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Original Research Article

Quantification of rutin and quercetin by HPTLC/HPLC and *in vitro* immunomodulatory and anticancer activities of *Capparis moonii* fruits extracts

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ABSTRACT

Background: The current research was undertaken on dried fruits of *Capparis moonii* to screen its potential for immunomodulatory and cancer indications with identification of phytoconstituents by chromatographic techniques.
Methods: Methanolic (MECN), hydro-methanolic (HMECN) and aqueous extracts (AQCN) of *Capparis moonii* were subjected to high performance thin layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) after studying the total phenolic and flavonoid content by using rutin and gallic acid as standards respectively as well as undertaking powder characteristics and preliminary phytochemical screening. Immunomodulatory activities covered were hemagglutination antibody titre and delayed-type hypersensitivity reaction with the aid of sheep red blood cells (0.5×109) as antigens. The extracts were studied for antioxidant potential. Anticancer prospects were focusing on in vitro cell lines screening (MCF 7 and HCT 15) by Sulforhodamine B assay method and potato disc assay.
Results: The total phenolic and flavonoid content of MECM, HMECM and AQCM fruits extracts were found to be 0.20, 0.11 and 0.47 mg of gallic acid/g and 78.3, 18.8 and 64.4 mg of rutin/g respectively. Rutin and quercetin were confirmed by HPTLC and HPLC showing well resolved peaks. IC50 values in antioxidant studies were found to be significant with all the extracts. Significant immunomodulatory effect was noticed at 200mg/kg in both models (high antibody titre levels and decrease paw volume after 48 h). Unsatisfactory results were observed with selected cell lines and disc assay.
Conclusions: Thus, selected fruits may probably have immunomodulatory potential due to presence of flavonols (rutin and quercetin).

Keywords: *Capparis moonii*, Delayed-type hypersensitivity, Haemagglutination antibody titre, Potato disc assay, Sulforhodamine B assay method

INTRODUCTION

Medicinal plants having ethnopharmacological relevance are been used since decades for treatment due to their phytoconstituents. Synthetic molecules pose multiple problems even leading to death.[1]

Witnessing the thrust area of immunology as the emerging field of research, the team has propagated the work in this paper.

Secondary immunodeficiencies are consequences caused by cytotoxic drugs, malnutrition, infections and metabolic disorders resulting in specific and nonspecific immunity *e.g.* cancer, AIDS *etc.*[2] Cancer is a disease characterized by uncontrolled multiplication of normal cells. As per Indian population census, rate of mortality due to cancer was alarming with about 8,06,000 existing cases by the end of last century. Developed countries have now changed their paradigm towards exploring herbal medicine.[3] As one-third population actually lack access to essential

lifesaving drugs, herbal therapies could provide a major breakthrough.

In Ayurvedic, plants considered under family Capparidaceae have been known for their immense rasayana property. Few of the widely studied plants are *C. sepiaria*, *C. spinosa*, *C. tomentosa*, *C. zeylanica* and *Capparis moonii* (CM). CM has been regarded as *Rasayani*, *Shoshghani*, *Jara Vinashnam* and *Rajyakshma* Shasyate for its therapeutic usefulness. The components present in the fruits are β-sitosterol, stachyhydrin, chebulinic acid derivatives and gallotannins. It has been reported for antitussive, antibacterial, hepatotoxicity and *in vitro* immunomodulatory activities.[4]

The selected family of plants is used in tuberculosis, rheumatism, cancer and diabetes but still elaborative research is required in other pharmacological fields.[5] In purview of above literature, the research group has attempted to study CM fruits extracts for their immunomodulatory and anticancer effects with supported phytochemical quantification of flavonols by UV, HPTLC and HPLC (Figure 1).

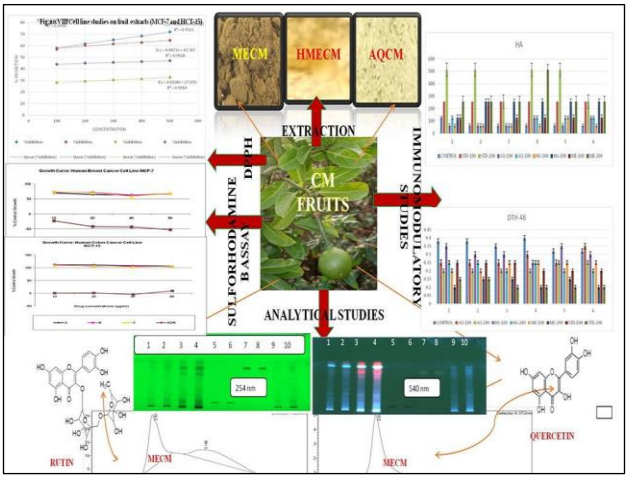

Figure 1: CM fruits extracts graphical abstract.

METHODS

Collection, authentication and extraction of CM fruits

The fruits of the healthy shrub were purchased from local market, Mumbai in May-June and dried in shade. The plant was authenticated at the Agharkar Research Institute, Pune, India. [Voucher specimen no. 138/15]. The dried fruits were grounded into coarse powder using a grinder which was subjected to three different extraction procedures (MECN, HECM and AECM) mentioned below and were stored at 2-8°C. The dried extracts were subjected to powder characteristics, flavonoid and phenolic estimation, TLC (Thin layer Chromatography), HPTLC (High Performance Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatography) quantification followed by immunomodulatory and cell line studies.

Methanolic extract of Capparis moonii (MECM)

The powder was macerated in an extraction bottle using methanol (100g in 500ml), kept in dark place at room temperature for 10-15 days with intermittent shaking. On the 16th day, the extract was filtered, and the residue was collected was dried at 60ºC on water bath in evaporating dish.

Hydromethanolic extract of Capparis moonii (HMECM)

The powder was extracted by soxhlet extraction at 55ºC with methanol and water (60:40). 50g pouch in 200ml was suspended in soxhlet thimble. The extract obtained in round bottom flask was filtered, evaporated and dried at 75ºC on water bath in evaporating dish.

Aqueous extract of Capparis moonii (AECM)

The powder was extracted at 100 ºC on water bath with demineralized water (50 g suspended in 500 ml). The extract was filtered with sintered glass filter, dried by applying vacuum pressure at 100 psi.

Determination of powder characteristics

CM fruits extracts were subjected to total ASH value, acid insoluble ash, water soluble extractive, ethanol soluble extractive, total solid content and loss on drying.[6]

Preliminary phytochemical screening

CM fruits extracts were studied for preliminary phytochemical screening.[7]

Total flavonoid and phenolic estimation

The amount of total phenolic and flavonoid contents in the CM fruits extracts were measured using rutin and gallic acid as standards respectively.[8] Results were expressed as rutin and gallic acid equivalents in mg/gm.

Analytical studies

TLC

In CM fruits extracts, mobile phases of methanol/chloroform/glacial acetic acid (1.5:2.5:1) and methanol/glacial acetic acid/formic acid/water (3:1.5:0.9:0.5) were used for identification of rutin and quercetin respectively. The standard and extract samples were dissolved in the methanol (10mg in 1ml) and were filtered through Whatman filter paper no.1 before spotting on the plates, exposed to ammonia and visualized under UV lamp.[9,10]

HPTLC

Standard (7mg/7ml of rutin and 5mg/5ml of quercetin) and fruit extracts (200mg/5ml) were dissolved in methanol. Quantification was done by application of the spots on HPTLC plate which were coated with silica gel (plate size of 20.0 x 10.0cm). The mobile phase used was ethyl acetate/formic acid/glacial acetic acid/water (10:0.5:0.5:1.3). The plates were developed at 254nm under UV visible wavelength but well resolved spots with images were obtained only after derivatization at 540nm. The details of the instrument used in studies was CAMAG LINOMAT 5 with 5 application parameters, inert gas (nitrogen) as spray gas, methanol as sample solvent with dosage speed 150nl/s and pre-dosage volume 0.2µl. Syringe size was 100µl with 10 tracks, 8mm application position and 8mm band length. The calibration parameters used was calibration mode multilevel, with CV statistics mode and the evaluation mode was based on peak areas. The results were calculated using single point calibration method for CM fruits extracts. The studies were undertaken at Anchrom Test Lab Pvt. Ltd., Mumbai.

Formula: Response factor = peak area of standard/Standard amount

HPLC

CM fruits extracts (5mg/5ml) and standard (5mg/5ml) were dissolved in methanol. Mobile phases used were water/acetonitrile/5% glacial acetic acid/methanol (75:10:5:10) and methanol/acetonitrile/phosphate buffer (pH 3) (42.5:42.5:15) for rutin and quercetin respectively. The column dimensions were C-18, 250 X 4.6mm X 5mm with flow rate of 1ml/min, run time of 10 min and injection volume of 20µl. Results were analysed by calibration curve, single point calibration method.

Formula: % of constituent =

$$\frac{\text{Area of sample x standard dilution x purity}}{\text{Area of standard x sample dilution}} \times 100$$

Standard drugs and chemicals

Ashwangandha churna is well known for its immunomodulatory potential in literature.[11] Ashwagandha churna (Dabur, India Ltd.) was used as a standard procured from local retailer supplier. Standard biomarkers (rutin and quercetin) from Sigma Aldrich Private Limited, Mumbai and all the chemicals used throughout the research were procured from local suppliers.

Preparation of antigens, standard and extracts

Fresh blood was collected from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×109 cells/ml for immunization and challenge.[12] Before dosing, CM fruits extracts and the standard were dissolved in 0.5% carboxy methyl cellulose.

Animal studies

Albino Wistar rats of either sex (120-150gm) were procured from Bharat Serums and Vaccines Ltd., Mumbai. Based on the body weight, dosing was done per oral route (*p.o*). The groups in studies are as follows:

- Group I: Served as control (0.5% sodium CMC solution at 1mg/kg).
- Group II, IV and VI: MECM, HMECM and AQCM extracts (100mg/kg).
- Group III, V and VII: MECM, HMECM and AQCM extracts (200mg/kg).
- Group VIII and IX: Ashwagandha churna (100mg/kg and 200mg/kg).

Experimental conditions

Rats were maintained under 12 h of light and dark cycles at temperature 22±1°C and relative humidity 65±10%. They were housed in groups of 3 in rectangular cages made from plastic Pyrex with paddy husk bedding and fed with standard pellet food. The animals were fasted approximately 16 h before study with free access to water.

Acute toxicity studies

The studies were undertaken as per OECD guidelines 423.[13] Female rats were administered a single dose at 2000mg/kg. The following conditions were recorded.

Mortality/viability/clinical signs/body weights

It was recorded for first 30 min and at 1, 2, 3 and 4 h after administration of CM fruits extracts on test day 0 (in common with the clinical signs) and twice daily during the acclimatization period (at least once on day of sacrifice). Body weights were determined on test days 0 (prior to administration) and on day 7 and day 14.

Animal models[3,14,15]

Hemagglutination antibody (HA) titre

Immunization was induced by 0.1ml of SRBCs suspension containing 0.5×109 cells intraperitoneally on day 0. Control, CM fruits extracts and standard were given orally for 7 days. On the 7th day, by retro-orbital puncturing technique, blood samples were collected in micro-centrifuge tubes, followed by centrifugation and the serum was pooled from each group. A two-fold serial dilution of pooled serum samples were made in 25µl volumes of normal saline in microtitration plates and to it was added to 25µl of 1% suspension of SRBCs in saline. Hemagglutination was observed under the microscope after incubation of the plates at 37°C for 1 h. Reciprocal of

the highest dilution of the test serum agglutination was treated as antibody result.

Delayed-type hypersensitivity (DTH) response[3,14,15]

Right hind foot pad was encountered with 0.5×10^9 SRBCs cells. After +24 and +48 h of this treatment period, foot thickness was monitored with vernier calipers.

Control, CM fruits extracts and standard were administered from 0 to 7th day. The treated foot measurement was done on 7th (prior to injection), 8th and 9th day. Difference between prior and post injection footpad thickness was noticed as DTH response.

Statistical analysis

The values were expressed as mean±SEM and *p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001 by applying one-way analysis of variance (ANOVA) followed by Dunnett test using Graph Pad Prism version of 6.

Antioxidant studies

CM fruits extracts (0.2ml) were diluted with methanol and 2ml of DPPH (1-Diphenyl-2-picrylhydrazyl) solution (0.5mM) were added. After 30 min, the absorbance was measured at 517nm. A graph of percent inhibition v/s concentration was plotted.[16]

In vitro screening of the extracts using cell lines[1,17,18]

The cell cultures and media consist of human colon cancer cell line HCT15, human breast cancer cell line MCF7 and standard drug Adriamycin (Doxorubicin) which were procured and maintained at ACTREC, Kharghar, Mumbai. The cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine.

For CM fruits screening, cells were inoculated into 96 well microtiter plates in 100µL at plating densities, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% with carbon dioxide, 95% air and 100% relative humidity for 24 h prior to addition. The extracts were initially solubilized in dimethyl sulfoxide at 100mg/ml, diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1mg/ml) was thawed and diluted to 100, 200, 400 and 800µg/ml with complete medium containing the CM fruit extracts. Aliquots of 10µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90µl of medium, resulting in the required final drug concentrations i.e. 10, 20, 40 and 80µg/ml.

After addition of CM fruits extracts and standard, the plates were incubated at standard conditions for 48 h and the assay was terminated by addition of cold TCA (trichloroacetic acid). Cells were fixed in situ by the gentle addition of 50µl of cold 30 % (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells and the plates were incubated for 20 min at room temperature.

After staining, the unbound dye was recovered, and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10mM trizma base and the absorbance was read on a plate reader at a wavelength of 540nm with 690nm as reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as:

Formula: % growth = Ti/C x 100

By using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)]

Potato disc assay method on CM fruits[19]

Russet potatoes were obtained from local grocery store. They were washed, peeled and immersed in sodium hypochlorite for 2 min. Different concentration of standard and CM fruits extracts were prepared. 5% DMSO was used as control solution. 1ml of 1.5% agar solution was transferred into a culture plate. Potato disc were kept in center of culture plate submerged nearly 2/3rd in agar solution. By using sterile micropipettes bacterial (*Agrobacterium tumefaciens*) solution, standard or control solutions were mixed, and each potato disc was incubated after applying 1 drop of test or control solution. After 7-21 days potato discs were analysed by using Lugol's solution.

RESULTS

Percent yield of the extracts

The maximum extraction yield of dried CM fruits extracts (MECM, HMECM and AQCM) was found to be 20, 32 and 38 % w/w respectively.

Powder characteristics of extracts

The total Ash value, acid insoluble ash, water extractive value, ethanol extractive value, total solid content and loss on drying for CM fruits extracts were found to be 6.4, 0.5, 26.5, 9.4, 26.6 and 15.4 % w/v respectively.

Preliminary phytochemical screening

CM fruits extracts were found to be present positive for flavonoids, steroids, alkaloids, saponins, glycoside, tannins and phenolic compounds, fats and oils.

Total phenolic and flavonoid content

The total phenolic and flavonoid content of MECM, HMECM and AQCM fruits extracts were found to be 0.20, 0.11 and 0.47mg of gallic acid/gm and 78.3, 18.8 and 64.4mg of rutin/gm respectively.

Table 1: HPTLC analysis on CM fruits extracts.

Track No	Application position	Application volume	Details of standard and sample	Maximum R_f value	Area
4	73.1 mm	20.0 µl	MECM (quercetin)	0.78	9785.2
2	37.7 mm	20.0 µl	HMECM (quercetin)	0.80	2863.9
9	161.6 mm	10.0 µl	AQCM (quercetin)	0.80	2319.2
8	143.9 mm	2.0 µl	Standard quercetin	0.78	10599.5
5	90.8 mm	2.0 µl	Standard rutin	0.09	5844.5
4	73.1 mm	20.0 µl	MECM (rutin)	0.12	3288.2
2	37.7 mm	20.0 µl	HMECM (rutin)	0.09	3106.1
9	161.6 mm	10.0 µl	AQCM (rutin)	0.09	1754.9

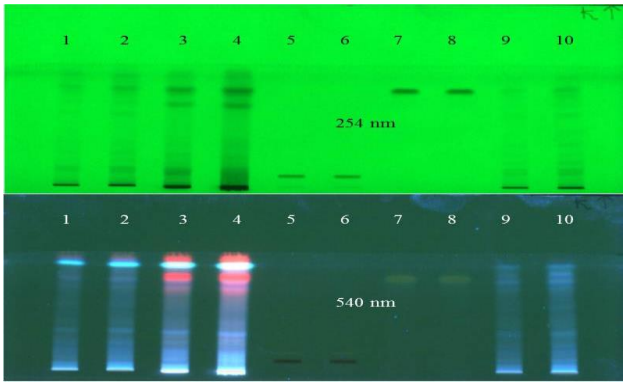

Figure 2: HPTLC analysis on CM fruits.

Analytical studies

TLC

R_f values were found to be 0.79 (MECM), 0.77 (HMECM) and 0.74 (AQCM) in comparison to the standard rutin

HPLC

having 0.79. Quercetin was found to have R_f values of 0.63 (MECM), 0.66 (HMECM) and 0.66 (AQCM) in comparison to the standard 0.64 for CM fruits extracts.

HPTLC

CM fruits extracts has shown well resolved spots at tracks 1-4 and 9, 10 in comparison to standard rutin (tracks 5, 6) and quercetin (tracks 7, 8). Maximum R_f value was found to be MECM (0.14), HMECM (0.12) and AQCM (0.12) in comparison with standard rutin at 0.09. The results were found to be 8.4, 3.6 and 2.8 % w/w respectively. Maximum R_f value of MECM (0.80), HMECM (0.78) and AQCM (0.80) was found comparable with standard rutin at 0.78. The results were found to be 0.8, 1.4 and 1.1 %w/w respectively (Table 1 and Figure 2).

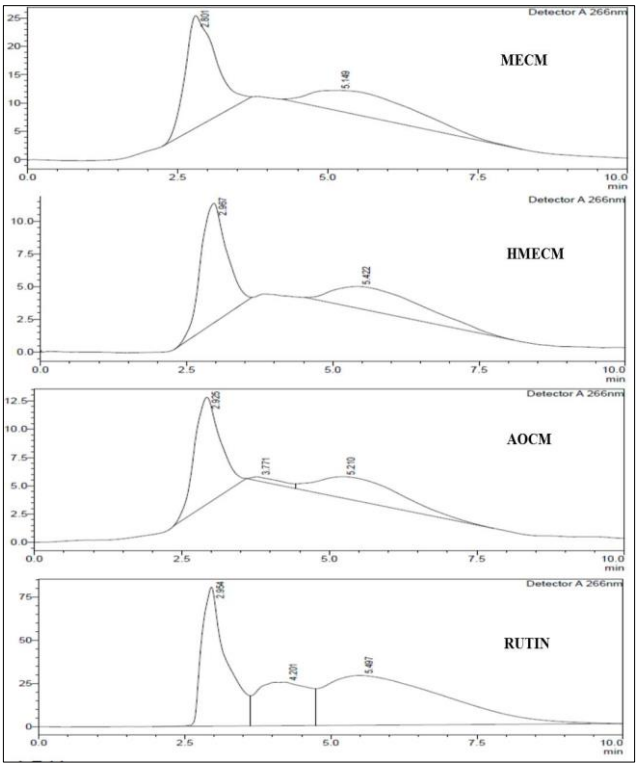

Figure 3: HPLC analysis on CM fruits extracts and standard rutin.

CM fruits extract at 266 nm showed well resolved peaks at retention time in minutes of MECM (2.801, 2.669), HMECM (2.967, 2.804) and AQCM (2.925, 2.669) in comparison to rutin (2.954) and quercetin (2.680) at 372 nm. Quantitative amounts of standards and extracts were found to be MECM (45.2, 8.6), HMECM (21, 8.9) and AQCM (31.2, 7.4) respectively in % (Figure 3, 4).

Acute toxicity

Mortality/Viability/Clinical signs/Body weights

CM fruits extracts were reported therapeutically safe up to 2000mg/kg B.W. and all rats had gained body weight by day 14 as compared to day 0.

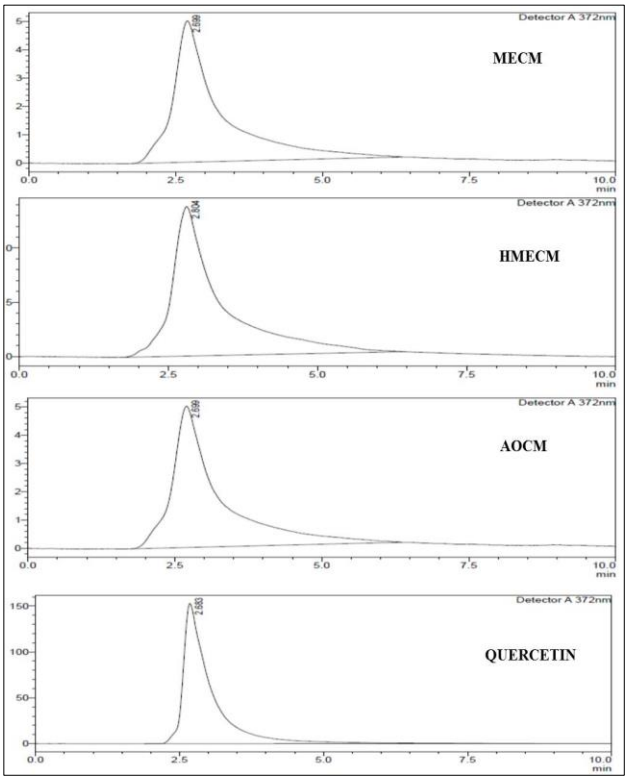

Figure 4: HPLC analysis on CM fruits extracts and standard quercetin.

Animal models

HA

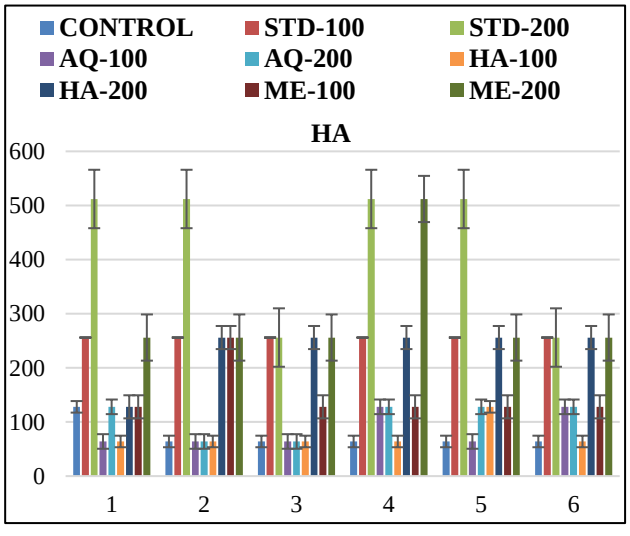

Figure 5: HA titre on CM fruit extracts.

CM fruits extracts at 200mg/kg showed significant activity and no activity at 100mg/kg as comparable to the standard (Figure 5).

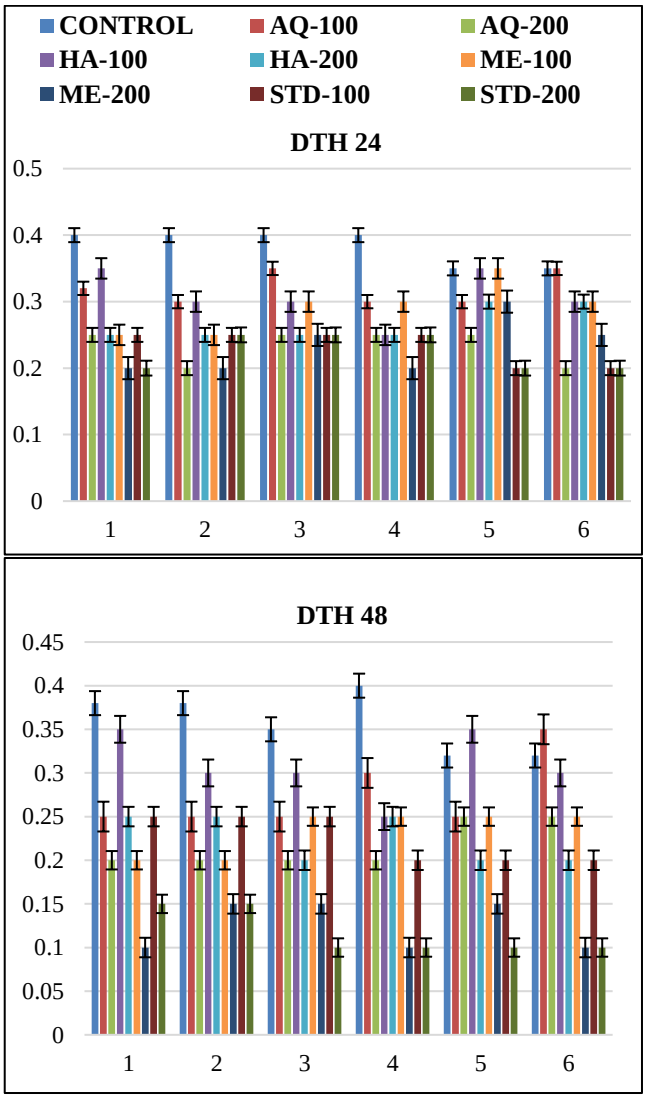

Figure 6: DTH studies on CM fruits extracts.

DTH

CM fruits extract showed dose dependent decrease in paw edema after 48 h of challenge at 100 and 200mg/kg when compared to control group (Figure 6).

Antioxidant activity

CM fruits extract showed IC_{50} value of MECM (0.338), HMECM (0.905) and AQCM (2.122) as compared to standard (0.132) (Figure 7).

In vitro screening of the CM fruits

CM fruit extracts were found to be unresponsive on selected cell lines at different concentrations studied as

compared to standard drug Adriamycin. A, B and C are AQCM, HMECM and MECM respectively (Figure 8).

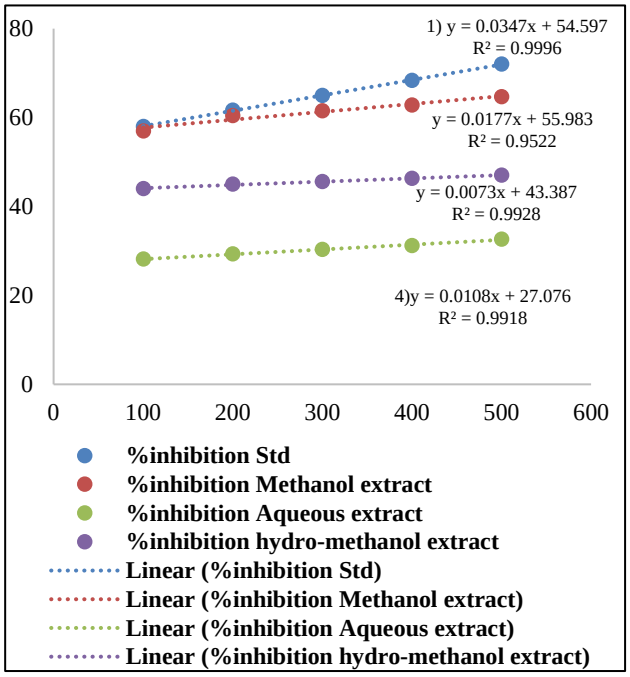

Figure 7: DPPH studies on CM fruits extracts.

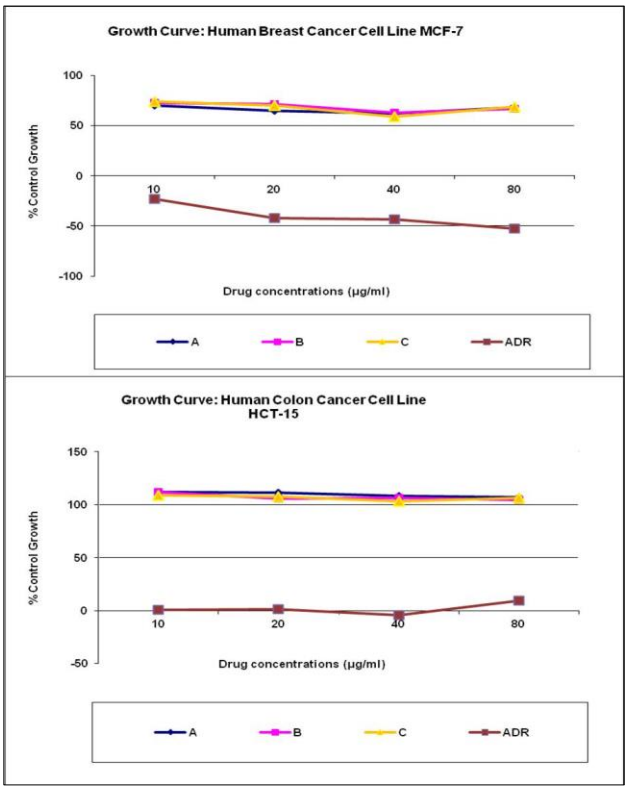

Figure 8: Cell line studies on CM fruits extracts.

Potato disc assay on CM fruits

CM fruits extract were found to be unresponsive on the selected assay method.

DISCUSSION

CM fruit extracts have been found positive for presence of phytoconstituents such as rutin and quercetin identified by combined chromatographic techniques such as HPTLC and HPLC. *In vivo* HA titre and DTH response immuno models were found satisfactory correlating to the above stated phytochemical results. However, an attempt for screening of the fruits extracts by potato disc assay and in vitro cell lines posed negative outcome (Figure 1).

In past research studies, aqueous and ethanolic extracts of dried fruits of *Capparis moonii* fruits showed significant in vitro phagocytic stimulation of lysosomal enzyme, myeloperoxidase activity and nitric oxide in peritoneal mouse macrophages, propagating the surge for in vivo studies.[4] The research in the paper has been one of its first attempts to screen the selected plant extracts (MECM, HMECM and AQCM) for its *in vivo* immunomodulatory potential with phytochemical identification of rutin and quercetin by chromatographic studies.

HPTLC has been regarded as one of the most versatile and result based technique in case of identification of phytoconstituents present in plants.[10,20] In current research studies, CM fruit extracts showed the presence of rutin and quercetin when compared with standard based on maximum R_f value, areas and formula calculations. HPLC has added an insight to the existing list of chromatographic techniques by providing clear view point of peaks compared on the basis of retention time.[10] In HPLC chromatograms, rutin and quercetin in the fruit extracts were interpreted based on its comparison with the standard.

Lymphoreticular system is a complex network of lymphoid cells and reticuloendothelial components. The lymphoid cells (lymphocytes and plasma cells) are basically concerned with the specific immune response. In response to the antigen attack irrespective of its nature, there is an exaggeration of humoral antibody (HAI) or cellular mediated (CMI) immunity. Lymphocytes are small, round cells located in peripheral blood, lymph, lymphoid organs and allied tissues nearby its vicinity area. The cortical follicles and medullary cods contain B lymphocytes whereas between these two regions there is an ill-defined intermediate zone which contains T lymphocytes. Activated T cells produce specific activation products (lymphokines) and initiate CMI and B-cells to divide, converting themselves into plasma cells which synthesize immunoglobulins. Due to the ethical considerations in the current decades, it is not possible to perform investigation of immune system related infections in humans and hence, alternative was to use rats species and SRBC's cells as antigens. B-cells when bind to SRBCs cells coated with antibody and complement leads to EAC rosettes formation due to the presence of C-3 receptor (CR 2) on B cell surface. T cells bind to SRBC's cells forming rosettes by CD2 antigen.[2,3,21-25] High antibody titre values (Figure 5) and marked decrease in the paw volume (48 h)

(Figure 6) correlates to B and T lymphocytes respectively suggesting probable immunomodulatory prospects of CM fruit extracts.

Witnessing the population statistics in India more patients noticed to be under the dilemma of breast and colon cancer. Further breast cancer was more prevalent among women and colon among men.[26-28] Considering these facts, an attempt has was being made to check whether the fruits extracts are having antioxidant potential and if results found to be positive (Figure 7), further screen them for its cancer potentiality. For this purpose, SRB assays were preferred (fluoresces with laser excitation at 448nm) which shows quick, stable, uniform and reliable results (based on single cell principle as compared to MTT assays.[29] The basic reason behind the selection of the cell lines were wide acceptance, utility in research area, hormone sensitivity through estrogen receptor and ability to activate p53 in order to judge the stage of cancer. CM fruit extracts results on selected cells lines (MCF 7 and HCT 15) were found to be unsatisfactory (Figure 8).

CONCLUSION

The present investigation suggests that CM fruits extracts exerts significant immunomodulatory activity.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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