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In vitro and in vivo anticancer studies of n-butanol fraction from ethanol extract of annona muricata leaves

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Abstract---Background: Cancer is a leading life threatening disease that adversely affects millions of people in a year worldwide. The growing concerns on the impact of cancer treatment such as surgery, radio & chemotherapy has been notably redirected to the usage of natural bioactive compounds having potential anticancer activity. *Annona muricata* Linn, a tropical plant belonging to Annonaceae family, was broadly investigated for the ethno medical property. The leaves of the plant was found to contain acetogenins that showed the significant anticancer activity. Objectives: To employ n-butanol fractionation to get the abundance of alkaloid phytochemicals from ethanolic extract of *Annona muricata* leaves and to study the extract In- vitro and In-vivo for anticancer activity. Methods: MTT assay was performed in-vitro to analyze the cytotoxicity of the extract. The in vivo studies were performed with albino mice and the effect of n-butanol extract was studied on the body weight, hematological & biochemical parameter, hepatic enzymes, antioxidant proteins of the experimental animals. Results: The cytotoxic potential of the n-butanol extract using MTT assay showed the IC₅₀ value of 50 µg/mL inferring 50% of inhibition to MCF-7 adenocarcinoma cells. The in-vivo results confirmed the tumor reducing potential and efficiency of the n-butanol fraction of leaf extract as anticancer agent. The results concluded that the n-butanol fraction of *Annona muricata* leaves could be potentially exploited for the treatment of cancer. Novelty: Though there are numerous studies on *Annona muricata* leaves, this work presents the n-butanol fractionation of the ethanolic extract to efficiently extract or enrich the alkaloids, a significant phytochemical

compound, which could serve as the anticancer agent in breast cell lines.

Keywords---Acetogenin, anticancer, medicinal plant, bioactive compounds, *Annona muricata*, invitro, invivo

Introduction

Cancer is one of the deadly diseases which remarkably favors 7.6 million deaths every year. It was estimated that the number of deaths from cancer would cross 13.1 million in the next decade [1]. Among all of the cancer types, breast cancer has become the most prevalent cancer among women throughout the world. [2]. Though there exists numerous treatments like surgical methods, radiation therapy, immune therapy and chemo therapy that available to cure cancer, the adverse side effects caused by the conventional methods affect the normal cellular metabolism of the individuals and also, the administration of anti-apoptotic drugs could favorably induce resistance to cancer [3]. Hence, there is an urgent need to employ the medicinal plants alternative to the synthetic drugs for the treatment of breast cancer. The abundance of acetogenins and alkaloid compounds present in the leaf extract contribute to vital properties like anticancer and antimicrobial activity. Therefore, herbal or plant mediated methods could potentially replace the existing treatment methods with no side effects [4].

Annona muricata is one among the plant that has been extensively used as an [anticancer agent](#), especially its leaves [5]. *Annona muricata* leaves were found to contain anticancerous compounds such as acetogenins that have the potency to inhibit cancer cell growth [6, 7]. Studies on *Annona muricata*-derived compounds exhibited a variety of anticancer effects including necrosis [8], stimulation of apoptosis [9], cytotoxicity [10], and inhibition of proliferation [11] on wide range of cancerous cell lines, including breast [12], lung, cervical and ovarian cancers [13]. The treatment of cancer by soursop leaves were found to be more promising and safer in comparison with chemotherapy or radiation therapy. However, most of the previous works on Soursop plant were performed not on the bioactive isolates which were responsible for the pharmacological property but were done on crude extracts of the plant. The toxicology of the plant was found due to the presence of annaceous acetogenins and benzyltetrahydro-isoquinoline alkaloids. Fewer research works attempted to extract the bio-active compound using chloroform and hexane but no such work has been carried out using n-butanol which significantly improved the abundance of alkaloids for anticancer activity. Further in vitro and in vivo studies were carried out to ensure the anticancer activity of the isolated bio-active phyto-compound [14]. Therefore, in the present study, the n-butanol fraction of the ethanol extract of *Annona muricata* leaves were obtained so as to enrich the bioactive compounds (alkaloids) present in the leaves. The n-butanol extract of the leaves were studied for the anticancer property both invitro using MCF-7 adenocarcinoma cells and invivo using albino mice.

Materials & Methodology

In vitro studies

Cell culture and maintenance

MCF-7 (human breast carcinoma, tumorigenic and non-invasive) adenocarcinoma cells were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were cultured in Eagle's Minimum Essential Medium (EMEM) media (Hyclone, GE Healthcare, USA), supplemented with 10% fetal bovine serum (HiMedia, India) & 10 ml of penicillin/streptomycin as antibiotics (Hyclone, GE Healthcare, USA) at 37°C in a humidified atmosphere of 5% CO₂ in a CO₂ incubator (Thermo Scientific, USA). All experiments were performed using cells cultured less than 10 passages.

MTT assay

The MTT [3-(4, 5- Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide)] (Sigma-Aldrich Cat.# M2128) cytotoxicity assay was performed to determine the anti-proliferative activity and cytotoxic potential of the powdered n-butanol fractionation of ethanol extract of *Annona muricata* leaves against MCF-7 breast adenocarcinoma cell line by which MTT effects on the mitochondrial reductase activity of viable cells. The assay is based on the cleavage of MTT, a yellow tetrazolium salt, which forms water insoluble dark blue formazan crystals. This cleavage occurs in living cells by the mitochondrial enzyme succinate-dehydrogenase. The water-insoluble dark blue formazan crystals are solubilized with dimethyl sulfoxide. While, the dead cells remains the same yellow colour and the viability of the cells can be determined with a UV visible spectrophotometer (Molecular Devices, UK)

Protocol

The MCF-7 Cells were cultured in 96-well microtitre plates with 8×10^4 cells/mL as initial concentration and incubated for 24 hours at 37°C in 5% CO₂ condition. Growth inhibition was compared with untreated controls to find the Inhibitory concentration (IC₅₀). The powdered n-butanol fraction of the ethanol leaf extract was added to wells at different concentrations ranging from 25µg/mL - 100 µg/mL. 10µL of MTT solution was added to each wells making final concentration of 0.45 mg/mL. The microtitre plate was incubated for 4 h at 37°C in CO₂ incubator. Later, 100µL of solubilization solution was added to each wells to dissolve the formazon crystals [16]. The absorbance was read at 570nm. Fig 1-6 represents the cytotoxic nature of the n-butanol fraction of ethanol extract of *Annona muricata* leaves. The percentage of cell viability was calculated using the formula,

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

In vivo studies

The *In vivo* studies were carried out to evaluate the tumor reducing potential of the powdered n-butanol fractionation of ethanol extract of *Annona muricata* leaves

on albino mice. An albino mice in each group was taken into study and the experimental design was given below. The cancer was induced with the tumor initiator called as DMBA (purchased from Sigma Aldrich, India) in mice belonging to all groups except group I. The DMBA induced mice were treated with different concentrations of n-butanol fraction of the ethanol extract of the leaves of *Annona muricata* plant. The mice belonging to group-III to group- V were treated with different concentrations of n-butanol fraction ethanol having concentration of 100mg /kg BW, 200mg /kg BW and 300mg /kg BW respectively. Group-VI was treated with standard drug 5- Fluorouracil having concentration of 10mg/kg BW. All investigations were conducted with approval from the ethical committee [17, 18]. Table 1 & 2 represents the effect of plant extract on hematological & biochemical parameters, and antioxidant proteins of experimental animals respectively.

Experimental design

- Group-I - Normal untreated mouse (Control)
- Group-II - DMBA Induced mouse (Tumor Control)
- Group-III - DMBA Induced mouse treated with 100mg/kg BW of n-butanol fractionation of ethanol extract of *Annona muricata* leaves.
- Group-IV - DMBA Induced mouse treated with 200mg/kg BW of n-butanol fractionation of ethanol extract of *Annona muricata* leaves.
- Group-V - DMBA Induced mouse treated with 300mg/kg BW of n-butanol fractionation of ethanol extract of *Annona muricata* leaves.
- Group-VI - DMBA Induced mouse treated with standard drug 5- Fluorouracil (10mg/kg. BW).

Also, the effect of n-butanol fraction of the ethanolic extract of soursop leaves on change in body weight, hematological & biochemical parameters, hepatic and antioxidant enzymes of experimental animals Fig 7, 8 represents the effect of plant extract on the body weight and hepatic enzymes of experimental animals respectively.

Results

The in vitro MTT assay was performed for 24 h to evaluate the anticancer activity of the different concentrations of the samples ranging from 25-100 µg/mL along with the control. 50 µg/mL concentration is more significant and relatively induces cellular death of approximately 50% (exactly 53.37%) on MCF-7 cells and this concentration is considered to be the inhibitory concentration IC₅₀ (concentration needed to kill 50% of cell population) concentration for the sample. The in vivo studies of table 1 indicates the tumor growth in the groups (II-VI) induced by the DMBA. It infers that in all the 5 groups, tumor was successfully induced and the final body weight was compared to the initial body weight of the animal. . The effect of extract on the hematological parameters such as RBC (10⁶ Cells/mm³), WBC (10³ Cells/mm³) and hemoglobin (%) and biochemical parameters such as blood glucose (mg/dl), protein (mg/dl), urea (mg/dl) and liver glycogen(mg/ g of tissue) revealed the n-butanol fraction of the leaves did not induce any changes to the experimental animals and the values were found compatible with the properties of normal mouse. The hepatic enzymes Aspartate

transaminase (AST) (U/L), Alanine aminotransferase (ALT) (U/L), and alkaline phosphatase (ALP) (U/L) were found within the normal range in comparison with the control group I. These enzymes are an indication of liver injury in blood due to drug toxicity. The group II belonging to untreated cancer induced by DMBA reveals increased level of these enzymes in blood stream and in the n-butanol treated groups (III-V) these levels are significantly reduced in the dosage concentration dependent manner. Hence, it confirms that the extract does not induce any renal injuries. Similarly, the antioxidant enzymes/proteins LPO (nmol MDA·mg⁻¹ (protein)), reduced glutathione (U·mg⁻¹ (protein)), glutathione peroxidase (mg·g⁻¹ (wet tissue)), superoxide dismutase (U·mg⁻¹ (protein)) and catalase (U·mg⁻¹ (protein)) lies within the normal range as in the control group I and tend to decrease in the untreated tumor group II. The n-butanol treated groups (III-V) showed significant increase in the antioxidant enzymes quantity in dosage dependent manner. It clearly shows that, antioxidant encountering tumor cells have been killed by the drug treatment and increased levels are observed.

Discussion

Extensive anticancer researches have been investigated on *Annona muricata* because of its ethnomedicinal uses against tumors and cancer [15]. Annonaceous alkaloids, acetogenins, and phenols are the most prominent bioactive metabolites isolated from the *A. muricata* leaves. These bioactive compounds exhibited a range of functional properties such as anticancer, immunomodulatory, anti-inflammatory, insecticidal, antimicrobial, and antioxidant. The leaves, and also the stems of *A. muricata*, exhibited active cytotoxicity against cancer cells, because of the presence of acetogenins and alkaloids, which did not show toxicity towards normal cells, but exhibited high toxicity to cancerous cells [16]. The antitumor properties of different fractions of *A. muricata* leaves using methanol, hexane and dichloromethane extracts were examined to isolate the bioactive compound present in the leaves [17]. In this study, the bioactive compound, alkaloid was predominantly isolated using n-butanol fractionation from ethanolic extract of *Annona muricata* leaves. The isolated alkaloid compound was used as the anticancer agent. Numerous studies suggest that the leaf extract of *Annona muricata* suppressed the growth of tumor in vivo in experimental animal models and also induced apoptosis of various cancerous cells in vitro [18]. The *Annona muricata* leaf extracts were analyzed for cytotoxicity against K562 (human bladder cancer cells), and also ECV304 (human leukemia cancer cells) which manifested a remarkable cytotoxic effects [19]. The MCF-7 (human adenocarcinoma) breast cancer cell lines treated with n-butanol fraction of the leaf extract also enlightened the cytotoxic potential of the extract against breast cancer having IC₅₀ value of 50 µg/mL showing 50% of cytotoxicity. These results were found to be consistent with those in other studies on breast cancer cell lines [20]. It reports that the main cytotoxic component responsible for anticancer activity are the acetogenins present in the leaf extract. The results were also tend to vary with the dosage dependent manner. This promising anticancer/antitumor effect was also reported in an in vivo study conducted on DMBA-induced cell proliferation in the breast tissues of mice [21]. The ethanol extract of soursop leaves, even at the low concentration of 30 mg/kg BW suppressed the initiation and proliferation stage of skin papilloma genesis in mice that was induced by DMBA [22]. This result was consistent with the present

study in which the n-butanol fraction of the ethanol extract of the leaves revealed the promising antitumor effects.

The hematological and biochemical parameters seem to lie within the normal range as group I (control) and alterations were observed in the tumor induced group II without treatment. The WBC value is slightly increased than the normal range which is an indication of the presence of high B lymphocytes which could be very useful for the effective treatment of cancer cells which is compatible with other studies on invivo models for anticancer activity [23-25]. Various research findings on the soursop plant suggests that the extracts were traditionally used for treating liver diseases. In the current study, effect of n-butanol extract on hepatic enzymes in blood showed the decreased levels of enzymes indicating that it does not favor renal injuries [25-29]. The seeds and leaves of the soursop plant are reported to contain enzymatic antioxidants such as catalase and superoxide dismutase, also non-enzymatic antioxidants like vitamin C and E [30-31]. The results of the current study confirms the increased level of expression of antioxidant enzymes for group III-V treated with n-butanol fraction of *Annona muricata* leaves.

Conclusions

Literature reviews on *Annona muricata* have showed and reported 117 isolates from the leaf, comprising mainly of annonaceousacetogenins, alkaloids and as well as phenolic compounds. Also, the pharmacological and biological studies conducted using the crude extracts of the species were at its preliminary stages. The specific bioactive compounds contributing to functional property have not been properly identified. Therefore, n-butanol fractionation was performed to isolate the bio active compound alkaloid and to utilize it for anticancer activity. Thewidely studied *Annona muricata* plant leaves were analyzed for the invitro anticancer studies with MCF-7 (breast cancer cell lines) showing IC₅₀ value of 50µg/mL which is quite efficient compared to other ethanolic extracts employed in the past literature works. The invitro studies of the extract revealed the significant cytotoxic activity against cancer cell lines. In addition, the relationship between the n-butanol fractions with the different parameters induced by cancer were investigated and the usage of the extract in cancer was found to be compatible with the normal mice thereby exhibiting improved antitumor activity. The present study proposes that the n-butanol fraction of the ethanol leaf extract of *Annona muricata* were found to be very efficient for the treatment of breast cancer compared to other solvents. Further, detailed toxicological studies and many clinical trials are necessary to verify and validate the efficacy of the plant extracts to be utilized as the therapeutic anticancer agent.

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Table 1: Effect of Plant extracts on Hematological and Biochemical Parameters of Experimental Animals

Groups	RBC (10 ⁶ Cells/m ³)	WBC (10 ³ Cells/ mm ³)	Hemoglo bin (%)	Blood Glucose (mg/dl)	Protein (mg/dl)	Urea (mg/dl)	Liver Glycogen (mg/ g of tissue)
I	7.99±0.2 3	8.27±0. 26	18.38±0. 49	112.94±2.08	7.65±0.5 3	35.09±0. 99	20.44±0.62
II	4.83±0.2 5	13.63± 0.58	11.31±0. 41	73.29±3.82	4.55±0.3 9	20.30±0. 67	7.74±0.21
III	6.05±0.1 8	11.23± 0.27	13.02±0. 37	89.28±0.96	5.39±0.3 5	25.93±0. 89	13.82±0.37
IV	6.59±0.2 0	10.59± 0.44	15.21±0. 61	96.22±1.44	6.2±0.34	30.15±0. 99	16.77±0.42
V	7.26±0.1 7	9.22±0. 34	16.33±0. 42	102.74±2.4	7.23±0.3 5	32.69±0. 73	19.67±0.39
VI	7.49±0.2 8	8.98±0. 13	17.8±0.2 8	108.94±2.51	7.40±0.4 3	34.18±0. 86	21.68±0.97

Table 2: Effect of Plant extracts on antioxidant proteins of Experimental Animals

Groups	LPO (nmol MDA·mg ⁻¹ (protein))	Reduced Glutathion (U·mg ⁻¹ (protein))	Glutathione peroxidase (mg·g ⁻¹ (wet tissue))	SOD (U·mg ⁻¹ (protein))	CAT (U·mg ⁻¹ (protein))
I	2.16±0.15	6.24±0.26	5.23±0.33	6.00±0.14	31.85±0.78
II	6.57±0.43	2.24±0.24	2.06±0.19	2.20±0.12	17.40±0.74
III	5.28±0.29	3.27±0.10	3.14±0.25	3.17±0.11	21.50±1.21
IV	4.49±0.15	4.57±0.16	3.89±0.35	4.72±0.15	26.67±0.80
V	2.56±0.27	5.50±0.12	4.42±0.21	5.41±0.30	29.88±0.82
VI	2.38±0.10	6.00±0.19	4.66±0.18	5.69±0.32	30.7±0.71

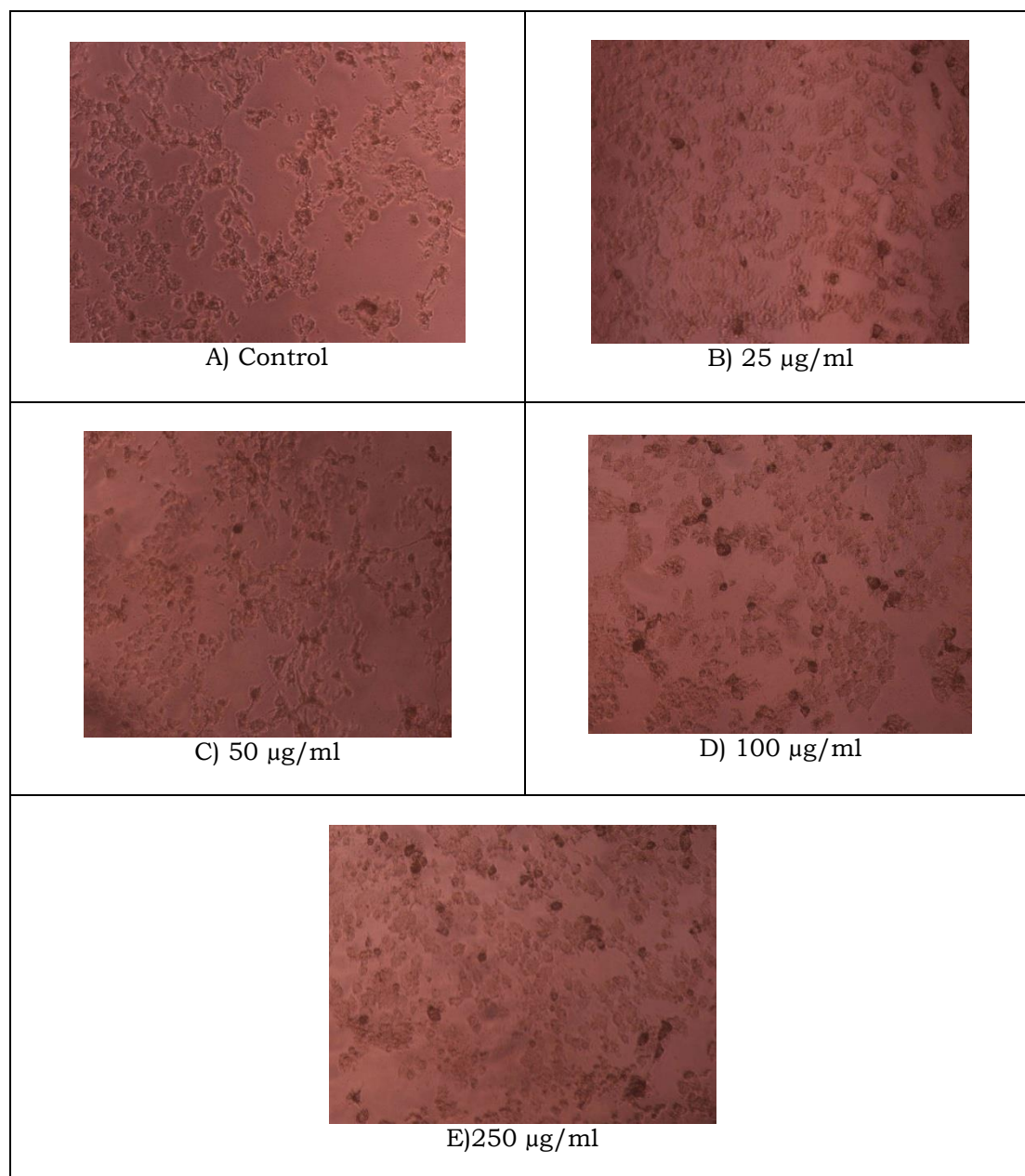


Fig 1-5 Cytotoxic potential of the n-butanol fractionation of ethanol extract of *Annona muricata* leaves on MCF-7 adenocarcinoma cells

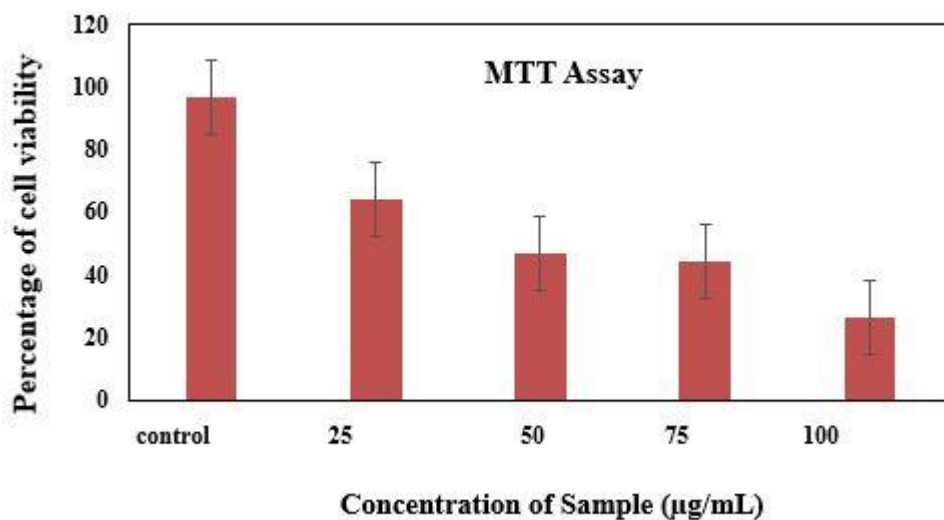


Fig 6 MTT assay of n-butanol extract of *Annona muricata* leaves

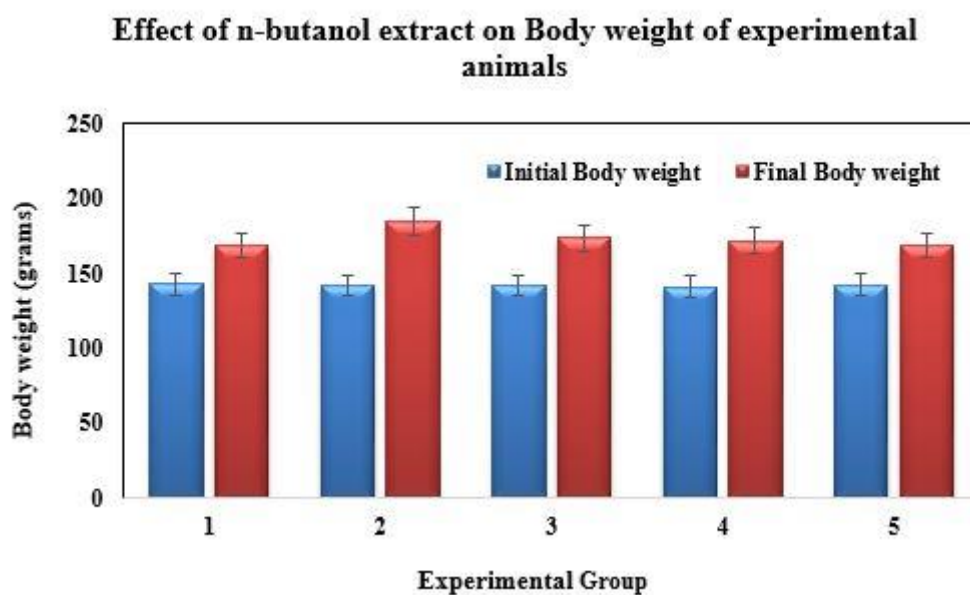


Fig 7 Effect of n-butanol extract of *Annona muricata* leaves on body weight of experimental animals

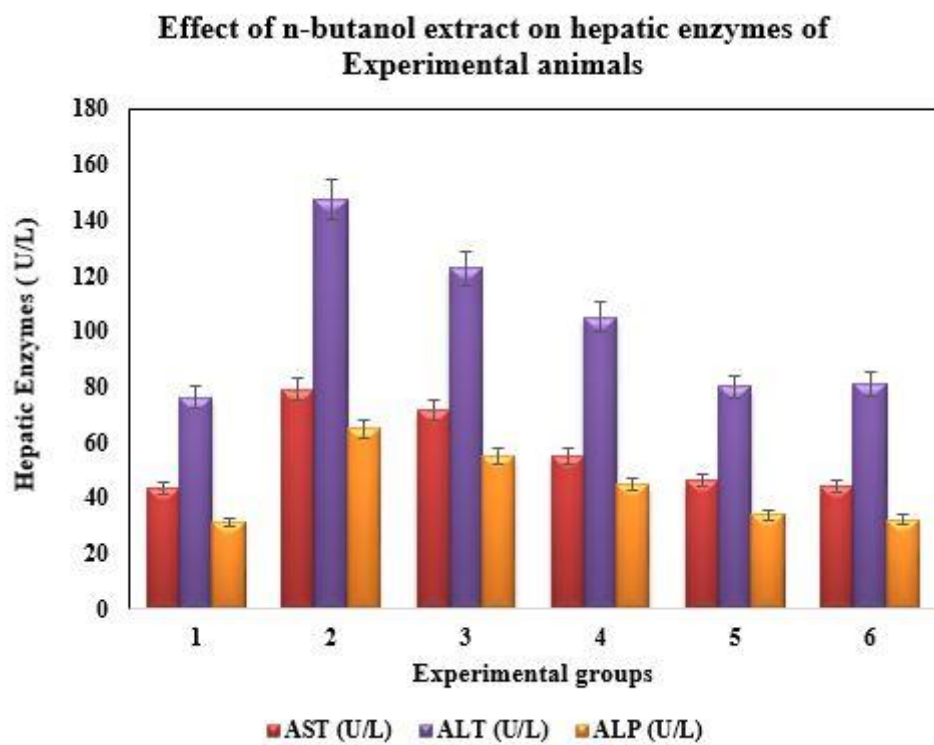


Fig 7 Effect of n-butanol extract of *Annona muricata* leaves on hepatic enzymes of experimental animals