nm at zero time point (t = 0). The plates were incubated at 37°C and the absorbance was recorded every 15 minutes until a significant difference in the readings occurred from those at t = 0. Percentage increase was calculated using the following formula:

Rate of percentage increase in caspase activity over the length of the reaction time = $(OD_{control/sample} - OD_{blank})/OD_{blank} \times 100$

All experiments were performed in triplicate and repeated at least three times.

Cell cycle kinetics

Cells grown in 12-well plates (5.0 X 10^5 cells/ml) were treated with fraction 6 for 24 hrs. Briefly, cell pellets were obtained by tripsinization, washed twice with PBS and fixed overnight with 70% ethanol at 4°C. After incubation, cells were centrifuged again at 5000 rpm for 10 minutes and washed twice with PBS. Cells were re suspended in 1 ml of PBS and in ribonuclease (100 µg/ml). Then cells were re-suspended in staining solution [50 µg/ml propidium iodide, 30 units/ml RNase, 4 mM/l sodium citrate, and Triton X-100 (pH 7.8)] and incubated at 37°C for 15 min. After incubation in the dark, fluorescence-activated cells were sorted in a FACScan flow cytometer (equipped with a 488-nm argon laser), and the data were analyzed on a MACS Quant analyser.

LDH cytotoxicity assay

LDH Assay is a colorimetric method of assaying cellular cytotoxicity. The assay quantitatively measures a stable cytosolic enzyme lactate dehydrogenase (LDH), which is released upon cell lysis. Cells treated with fraction 6 of mango for 24 hrs were collected by tripsinization, centrifuged at 1000 rpm for 10 mins, and 10 µl of lysis buffer was added and plated in triplicates in a 96 well plate along with the controls, positive (1% Triton X-100) and negative (untreated). 50 µl of substrate was added to the wells and incubated in the dark for 20 mins. After the incubation period, 50 µl of stop solution was added to all the wells to stop the reaction and readings were noted down at 490 nm. Percentage cytotoxicity was measured using the following formula:

Percentage cytotoxicity = $(OD_{treated} - OD_{negative\ control})/OD_{positive\ control} \times 100$

HPLC analysis of the active fractions

To further purify the active fractions, the TLC purified bioactive fraction 6 of mango seed was subjected to high performance liquid chromatography. The HPLC system with 2487 dual λ U-V detectors (258 nm), 1525 Binary pumps and ODS –C18 analytical column (4.6 I. D X 150 mm) was used with 5µm particle size. The mobile phase consisted of solvent A: solvent B (1:1). Solvent A is 2% acetic acid

and Solvent B acetonitrile: 0.5% acetic acid (1:1, v/v). The separation was performed using isocreatic elution with a flow rate of 1.0 ml/ min. The injection volume was 20 µl and the column temperature was 25°C. The sample and mobile phase was filtered through 0.22 µm PVDF filter before injecting to the column.

Spectroscopic analysis

The ESI mass spectra was recorded using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). Spectra were acquired over the mass range 50-1500 m/z.

Statistical analysis

All experiments were carried out in triplicates. The results were expressed as mean±standard error values. Statistical significance was calculated using one-way analysis of variance (ANOVA). A value of $p<0.05$ was taken as statistically significant.

RESULTS

Viability of HeLa cells by MTT assay

When HeLa cells were treated with the ethanolic extracts of mango seeds, there was a time and dose dependent inhibitory effect on cell proliferation. Percentage viability decreased as the period of exposure to the extract increased from 24 hrs to 48 hrs and then to 72 hrs. At 5µg/ml concentrations of the extract, the percentage viability of 24 hr treated HeLa cells were 65% and it decreased to 40% by the end of 72 hrs. As the concentration increased from 5 to 20 µg/ml, the percentage viability of HeLa cells decreased (fig. 1).

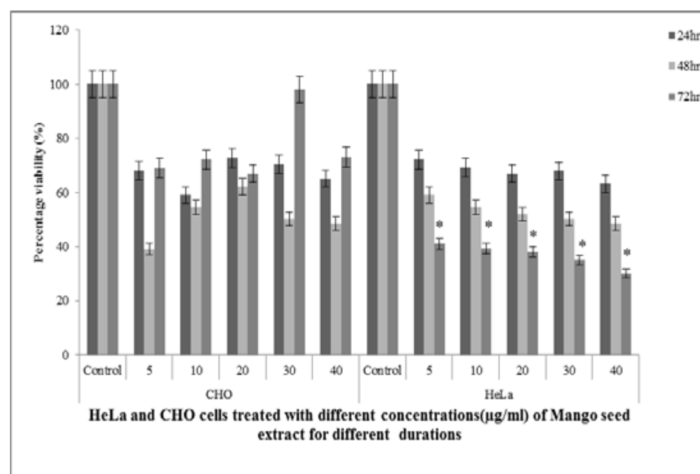

Fig. 1: Effect of *Magnifera indica* seed ethanol extract on HeLa and CHO cell Lines.*p<0.05 compared with control

When CHO cells were treated with the ethanol extract of mango seeds, we could not see any correlation between the concentration and time duration of treatment to percentage inhibition of viability (fig. 1). At all the treated concentrations, percentage viability remained approximately the same and as the duration of exposure to the extract increased from 24 hours to 72 hours, the percentage viability of the cells increased. At a concentration of 40 µg/ml, after 72 hr of treatment, the percentage viability was 72% as compared to 65% after 24 hrs. This indicates that the extract was not toxic to CHO cells as compared to the HeLa cells.

Chromatographic separation of the bioactive compound

When thin layer chromatography (TLC) was performed using Toluene as the solvent, six major fractions were separated from the mango seed extract, which were visualized as distinct bands (fig. 2) with the help of a UV transilluminator (254 and 366 nm).

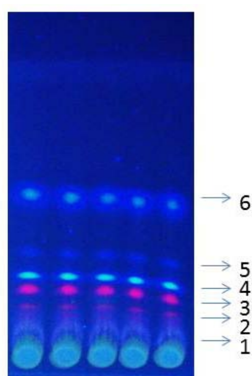

Fig. 2: TLC of mango seed extract as observed under UV light

Fractions recovered from TLC were further tested for cytotoxicity by the MTT assay. The 6thfraction of the ethanol extract of Mango seeds exhibited highest cytotoxic effects than the other fractions. Fraction 6 reduced the percentage viability of CHO cells to 40% (fig. 3) and HeLa cells to 33% at 20 µg/ml concentrations, after 72 hrs of treatment. IC_{50} value on HeLa cells was found to be<10µg/ml. When the bioactive fraction 6 from the seed extract was checked for its cytotoxicity on normal human peripheral lymphocytes, the percentage viability of the treated lymphocytes remained approximately 100 at all the tested concentrations (fig. 4) except at 20µg/ml, where there was a proliferation of lymphocytes. Here the percentage viability of lymphocytes was 270 as compared to the controls, indicating that at higher concentrations this fraction would be having a stimulatory effect on lymphocytes and comparatively safer on humans.

Phytochemical analysis

By phytochemical screening, among the different tests performed, the samples (crude extract and the bioactive fraction 6) indicated the presence of phenols and also gave confirmatory results to the presence of flavonoids (table 1). Based on the observation, the functional group of the active component was tentatively identified as a flavonoid.

Table 1: Chemical screening of the crude extract and the bioactive fraction 6

Compound	Crude extract	Fraction 6
Alkaloids	-	-
Phenols	+	+
Steroids	+	-
Reducing sugars	+	-
Tannins	+	-
Saponins	-	-
Proteins	+	-
Flavonoids	+	+

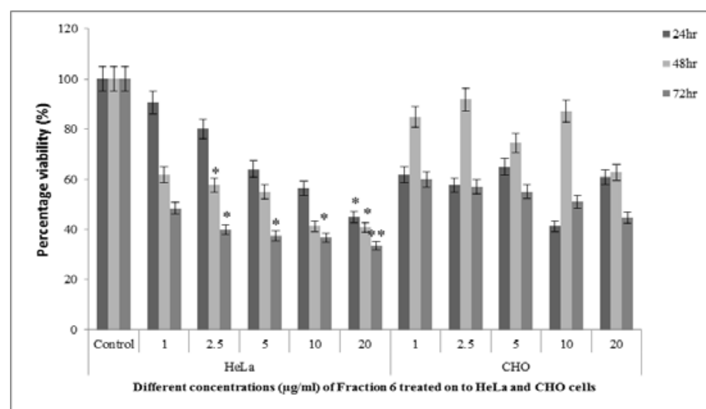

Fig. 3: Percentage viability of HeLa cells and CHO cells treated with the bioactive fraction 6 of mango seed extract. *p<0.05 compared with control

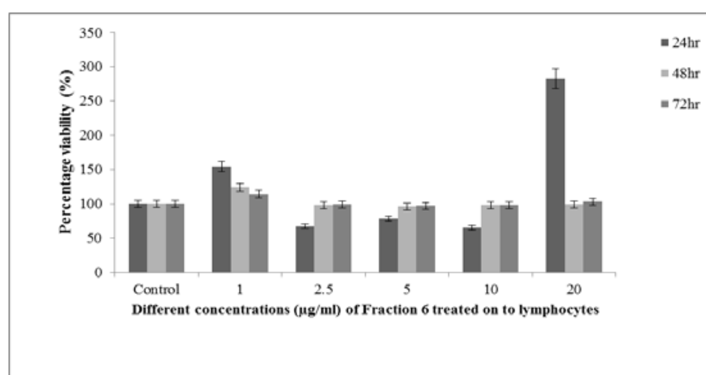

Fig. 4: Percentage viability of lymphocytes treated with the bioactive fraction 6 of mango seed extract

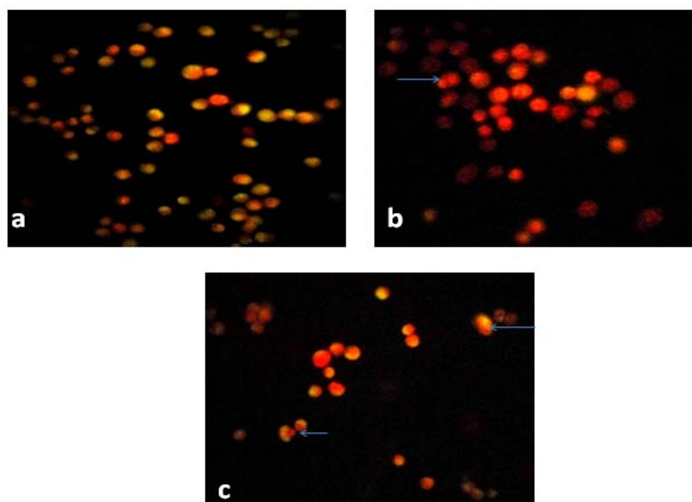

Fig. 5: Fluorescence Microscopic photographs. 5(a) Control HeLa cells 5(b) HeLa treated with 10 µg/ml of bioactive fraction 6. 5(c) HeLa cells treated with 20 µg/ml of bioactive fraction 6. Arrows indicate the breaking up of the nuclei in apoptotic cells. The treated cells are bright orange in color and fewer in number as compared to the control HeLa cells which are greenish in color and more in numbers

Ethidium bromide/acridine orange staining

Staining with EB/AO of HeLa cells treated with the bioactive fraction from the mango seed extract, showed viable cells greenish orange in color (fig. 5a) with intact nuclei and the non-viable cells were bright orange (fig. 5b & 5c). Apoptosis was visible by the appearance of breaking up of the nuclei. Also, there was fewer number of cells in the treated flask as compared to the controls which is clearly seen in the fluorescence microscopic photographs. The effect of treatment with the bioactive fraction was more profound on HeLa cells rather than the CHO cells (results not shown).

Caspase-9 activity assay

The effect of fraction from mango seed extract on the activity of initiator caspase-9 is shown in fig. 6. The fraction was found to significantly increase the activity of caspase-9 in HeLa cells treated for 48 hrs, rather than the CHO cells. There was a 2-fold increase of caspase activity in the treated HeLa cells as compared to the control

HeLa cells or the CHO cells, confirming the apoptotic induction of cell death in the cervical cancer cell line HeLa.

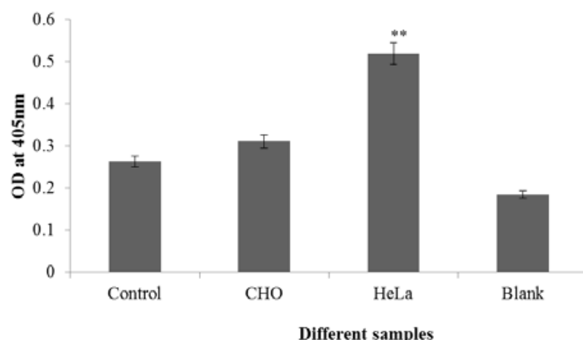

Fig. 6: Comparision of Caspase-9 levels between Control and samples. **denotes 0.01 level of significance

Cell cycle analysis

The bioactive fraction 6 of mango seeds demonstrated anticancer activity by inhibiting the growth cycle of an immortal cancer cell line HeLa. The addition of the bioactive fraction resulted in decreasing the total percentage of viable cells to 42.7% with the cell population in the G_0/G_1 phase at 28.98% (fig. 7), S phase at 2.11% and G_2/M phase with 4.72% when assessed after 24 h of treatment. There were significantly fewer cells in the S and G2/M phases as compared to the controls.

LDH cytotoxicity assay

The cytotoxicity, as assessed by LDH release by the HeLa cells treated with 10 µg/ml of fraction 6 for 24 hrs, was 37% (fig. 8) when compared with that of the positive control i.e., 1% Triton-X 100, which could induce all of the HeLa cell death through cytotoxic effects. This indicates that the bioactive fraction has released LDH when added to the HeLa cells, and LDH release assays are an appropriate and possibly preferable means of measuring cellular cytotoxic reactions.

LC-MS Analysis

The bioactive fractions separated by TLC and identified by bio-assay were further characterized by LC-MS analysis. When the bioactive fraction 6 was subjected to LC-MS, there were two peaks, one small peak at a retention time of 4.07 mins and a second larger peak at a retention time of 6.72 mins, and this larger peak has shown a mass to charge ratio of 701.73 by mass spectrometric (MS) analysis (fig. 9) with a base peak value of 680. This molecular weight is not corresponding to that of mangiferin, which is a well-known bioactive compound isolated from various parts of the mango plant and also proven to have anticancer property on to several cancer cell lines [23, 32].

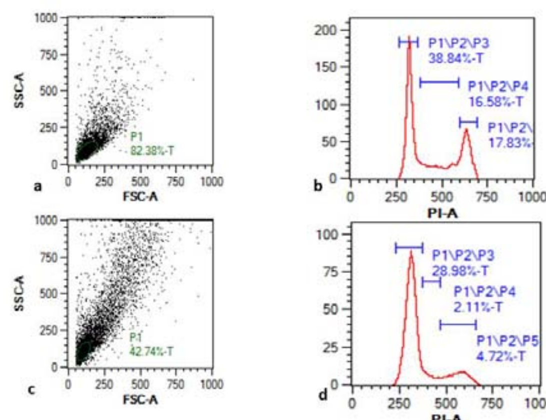

Fig. 7: Cell cycle analysis of the HeLa cells. 7a&b: control HeLa cells. 7c&d: HeLa treated with fraction 6

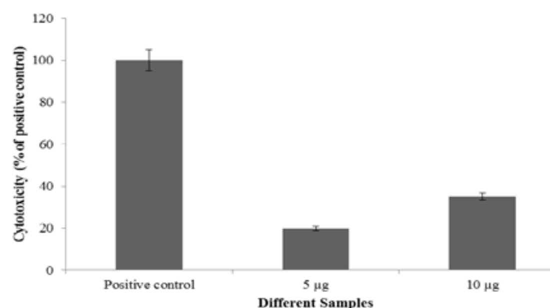

Fig. 8: Cytotoxic effects of fraction 6 of mango seed extract determined by LDH leakage experiment using the cytotoxicity detection kit

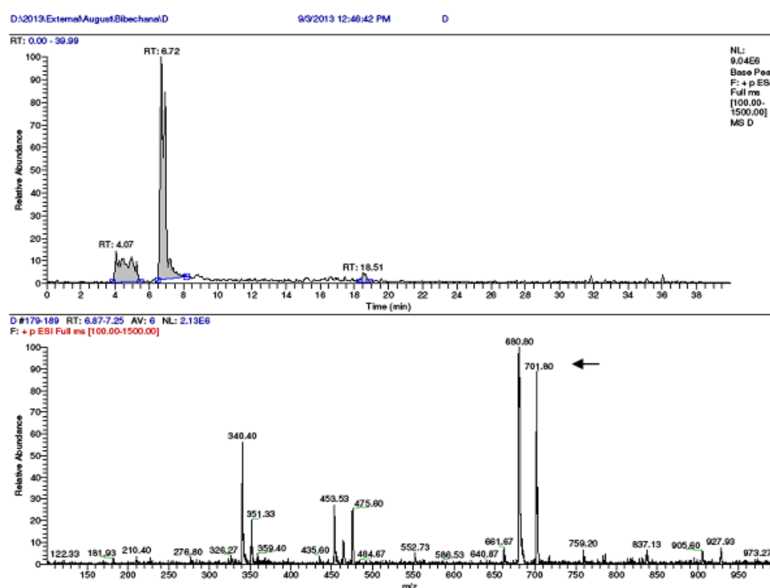

Fig. 9: LC-MS analysis of TLC purified fraction 6 of mango seed extract.
Upper panel: HPLC chromatogram of fraction 6 showing 2 peaks, one at RT 4.07 min. and second larger peak at RT 6.72 min.
Lower panel: LC-MS spectra of the second fraction, m/z 701.8

DISCUSSION

Recently much attention has been given to phyto chemicals and the distinctive roles they play in anti-inflammatory and anti-cancer properties related to the consumption of fruits and vegetables [33]. Mangoes (*Mangifera indica* L.) are one of the most important tropical foods. It is a rich source of nutritive and nonnutritive compounds, including ascorbic acid, carotenoids, and polyphenols. In theory, these phytochemical compounds, contribute to disease-risk reduction [34]. During mango processing, one of the main by-products is the seed. The seeds are not currently utilized for any commercial purpose and are discarded as a waste. Mango seeds contain significant amounts of polyphenols including mangiferin, catechin, flavonoids, epicatechin, and tannins [26]. Mango seed extract has been shown to have antioxidant properties [35] reduced adipogenesis [27], but anticancer activity was not reported. By *in vitro* cytotoxicity assay, mango leaf extract, stem-bark extract, pulp extract and peel extract have exhibited potential anticancer properties and inhibited proliferation of multiple cancer cells [36, 33]. Animal studies have demonstrated that the flavonoid mangiferin isolated from different parts of the mango tree (like leaves, bark, fruit pulp and peel) exhibits anticancer activity in animal models [19, 20]. Ahmed *et al.,* [26] have studied and quantified the different polyphenols present in various parts of the mango tree. Compared to other parts, seeds of mango were having higher amounts of phenolics. Based on the potent antioxidant activity and its major components of polyphenolic compounds and flavonoids [22], it would be assumed that the bioactive compounds present in mango seed extract might also have anticancer activity against cancer cells. But to the best of our knowledge, a systematic study involving the mango seeds, which is a by-product of mango food processing industries, as a potential source for the isolation of anticancer compound was found lacking. In this study, we demonstrated that the ethanol extract of mango seed had anti-proliferative effects to the cervical cancer cell line with an IC_{50} of 25 µg/ml and the flavonoid bioactive compound (fraction 6) isolated from the ethanol extract also had strong dose and time dependent anticancer activity against the HeLa cell line with an IC_{50} of 10 µg/ml.

Cancer cells cultured *in vitro* are valuable means for rapid screening of potential anticancer agents as well as for elucidation of their mechanism of activity. On analysis of the treated HeLa cells for caspase 9 enzyme, we could demonstrate that the bioactive compound not only had strong inhibitory effect on the proliferation of the cervical cancer cell line HeLa, but did so by inducing apoptosis in the treated cells as evidenced by the enhanced level of caspase 9 enzyme activity in them, without any untoward toxic effects on the normal human lymphocytes. Further, more experimental studies on *in vivo* mice models are required to confirm its anticancer activity.

The anticancer activity of the bioactive fraction 6 from the mango seed extract might result partly from inhibition of DNA synthesis (as evidenced by the results of the flow cytometry), as well as apoptosis induction of cancer cells (as evidenced by fluorescence microscopic observation and increased level of caspase enzyme activity) and partly from direct cytotoxic effects (as evidenced by LDH cytotoxicity assay results). Cell death, due to necrosis (caused by cytotoxic effect) and apoptosis, was involved in fraction 6 treatment. The cytotoxic effects were demonstrated by LDH production assay. The bioactive fraction of mango seeds at a concentration of 10 µg/ml was shown to have about 37% of cytotoxicity of Triton X-100 (1%), which can cause almost all cell death through the cytotoxic effect.

The potential anticancer activity of ethanol extract of mango seed was investigated in this experimental study for the first time, and in our study we found that mango seeds can serve as a promising natural source for the development of an anticancer lead molecule with lesser side effects.

CONCLUSION

In conclusion we can say that the ethanol extract from the seeds of mango (*Magnifera indica*) and the bioactive fraction isolated from the extracts have exhibited strong inhibitory effects on the proliferation of HeLa and moderate toxicity to CHO cells, without much adverse effects on the survival of normal human lymphocytes. This experimental study suggested that the bioactive fraction has potential anticancer properties and would be useful for anticancer drug discovery. The LC-MS analysis of the bioactive fraction revealed that the mass/charge ratio (701.8) does not correspond to any one of the earlier reported phyto-constituents with anticancer activity from this particular plant species. However, these findings warrant extensive studies on the chemical profiles and mechanistic action of anti-proliferative and anticancer activities of the bioactive fraction from the mango seeds and such studies are currently underway in our lab.

CONFLICTS OF INTEREST

We declare that we have no conflicts of Interest

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