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Preparation and Evaluation of Phytosomes Containing Methanolic Extract of Leaves of *Aegle Marmelos* (Bael)

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Abstract: Lipid compatible molecular complex of extract is called as phytosomes. Phytosome technology is applied to water soluble phytoconstituents tannins, phenols, terpenoids etc.
Phytosomes was prepared by solvent evaporation method. Firstly leaves of *A.marmelos* were extracted with pet ether and then with methanol by soxhlet extraction. Then phytosomes batches were prepared, solvent evaporation method was used for preparation of phytosomes. F3 formulation selected as optimized formulation and futher evaluated it for particle size, digital microscopy, SEM, TEM, FTIR, DSC, XRD analysis. Comparative evaluation of antioxidant, antiproliferative and anticancer activity of extract and phytosome was carried out.
From above studies we are concluded that phytosomes has better physical characteristics as compared to that of methanolic extract of leaves of *A.marmelos*. Phytosomes has nearly same antioxidant, antiproliferative and anticancer activity as that of methanolic extract of leaves of *A.marmelos*.
Keywords : phytosome, bael, lipid, anticancer.

Introduction:

Phytosome-

The Phytosome technology, developed by Indena S.P.A. of Italy. [1] Phytosome is a patented technology including, to incorporate standardized plant extracts or water soluble phytoconstituents into phospholipids to produce lipid compatible molecular complexes. The phytosomes process produces a little cell because of that the valuable components of the herbal extract are protected from destruction by digestive secretions and gut bacteria. Phytosomes are better able to transition from a hydrophilic environment into the lipid-friendly environment of the enterocyte cell membrane and from there into the cell finally reaching the blood. Phytosomes have improved pharmacokinetic and pharmacological parameter. [2] Phytosomes are more bioavailable as compared to herbal extract owing to their enhanced capacity to cross the lipid rich biomembranes and finally reaching the blood. [3]

Soyalecithin (phosphatidylcholine) is a phospholipid. It is key component of phytosome process. Phospholipids are employed as natural digestive aids and carriers for water soluble and lipid soluble nutrients. [4]

Preparation of phytosomes-

Solvent evaporation, mechanical dispersion, salting out, lyophilization methods are used for preparation of phytosomes. [5, 6]

Evaluation of phytosomes-

The phytosomes are evaluated for visualization, vesicle size and zeta potential, entrapment efficiency, DSC, TEM, SEM, vesicle stability, drug content, spectroscopic evaluation.[7]

Plant profile-

Figure 1 Bael plant

Kingdom	**Plantae**
(Unranked)	Angiosperms
(Unranked)	Eudicots
(Unranked)	Rosids
Order	Sapindales
Family	Rutaceae
Subfamily	Aurantioideae
Tribe	Clauseneae
Genus	*Aegle*
Species	*A.Marmelos*

Table 1 Scientific classification

Chemical constituents-

Coumarins, alkaloids, tannins, phenolic compounds, carotenoids, oils, etc. [8]

Pharmacological activity-

Antidiabetic, antiulcer, antiviral, antibacterial, antifungal, antidiarrheal, antidysentric, demulcent, antipyretic, astringent, anticancer, etc. [9]

Material and method:

Material-

Plant- Leaves of bael (*A.marmelos*) collected from Chikalthana area, Aurangabad.

Solvents- Pet ether, methanol, chloroform purchased from Dipa lab, Aurangabad.

Chemicals- Soyalecithin (phospholipon® 90H as a gift sample from Lipoid pharma, Germany, and Cholesterol purchased from Loba Chem lab.

Method-

Extraction of leaves-

The plant material required for the study was fresh. Tender leaves were harvested as it has 0.15-0.2% yields from the month of November to March. These leaves were kept in sunlight and the dry leaves were powdered and stored.

The leaves (2 kg) were dried on the laboratory bench for 10 days. The dry sample was milled and ground into powder (1.3 kg). The powdered plant sample (1kg) was packed into a soxhlet apparatus and extracted firstly with petroleum ether to remove fatty material and chlorophyll for 12hrs. Then after that marc was collected and placed it for complete removal of petroleum ether and again marc was extracted with methanol using same soxhlet apparatus assembly.[10]

Preparation of phytosomes-

Procedure-

Accurately weighed quantity of phosphatidylcholine and cholesterol were dissolved in 10 ml of chloroform in round bottom flask (RBF) and sonicated for 10 min using bath sonicator. Organic solvent removal is done by Rotary evaporator (45-50°C). After complete removal of solvent thin layer of phospholipids

mixture was formed. This film was hydrated with methanolic extract of bael leaves in rotary evaporator (37-40°C for 1 hour). After hydration, mixture of lipid and plant extract was sonicated for 20 minutes in presence of ice bath for heat dissipation. Then prepared phytosomes were filled in amber colored bottle and stored in freezer (2-8^{0}C) until used. [11]

Table 2 Composition of phytosome formulation of ME bael leaves

Formulation Code	Chloroform (ml)	ME (ml)	CL:PL ratio	Sonication time (min)
F1	10	10	1.5:4	5
F2	10	10	1.5:4.5	10
F3	10	10	1.5:5	15
F4	10	10	1.5:5.5	20
F5	10	10	1.5:6	25
F6	10	10	1.5:6.5	30
F7	10	10	1.5:7	35

Evaluation of phytosomes-

a. Visualisation-

The morphology of phytosomes was observed by digital microscopy, transmission electron microscope and scanning electron microscope.

i. Digital microscopy-

Phytosome formulation shaken in distilled water and viewed under digital microscope at 400X objective lens.

ii. TEM analysis-

The complex was shaken in distilled water and viewed using Transmission Electron Microscope (Hitachi, Japan).

iii. SEM analysis-

Approximately 5 µL of the phytosomal suspension was transformed to a cover slip, which in turn was mounted on a specimen tab. The samples were allowed to dry at room temperature. Then the particle size of the formulation was viewed and photographed using Scanning Electron Microscope (Sigma, Carl Zeiss). The particles were coated with platinum by using vaccum evaporator and thus, the coated samples were viewed and photographed in JEOL JSM-6701F Field Emission SEM.

b. Particle size analysis-

Diameter of particles and polydispersity index was noted down by BECKMAN COULTER, Delsa^{TM} Nano. Phytosome formulations were diluted with solvent methanol and then evaluated.

c. FTIR-

FTIR (SHIMADZU, Japan) spectral data were taken to ascertain the structure and chemical stability of extract, PC and phytosome. Spectral scanning was done in the range between 4000 and 500 cm^{-1}.

d. DSC-

Bael leaves extract, phospholipon and phytosome were placed in the aluminum crimp cell and heated at 10^{0}C/min from 0 to 400 ^{0}C in the atmosphere of nitrogen (TA Instruments, USA, Model DSC Q10 V24.4 Build 116). Peak transition onset temperatures were recorded by means of an analyzer.

e. XRD analysis-

XRD was done on pure extract, PC and phytosome to see the crystallinity in the substance. Sample was scanned in the angular range of 5^0 - 80^0 in a PHILIPS XPert Pro X-Ray Diffractometer. Dried powder sample was kept in sample holder (20 mm × 15mm × 2mm) which was fitted into the instrument and X-ray was passed through the sample. The powder X-ray diffraction patterns of extract, PC and phytosome are shown in figure.

f. *In-vitro* evaluation-

i. Antioxidant activity:

The ability of the compound to scavenge H_2O_2 was determined using spectrophotometric method by measuring the absorption with extinction coefficient for H_2O_2 of $81M^{-1}cm^{-1}$. For assay 40mM solution of H_2O_2 was prepared in phosphate buffer (pH 7.4). Solutions of ME of leaves of *A.marmelos* at different concentrations (10µg/ml to 100µg/ml) were prepared in phosphate buffer. H_2O_2 solution (40mM) 0.6ml added to solutions of extract. Absorbance of all solutions determined at 230nm after 10min incubation against a blank solution containing phosphate buffer without H_2O_2. Ascorbic acid was taken as reference. Same procedure was repeated for phytosomes.

The percentage of scavenging of H_2O_2 was calculated by following equation: [12]

$$\%\ scavenged = \frac{AB - AA}{AB} \times 100$$

Where, AB- Absorbance of blank, AA- Absorbance of sample

ii. Antiproliferative activity:

Preparation of yeast inoculums

Yeast was inoculated in a conical flask containing 100 ml sterilized nutrient broth and incubated at 37°C for 24hrs. This was referred as seeded broth. 1ml of seeded broth was taken and diluted with sterilized distilled water to contain 25.4 x 10^4 cells.

Preparation of potato dextrose broth

The sliced potatoes (200g) were boiled in 1L of distilled water for 1 hour and then filtered through muslin cloth. The volume of filtrate was made up to 1000ml with distilled water and then glucose (20g) was added. The medium was sterilized by autoclaving.

Cell viability count

0.5 ml of yeast inoculum and 2.5ml of potato dextrose broth was treated with each 1 ml of various concentration methotrexate (50ng/ml), ME of leaves of *A.marmelos*(4mg/ml), phytosomes(4mg/ml). It was then incubated for 24 hours at 37^0C with control. This cell suspension was then mixed with 0.1% methylene blue and examined under low-power microscope. The number of viable cells (those transparent, oval shape and do not take stain) and dead cells (those get stained and stained blue) were counted in hemocytometer. The mean was calculated.

The cells per ml and percentage of cell viability were calculated by following formula: [13]

Viable cells/ ml = average no of viable cell in one square x dilution factor x 10^4

Percentage of cell viability = Total viable cells / Total cells x 100

iii. Anticancer activity:

Cytotoxic effect on human breast (MCF7) cell lines by SRB assay-

Method:

The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 10, 20, 40, 80µg/ml of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37^0C for 72 hours in 5% CO2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4^0C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then airdried. 100µl of 10mM Tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540nm. [14]

%cell inhibition = 100-{(At-Ab)/ (Ac-Ab)} x100

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

Note: The samples for anti-cancer activity were tested in TATA MEMORIAL ADVANCED CENTRE FOR TREATMENT, RESEARCH AND EDUCATION IN CANCER.

Results and discussion:

Preparation of phytosomes-

Solvent evaporation method was used. F3 formulation having Cholesterol: PC ratio 1.5:5 was selected as optimized formulation on the basis of morphology and particle size and then evaluated further.

Evaluation of phytosomes-

a. Visualisation-

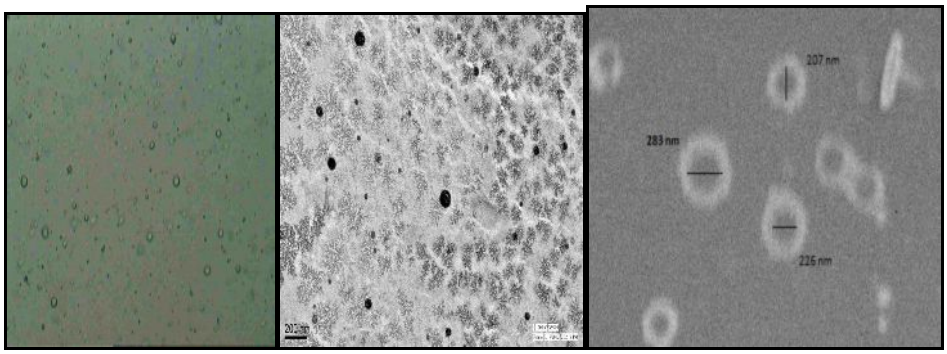


Figure 2 Digital micropic, TEM, SEM view of phytosome

Uniform, regular and rigid vesicles were observed in digital microscopic view. Vesicles between size range of 200nm-300nm were observed in scanning electron microscopic view.

b. Particle size-

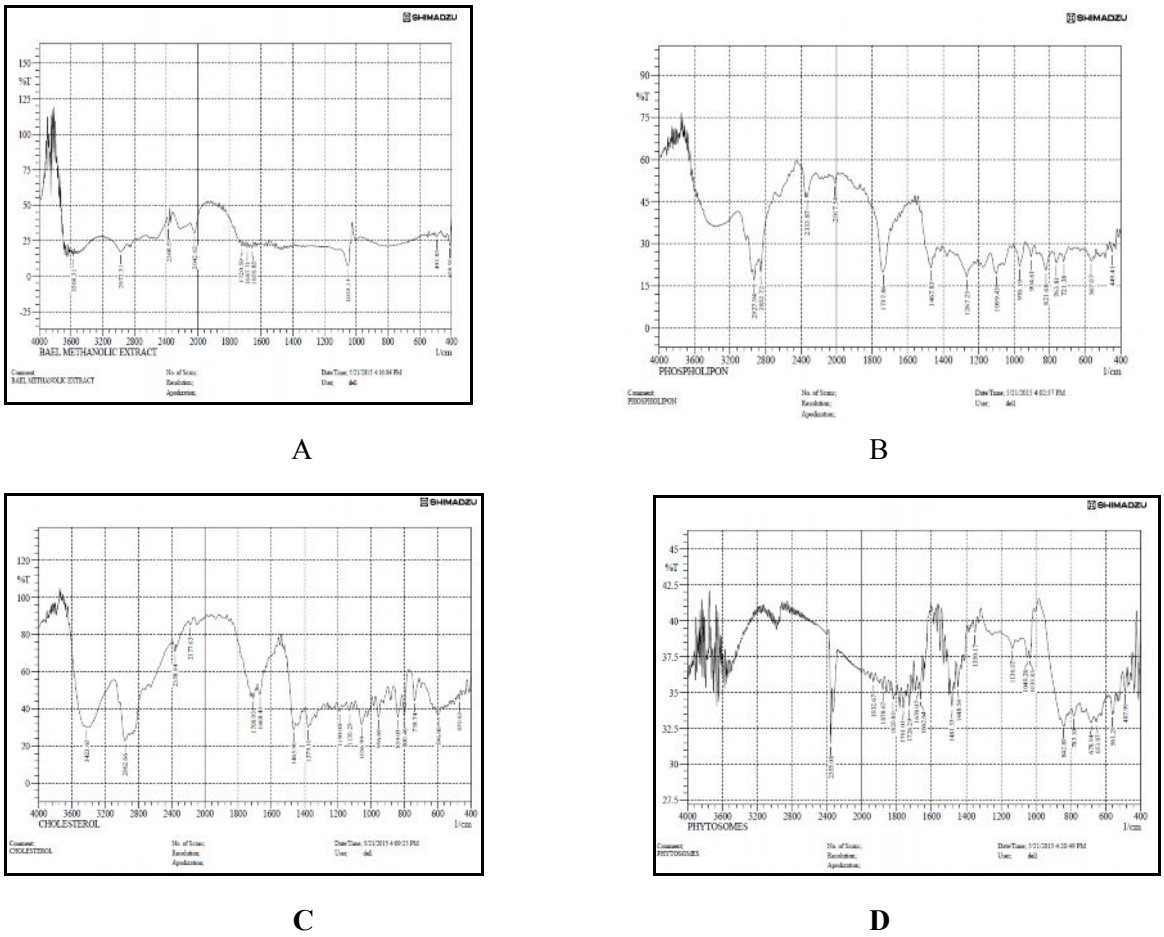
Table 3 Particle size of various batches of phytosomes

Sr. No.	Formulation	Particle size(nm)	Polydispersity index
1)	F1	998.1	0.569
2)	F2	396.6	0.353
3)	F3	212.6	0.337
4)	F4	1570.2	0.456
5)	F5	292.7	0.319
6)	F6	5427.0	0.673
7)	F7	3347.2	0.528

Figure 3 Particle diameter and polydispersity index of F3 formulation.

The average particle size and polydispersity index of optimized phytosome formulation was found to be 212.6nm and 0.337 respectively.

c. FTIR-



A B

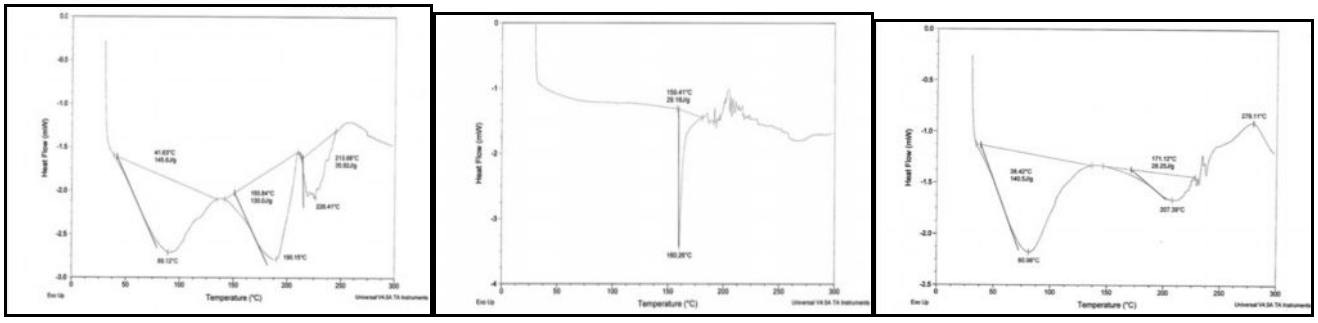
C D

Figure 4 FTIR of A. Extract, B. Soyalecithin, C. Cholesterol, D. Phytosome

- The FTIR spectroscopy revealed shifting of hydroxyl group (OH) to a lower frequency in phytosome spectra (3625cm-1 to 3560cm-1) as compared to spectra of ME of leaves of *A.marmelos*, indicating the formation of strong hydrogen bonding between hydroxyl group of phospholipon and extract phytoconstituents in phytosome form.
- The band of choline N-(CH3)3 groups in phospholipon spectra is shifted to higher frequency in phytosome spectra (1099.43cm-1 to 1136.07-1) with decreased intensity, indicating that the interaction between PL and extract constituents is also at the level of the choline moiety.

d. DSC-

Methanolic extract of bael leaves shows a broad endothermal peaks and its beginning melting point was 41.6^{0}C, 150.84^{0}C and 226.4^{0}C. PC gives endothermal peak at 159.4^{0}C. Phytosome complex gives endothermal peak at 80.9^{0}C and 171.1^{0}C.

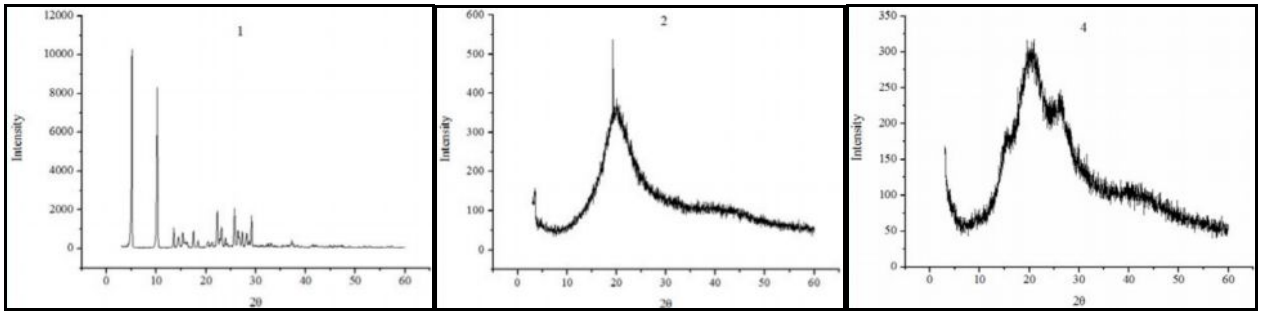


A B C

Figure 5 DSC of A. Extract, B. Soyalecithin, C. Phytosome

e. XRD analysis-

Powder diffraction pattern of extract displayed sharp crystalline peak, In contrast, phospholipid showed an amorphous state lacking crystalline peak. Crystalline peaks of extract had disappeared in the complex.



A **B** **C**

Figure 6 XRD analysis of A. Extract, B. Soyalecithin, C. Phytosome

f. In-vitro evaluation-

i. Antioxidant activity:

Table 4 Comparative H_2O_2 scavenging activity of ME of leaves of A.marmelos and its phytosome

Sample	Concentration (µg/ml) and % inhibition						IC50 value
	10	20	40	60	80	100	
Ascorbic acid	10.70	16.65	20.32	32.92	41.46	51.32	98.97
ME	8.63	17.30	20.63	29.60	31.79	36.23	95.32
Phytosome	8.92	18.13	20.85	31.23	33.21	37.10	96.12

ii. Antiproliferative activity:

Table 5 Comparative % inhibition of cell viability by ME and its phytosome on yeast

Sample	Total number of viable cells per ml(10^6) (unstained)	Total number of cells per ml(10^6) (unstained and stained)	% of cell viability (%)	% inhibition of cell viability (%)
Control	517	531	97.36	2.64
Methotrexate (50ng/ml)	217	531	51.04	48.96
Methotrexate(100ng/ml)	221	531	49.02	49.52
ME(4mg/ml)	276	531	51.98	48.02
ME(5mg/ml)	285	531	51.85	49.57
Phytosome(4mg/ml)	183	531	34.46	52.05
Phytosome(5mg/ml)	189	531	42.06	52.92

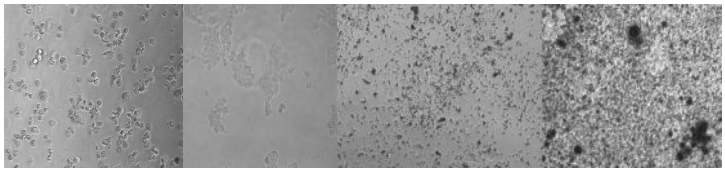
iii. Anticancer activity:

Table 6 Result for % control growth of MCF 7 cell lines by ME and its phytosome

Sample	Concentration(µg/ml) and % control growth			
	10	20	40	80
ADR	-56.3	-49.8	-58.3	-71.5
ME	-39.0	-62.9	-65.4	-67.0
Phytosome	-62.9	-64.3	-62.1	-51.6

Table 7 LC50, TGI, GI50 values of samples

Sample	LC50	TGI	GI50
ADR	3.7	<10	<10
ME	8.1	<10	<10
Phytosome	<10	<10	<10



A B C D

A)MCF 7 positive control, B)MCF 7 control, C)Activity shown by ME, D)Activity shown by phytosome

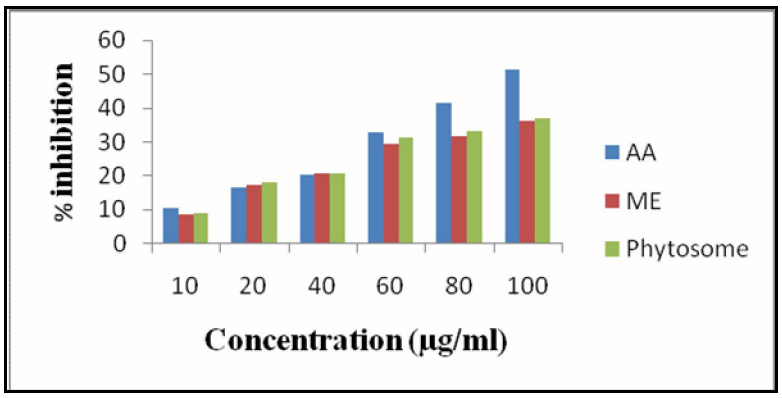


Figure 7 *In-vitro* antioxidant activity of ME of leaves of *A.marmelos* and its phytosome

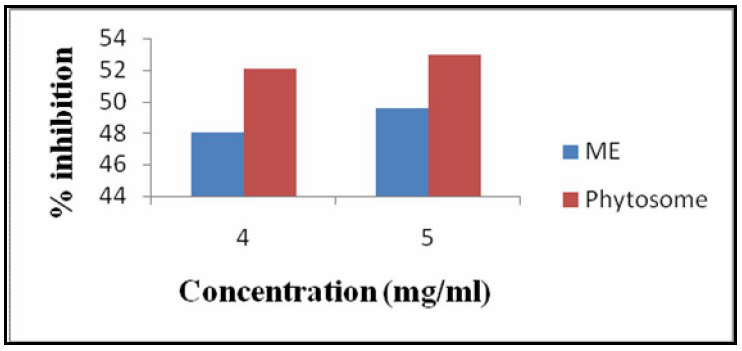


Figure 8 Antiproliferative activity of ME of leaves of *A.marmelos* and its phytosome

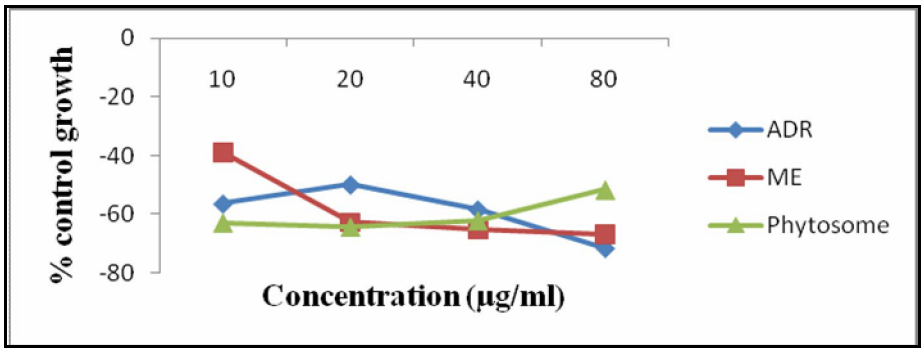


Figure 9 Comparative % control growth by ME of leaves of A.marmelos and its phytosome

Conclusion:

From above studies we are concluded that phytosomes has better physical characteristics than that of extract. In-vitro studies revealed that phytosomes showed same antioxidant, antiproliferative and anticancer activity as that of methanolic extract of leaves of *A.marmelos*.

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