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Evaluation of In Vivo Antitumour Activity of Various Extracts of Fruit of *Punica granatum* against HT 29 Cell Line

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Abstract: The main aim and objective of the present research work was the preliminary phytochemical screening and evaluation of in vivo anti tumour activity of mthanolic and ethyl acetoacetate extracts of fruit of Punica granatum (ME-FPG and EAAE-FPG). Acute oral toxicity of ME-FPG and EAAE-FPG were performed according to the OECD guideline 423 method. The experimental results of acute oral toxicity studies revealed that all the extracts proved to be non toxic at tested dose levels and well tolerated by the experimental animals as there LD_{50} cut of values > 2000 mg/kg b. w. The in vivo antitumor activity of ME-FPG and EAAE-FPG was carried out against human colon cancer cell line HT 29. The present in vivo experimental data displayed that both ME-FPG and EAAE-FPG significantly increased the PILS. While 5-FU increased the life span of 85%, ME-FPG increased it by 75% and 67.5% by EAAE-FPG respectively ME-FPG. So ME-FPG and EAAE-FPG at dose 300 mg/kg significantly improved the overall survival of treated animals in comparison to tumour control group and 5-FU was not significantly differed from each other in improving the overall survival of animals. Histopathological study displayed a greater degree of tumour apoptosis and necrosis in the ME-FPG and EAAE-FPG treated group than in the control group. In addition, PCNA showed that administration of ME-FPG and EAAE-FPG decreased the proliferation of tumour tissues. Proliferating cell nuclear antigen (PCNA) is a DNA clamp that acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication.

Keywords: antitumor activity; LD_{50}; human colon cancer; PILS; apoptosis and necrosis etc.

INTRODUCTION [1]

The development of modern Pharmacognosy took place a simultaneous advancement in the area of Organic chemistry, Biochemistry , Medicinal chemistry, Biosynthesis and modern methods and techniques of analysis like TLC, Paper chromatography, HPLC, UV-Visible, IR, NMR and Mass spectroscopy etc. Thus a wide variety of active principles were isolated from different parts of various plants and established to possess a wide range of pharmacological and antimicrobial activities.

The huge number of chemical substances that are present in the plant kingdom and animal kingdom in one form or the other are termed as constituents. These constituents may be further divided in to two main categories namely: (a) Active constituents: The chemical entities that are solely responsible for existing pharmacological, microbial or in a broader-sense therapeutic activities usually termed as Active constituents. Most drug like alkaloids, glycosides, steroids, terpenoids, bitter principles are the bonafide members of this particular category. (b) Inert Constituents: The chemical compounds though present in plant and animal kingdom. Which do not passess any definite theraputicvalues as such but useful as an adjunct either in the formulation of a drug or in surgery are collectively known as "Inert Constituents". It has been observed that the very presence of 'Inert constituents' either act towards modifying or check the absorbance and the therapeutic index of the active constituents. Obviously to get the right active constituents one has to get rid of the host of 'Inert constituents' by adopting various known method of separation, Purification and crystallization. There fore, most literatures, invariably refer to the former as, Secondary' plant products. The presence of these secondary plant products (Active constituents) are governed by two school of thoughts namely;

Superfluous Metabolites: i.e., substances that have no values as such and perhaps their presence are due to the lack of exceretory mechanism in them and ultimately result as the 'residuai lock-up' superfluous metabolites, and

Characteristic Survival Substances: i.e., substances which exert a positive survival value on the plant wherein they are actually present. They offer more or less a' natural defence-mechanism' where by these host plants are survived from destruction owing to their astringent, odorous and unpalatable features.

Drug Biosynthesis (or Biogenesis): In the recent past, a good deal of well-deserved importance and recognition have been attributed to the exclusive study of the biochemical pathways that precisely lead to the formulation of' active constituents' otherwise referred to as the secondary constituents mostly employed as drugs. This specific study is normally termed as Drug Biosynthesis or Biogenesis. As a 'medicinal chemist' is required to know the synthesis of chloroquine-an anti malarial drug from pure synthetic compounds, a 'phytochemist' is supposed to know the biogenesis of quinine in the cinchona bark. With the advent of isotopically labeled organic compounds known in the early fifties it was quite possible to establish scientifically that the host of amino acids along with their corresponding derivatives more or less acted as precursors of complex alkaloids.

Pomegranate [2-9]: The pomegranate botanical name *Punica granatum* is a fruit-bearing deciduous shrub or small tree growing between 5–8 meters (16–26 ft) tall. The pomegranate is widely considered to have originated in Iran and has been cultivated since ancient times**.** Today, it is widely cultivated throughout the Mediterranean region of southern Europe, the Middle East and Caucasus region, northern Africa and tropical Africa, the Indian subcontinent, Central Asia and the drier parts of southeast Asia. Introduced into Latin America and California by Spanish settlers in 1769, pomegranate is also cultivated in parts of California and Arizona. In the Northern Hemisphere, the fruit is typically in season from September to February**.** In the Southern Hemisphere, the pomegranate is in season from March to May. The pomegranate has been mentioned in many ancient texts, notably in Babylonian texts, the Book of Exodus, the Homeric Hymns and the Quran. In recent years, it has become more common in the commercial markets of North America and the Western Hemisphere Pomegranates are used in cooking, baking, juices, smoothies and alcoholic beverages, such as martinis and wine.

Scientific classification

Kingdom	Plantae
(Unranked)	Angiosperms
(Unranked)	Eudicots
(Unranked)	Rosids
Order	Myrtales
Family	Lythraceae
Genus	Punica
Species	*P. granatum*
Binomial name	*Punica granatum*

Description [10, 11]: The *Punica granatum* leaves are opposite or sub-opposite, glossy, narrow oblong, entire, 3–7 cm long and 2 cm broad. The flowers are bright red, 3 cm in diameter, with four to five petals (often more on cultivated plants). Some fruitless varieties are grown for the flowers alone. The edible fruit is a berry and is between a lemon and a grapefruit in size, 5–12 cm in diameter with a rounded hexagonal shape, and has thick reddish skin. The exact number of seeds in a pomegranate can vary from 200 to about 1400 seeds, contrary to some beliefs that all pomegranates have exactly the same number of seeds. Each seed has a surrounding water-laden pulp—the edible sarcotesta that forms from the seed coat—ranging in color from white to deep red or purple. The seeds are embedded in a white, spongy, astringent membran.

Fig-1A: Pomegranate plant

Fig-1B: Ripened Pomegranate fruit

Clinical trial rationale and activity [12-14]: Metabolites of pomegranate juice ellagitannins localize specifically in the prostate gland, colon, and intestinal tissues of mice[35], leading to clinical studies of pomegranate juice or fruit extracts for efficacy against several diseases. prostate cancer, prostatic hyperplasia, diabetes, lymphoma, rhinovirus infection, common cold, oxidative stress in diabetic haemodialysis, atherosclerosis, coronary artery disease, infant brain injury, haemodialysis for kidney disease, male infertility, aging, memory, pregnancy complications, osteoporosis and erectile dysfunction.

MATERIALS AND METHOD

Drugs and chemicals used: The standard drug 5-FU was purchased from Local Retail Pharmacy Shop and solvents and other chemicals which were used for the extraction and phytochemical screening provided by Institutional store and were of AR and LR grade.

Experimental animals: White male albino mice weighing about 15-20 gm were used. They were obtained from the animal house of C. L. Baid Metha College of Pharmacy, Chennai. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of CPCSEA: IAEC/XXIX/01/2017.

Cell culturing: HT 29 human colorectal cancer cells were purchased from Amala Cancer Research Centre, Thrissur, and Kerala. HT29 cells were cultured in McCoy's 5A media containing 10% FBS and 1% penicillin and streptomycin. The cell line was grown and maintained at 37 °C in humidified 5% CO2, 95% air. When cells reached 70% confluence, the medium was discarded and the cells were washed three times with Dulbecco's Phosphate-Buffered Saline (D-PBS). The cells were then incubated with 4 ml of trypsin at 37 °C in humidified 5% CO2, 95% air for 5 min. Next, the cells were harvested in 10 ml of their respective media without FBS containing 1% penicillin and streptomycin media. The harvested cell mixture was centrifuged at 300 g for 10 min. Cell pellets were re-suspended in those media and cells were then labelled in 6 µM Calcein AM at 37 °C for 2 h. Labelled cells were then centrifuged at 300 g for 10 min and the label solution was discarded. The cell pellet was finally re-suspended in 1.5 ml PBS for introduction to animals.

Methodology for extraction [15]: Weigh 20 g of fruits of *Punica granatum* paste (ripen can be mashed to prepare a paste) transfer into two 250 ml round-bottomed flask separately. Add 50 ml of methanol and 60 ml of dichloromethane in first round bottomed flask and add 50 ml of ethyl acetoacetae and 60 ml of dichloromethane in second RBF. Heat the mixture under reflux for 5 min on stem-bath with frequent shaking. Filter the mixture under suction and transfer the filtrate to a separating funnel. Wash this mixture containing bioactive compounds with three portions of 150 ml each with sodium chloride solution. Dry the organic layer over anhydrous magnesium sulphate. Filter and evaporate most of the solvent in vacuum without heating and obtained ME-FPG and EAAE-FPG (methanolic extract and ethyl acetoacetate extract of fruits of *Punica granatum*).

Phytochemical screening [16-18]: Preliminary Phytochemical screening of ME-FPG and EAAE-FPG had shown the presence of various bioactive compounds such as carbohydrates, amino acids and peptides, phytosterols, carotenoids, and polyphenols etc.

Protocol for the study of acute oral toxicity of ME-FPG and EAAE-FPG [19]:

In the present study acute oral toxicity of the extracts were performed by acute toxic class method according to OECD guideline-423. In this method the toxicity of extracts were tested using a step wise procedure, each step using three rat of single sex (female/male). The Swiss albino mice were fasted prior to dosing (food but water should be with held) for three to four hours. Following the period of fasting the animal should be weighted and the test compounds were dissolved in 3% CMC, administered through intra peritoneal to the different groups with 2000 mg / kg body weight. Animals were observed individually after dosing at least once during the first 30 min; periodically during the first 24 h with special attention giving during the first 4 h and daily thereafter, for total of 14 days. As

know mortality observed with the above dose. Test compound dose reduced by specific intervals. The mortality was not observed at the dose 2000 mg / Kg. So 300 mg /Kg body weight was selected for their pharmacological evaluation (since LD_{50} cut of value is > 2000). Mortality was determined after 24 hours of treatment. The dose at which 50% of the mice survived was considered the LD_{50} value of the compound. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of CPCSEA: IAEC/XXIX/01/2017.

Evaluation of *in vivo* antitumor activity of the ME-FPG and EAAE-FPG [20]

The in vivo antitumor activity of ME-FPG and EAAE-FPG was carried out against human colon cancer cell line HT 29.

Study design

An investigational study was designed to evaluate the *in vivo* antitumor activity of various extract of fruit of *Punica granatum* on mouse tumour models. Study was carried out with HT 29 cell line induced malignant ascites on mouse models. The dose of extracts 300 mg/kg were chosen based on the results of a toxicity study done previously. The animals were divided into five different groups as followed. The animals were divided into five different groups (each group contains 5 mice) as follows:

A. Group I: Normal Control Group [only the vehicle (1 ml/kg/day of 1% CMC orally)]

B. Group II: T. Control (1% CMC orally + HT 29 = 2×10^6 i. p.)

C. Group III: Standard (HT 29 = 2×10^6 i. p + 5-FU 25 mg/ml inj.)

D. Group IV: ME-FPG (HT 29 = 2×10^6 i. p + 300 mg/kg orally)

E. Group V: EAAE-FPG (HT 29 = 2×10^6 i. p + 300 mg/kg orally)

Table-1: Experimental design

Days	Activity was carried out	No. of mice / group (5)
Day 1	Collection of 0.3 ml of blood sample	Group-I-V
Day 2	Tumour cell injection, HT 29 = 2×10^6 i. p.	Group-II-V
Day 3-12	Treatment of CMC	Group-II
	Treatment of std. drug 5-FU	Group-III
	Treatment of ME-FPG and EAAE-FPG	Group-IV -V
Day 15	Collection of 0.3 ml of blood sample	Group-II-V
Day 16-35 follow up	Observed till death/35th day	Group-II-V

Experimental procedure: On day 1, blood collection from retro-orbital plexus was carried out and the samples (0.3 ml) in EDTA were used for the assessment of haematological parameters such as haemoglobin (Hb) content, red blood cell (RBC) count, total white blood cell (WBC) count, DLC and platelet count. On day 2, HT 29 cell suspension was withdrawn from 1.5 ml of PBS solution and the tumour cell count was done by using Neubauer chamber under the light microscope. The PBS was added to make a concentration of 1×10^6 cells in 0.1 ml. For tumour induction in study each experimental animal (Group-II to Group-V) was injected with 2×10^6 HT 29 cells i.e. 0.2 ml by intra peritoneal route. After 24 h of the tumour cells inoculation, the animals were treated with STD drug and tests compounds according to their respective groups once daily for next 10 days. On day 15, the retro-orbital blood collection was done again for haematological assessment, if the animal was alive. The animals were followed till death or up to 35 days. The parameters for antitumor activity in study were recorded as followed. Determination of the percentage increase in life span (PILS): It is calculated from the mean survival time (MST) values[4]. The MST for each group was calculated as:

MST (days) =Total number of days survived by all animals in the group/Number of animals in the group

For each group, Percent increase of lifespan (% ILS) was determined by the following formula:

PILS (%) = [(MST of treated group/MST of control group) −1] × 100

The haematological parameters of all surviving animals such as haemoglobin, RBC, WBC, neutrophils, lymphocytes and platelets were assessed for all. A group of four normal mice was studied for assessing their haematological parameters. These normal (control) values were used for comparisons. The tumour bearing animals alive at the end of the study were sacrificed by cervical dislocation.

RESULTS AND DISCUSSION

Effect on the hematological parameters: All 5 animals were in each group. So mortality was less in all groups. The Hb and RBC count were significantly lower in tumor control group compared to normal control group and significantly raise nearly to normal in all treatment groups when compared with control group. The WBC counts were significantly increased in

tumour control, and it came down to nearly normal range in all treatment groups. The neutrophils were increased and lymphocytes were decreased significantly in tumour control groups and significantly decreased neutrophils and increased lymphocytes in all treatment groups. The platelet count was significantly increased in tumour control and ME-FPG and EAAE-FPG at dose 300 mg/kg group compared to normal group.

Effect on the survival: ME-PG and EAAE-PG significantly increased the PILS. While 5-FU increased the life span of 85%, ME-FPG increased it by 75% and 67.5% by EAAE-FPG respectively ME-FPG. So ME-FPG and EAAE-FPG at dose 300 mg/kg significantly improved the overall survival of treated animals in comparison to tumor control group and 5-FU was not significantly differed from each other in improving the overall survival of animals.

Effect on proliferation: H&E staining displayed a greater degree of tumor apoptosis and necrosis in the ME-FPG and EAAE-FPG treated group than in the control group. In addition, PCNA showed that administration of ME-FPG and EAAE-FPG decreased the proliferation of tumour tissues. Proliferating cell nuclear antigen (PCNA) is a DNA clamp that acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication.

Table-2A: for the assessment of haematological parameters

Group	Treatment	Hb (g/dl)	RBC(1×10^6/mm^3)	WBC(1×10^3/mm^3)
I	N. Control	13.7	9.25	60.25
II	T. Control	7.9	6.41	77.23
III	5-FU	11.1	8.88	61.79
IV	ME-FPG	10.4	8.96	61.71
V	EAAE-FPG	9.6	8.44	63.81

Table-2B: for the assessment of haematological parameters

Group	Treatment	Neutrophils (%)	Lymphocytes (%)	Platelets (1×10^3/mm^3)
I	N. Control	14.1	88.9	449.6
II	T. Control	84.7	13.2	1316.1
III	5-FU	13.1	85.85	459.1
IV	ME-FPG	13.8	85.55	470.5
V	EAAE-FPG	14.87	86.61	517.4

Table-3: for PILS

Group	Treatment	Total number of days survived by all animals in each group	MST (days)	PILS (%)
II	T. Control	20	4	-
III	5-FU	37	7.4	85.00
IV	ME-FPG	35	7.00	75.00
V	EAAE-FPG	33 and half day	6.7	67.5

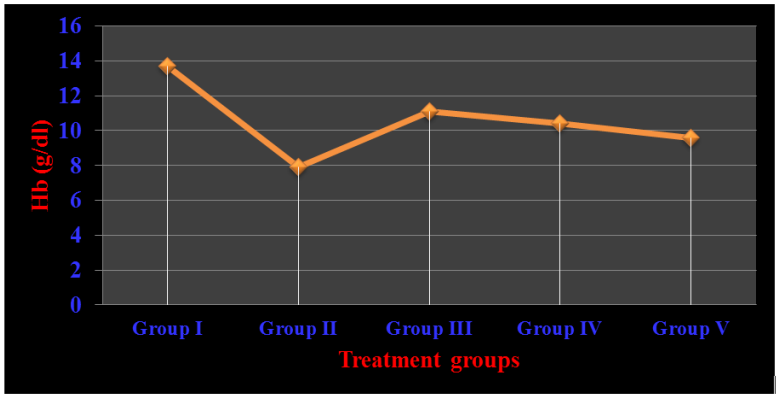

Fig-2: Comparison of level of hemoglobin in different treatment groups

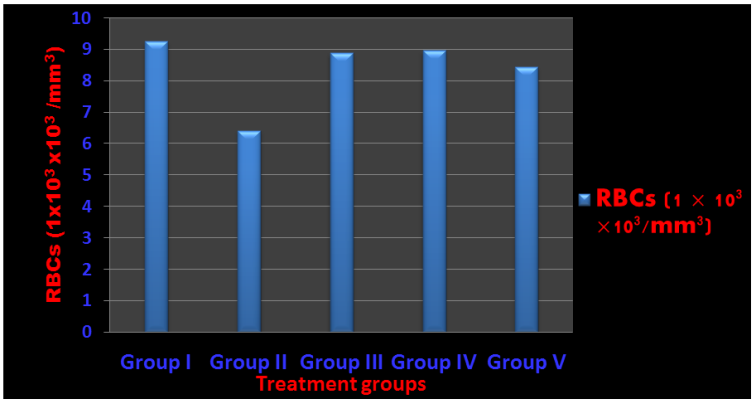

Fig-3: Comparison of RBCs count in different treatment groups

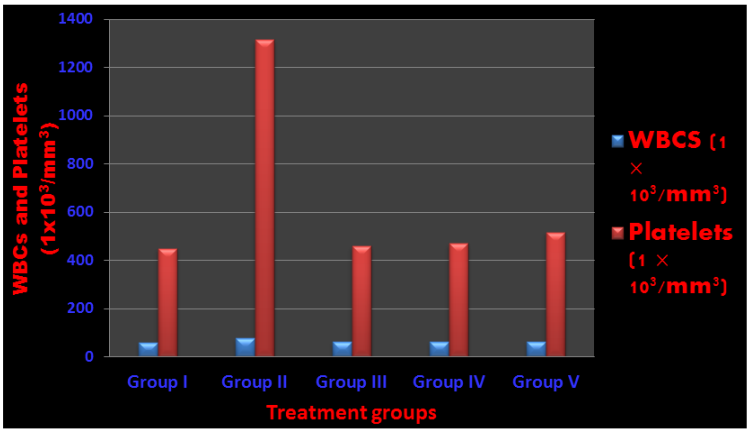

Fig-4: Comparison of WBCs count in different treatment groups

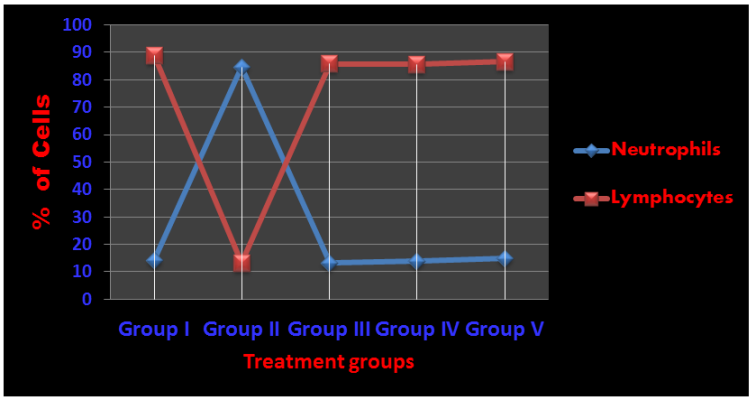

Fig-5: Comparison of % of neutrophils and lymphocytes in different treatment groups

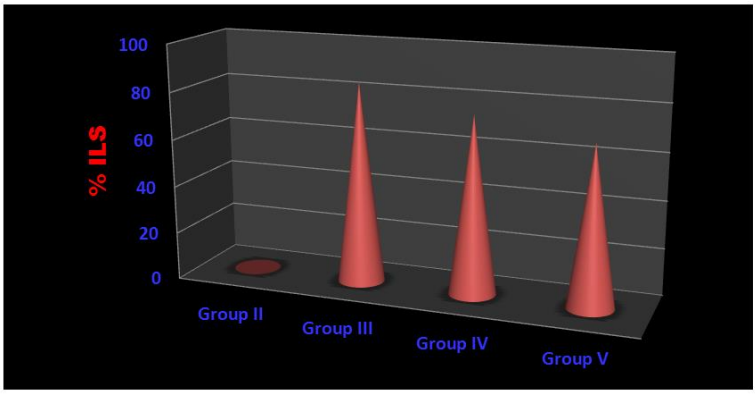

Fig-6: Comparison of % of ILS in different treatment groups

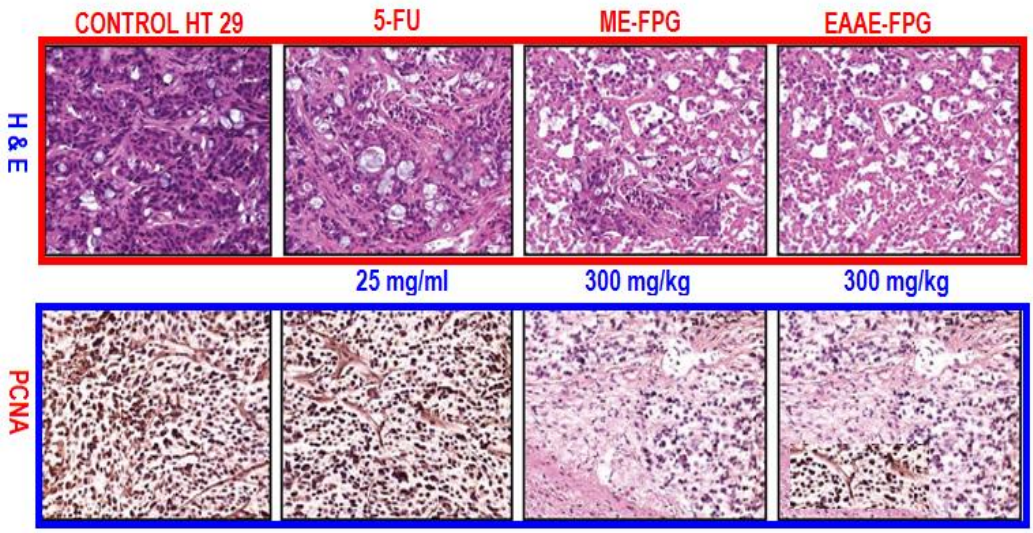

Fig-7: H&E staining and PCNA study of HT 29 Cells

CONCLUSION

From the above experimental data, here we concluded that the MEF-PG and EAAE-FPG contained various bioactive molecules which were confirmed by their qualitative confirmatory chemical tests and executed good antitumor activity against human colon cancer cell line HT 29 at dose of 300 mg/kg b. w. In addition, PCNA showed that administration of ME-FPG and EAAE-FPG decreased the proliferation of tumour tissues.

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