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ARTICLE

Anticancer Activity of Active Constituents Isolated from *n*-Butanolic Extracts of *Flacourtia jangomas* (Salicaceae)

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ABSTRACT

The present study explores the active bioconstituents of *n*-butanolic extracts of *Flacourtia jangomas* fruits and evaluate its anticancer potentiality based on the evidences from the ethnomedicianl practice of the plant. In this work, in *vivo* model was used to evaluate the anticancer activity. Hematological profiles were found to be nearly normal level in extract treated mice compared with tumor bearing control mice.

KEYWORDS

Flacourtia jangomas, Dalton cell line, Flavonoids.

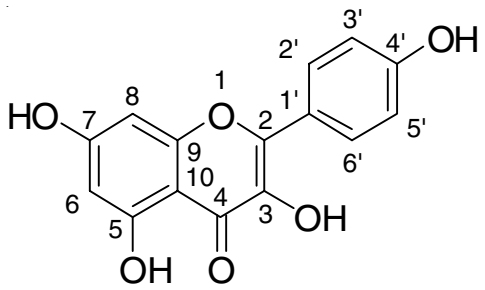
Asian Journal of Organic & Medicinal Chemistry

Volume: 3 Year: 2018
Issue: 3 Month: July–September
pp: 89–94
DOI: https://doi.org/10.14233/ajomc.2018.AJOMC-P121

Received: 31 May 2018
Accepted: 25 July 2018
Published: 25 September 2018

INTRODUCTION

Phytochemicals represents an enormous reservoir of biologically active compounds with various chemical structures obtained from the plant kingdom. These includes often some secondary metabolites present in smaller quantities in higher plants, including the alkaloids, steroids, flavonoids, terpenoids, tannins, *etc*. Approximately 50% of drugs obtained from the plant origin are used in medicine but only a small fraction of plants with medicinal activity has been scientifically proved [1]. In 20th century, cancer is one of the most dreaded diseases and is spreading further continuously with an increasing rate in 21st century. Cancer is a group of more than 100 different diseases, characterized by uncontrolled growth of the cell, local tissue invasion and distant metastases. Different scientific investigations have been performed for making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into the world medicine [2].



Structure of 3,5,7-trihydroxy-2-(4 hydroxyphenyl)-4*H*-chromen-4-one (**1**)

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Herbal drug formulations have been playing a significant role in the last three decades for the prevention and treatment of cancer and the interest on natural sources of potential chemotherapeutic agent is continuing. The discovery of effective herbs and elucidation of some novel bioactive secondary metabolites could lead to the development of an alternative and complementary method for cancer prevention and treatment [3].

Flacourtia jangomas Lour. (Salicaceae) draws the attention of the researchers through worldwide for its traditional use in various parts of the world. In Assam, eastern part of India, it is known by the name "Poniol" and traditionally used in cancer, stimulant, antioxidant and for antibacterial activity. Phytoconstituents such as alkaloids, sterols, flavonoids and tannins together with other compounds such as palmitic acid, oleic acid, tartaric acid and linoleic acids were already reported from this plant. Although some studies are reported on anticancer activity of leaves and aerial plant parts extract of *Flacourtia jangomas*, no studies were conducted on the anticancer activity of fruit parts of the plants so far. This study was undertaken to explore the anticancer property of fruits of *Flacourtia jangomas* using *in vivo* models [4].

EXPERIMENTAL

Fruits of *Flacourtia jangomas* were collected from local areas of Guwahati (Azara) Assam, India in the month of May 2013. The plant material was authenticated by Dr. P.P. Baruah, Prof. and Head, Department of Botany, Gauhati University, Guwahati, India and deposited in the herbarium of Gauhati University (voucher specimen no. 18229). Trypan blue was purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India. Methotrexate (Biotrexate) of Biochem Pharmaceutical Industries was purchased from Guwahati, Assam, India.

Preparation of extracts: The collected plant parts were washed with water, shade dried in open air and pulverized by using mechanical grinder. About 200 g of *Flacourtia jangomas* powder was packed into Soxhlet apparatus and subjected to successive extraction using petroleum ether, ethyl acetate, dichloromethane and *n*-butanol as the solvent. The preliminary phytochemical and pharmacological screening shows a good result for *n*-butanolic extract, therefore, it is selected for further studies. The extract was filtered through Whatman filter paper No. 40, evaporated using vacuum rotary evaporator (Buchi) and heated on a water bath at 45 ± 5 ºC and stored in vacuum desiccators for further use [5,6].

Isolation of compound: Isolation of active components from the plant was performed incorporating Teledyne ISCO Combi Flash Rf 150 model. Stationary phase used for separation of the active constituents is composed of silica gel (60-120 mesh) for column chromatography grade (Merck Pvt. Ltd.). Packing of the stationary phase is done by dry packing over a plastic column. A mixture of powdered extract (*n*-butanol extract, 10 g) and column grade silica gel 60-120 mesh is thoroughly mixed and pre-packed over the plastic column, which is fitted to the instrument. Solvent system used for running the column is composed of a mixture of chloroform and methanol. Before starting the process, the instrument was set auto prime with polar and non-polar solvent to avoid any contamination with previously used solvent system. After auto prime the process of separation is started.

For the isolation non-polar solvent was elute first the gradually the polarity was increased. About 12 mL of fractions were collected in each test tube. The compound was obtained in the form of a crystal after filtration and evaporation. It was identified by ultraviolet, Fourier-transform infrared, NMR (^{1}H and ^{13}C), mass spectroscopic analysis and elemental analysis. The compound was identified as 3,5,7-trihydroxy-2-(4 hydroxyphenyl)-4*H*-chromen-4-one (**1**) [7-9].

Ultraviolet-visible analysis: The crystalline isolated compound was dissolved in spectroscopic grade methanol and the absorption spectra was taken in Shimadzu 1601 double beam UV-visible spectrophotometer from 200 to 500 nm [10]. Sodium acetate, aluminium chloride and other analytical solvents were used as shifting reagent for the characterization process.

Infrared analysis: The infrared absorption spectra of the isolated compound were taken with Bruker FTIR spectrophotometer, in potassium bromide discs. The spectra were recorded in the region of 4000-400 cm^{-1} [10].

NMR analysis: The ^{1}H NMR spectra of the isolated compound from *n*-butanolic fraction of *Flacourtia jangomas* fruits were undertaken in Brucker Avance II 400 MHz Spectrophotometer in DMSO-d_6 solution [11].

Mass analysis: Mass spectrum (ESI, APCI) of the isolated compound from the fruits of *Flacourtia jangomas* was recorded on a T: ITMS-c ESI Full MS (125.00-1000.00) spectrophotometer [11].

Experimental animal: The guidelines set by CPCSEA were approved by the Institutional Animal Ethical Committee (GIPS/IAEC/Phd/2015/01) were followed throughout the experimental process. Swiss albino mice (20-25 g) of both sexes were used for the study and housed following the standard laboratory conditions as per the CPCSEA guideline such as 22±3°C of temperature, 50 ± 10 % humidity, 12 h interval of light and dark phase and were fed with standard pellet diet throughout the entire experiment [12].

Acute toxicity study and dose optimization: The acute toxicity study was conducted as per the OECD guidelines 425. Initially, the dichloromethane extract was administered orally at a limit dose of 2000 mg/kg for the extract and 2.5 mg/kg for the isolated compound to single mice. The animals were observed closely for the first 4 h and then periodically upto 24 h for any toxic symptoms and mortality. After 24 h, same dose was administered to four more female mice. This study was approved by Animal Ethics Committee of Girijananda Chowdhury Institute of Pharmaceutical Sciences, Guwahati, Assam, India (GIPS/IAEC/08). The acute toxicity study was conducted for 24 h.

Anticancer activity of *n*-butanolic extract of *Flacourtia jangomas*: For this study, animals are divided into seven groups each containing six animals. All the animals except the normal group received Dalton cells 1×10^{6} cells/mouse Group I (normal) received 0.9% normal saline (p.o.), Group II (control) 0.9 % normal saline (p.o.), Group III treated with standard methotrexate at 2.5 mg/kg/day p.o, Group IV treated with 250 mg/kg/day p.o. of *n*-butanolic extract, Group V with 500 mg/kg/day p.o of *n*-butanolic extract, Group VI with 1000 mg/kg/day p.o of *n*-butanolic extract and Group VII treated with 2.5 mg/kg/day p.o of isolated compound. All treatments were given for 9 days.

Determination of body weight and mean survival time of *n*-butanolic extract treated mice: The body weight and mean survival time of each group consisting of six mice were noted. The antitumor efficacy of *Flacourtia jangomas* was compared to that of methotrexate. The percentage increase life span (% ILS) of each mouse was calculated using the following equation:

$$\text{Increase in life span (\%)} = \frac{T - C}{C} \times 100$$

where T = number of days the treated animals survived and C = number of days control animals survived.

Determination of viable and non-viable cell count of *n*-butanolic extract treated mice: The viability and non-viability of the cells were evaluated as per trypan blue assay. The cells were stained with trypan blue (0.4 % in normal saline) dye. On staining, the viable cells did not take the stain while non-viable cells were stained blue and counted using Invitrogen auto cell counter.

Determination of hematological parameters of *n*-butanolic extract treated mice: At the end of the experimental period, the next day after an overnight fasting, blood was withdrawn from the retro-orbital plexus and used for the estimation of hemoglobin (Hb) content, red blood cell (RBC) count and white blood cell (WBC) count using an automatic analyzer. Half of the animals from each group were sacrificed and checked for tumor volume [13,14].

Statistical analysis: Data analysis was performed by using Graph Pad Prism software and the data were expressed as a mean ± standard error. The significance level of treatment effect was determined by one-way analysis of variance (Dunnett's post test); $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The present study was undertaken to isolate the active bioconstituent from *n*-butanolic extract of *Flacourtia jangomas* fruits and to evaluate its anticancer activity at the different doses in EAC tumor bearing Swiss albino mice.

Preliminary phytochemical screening: The preliminary phytochemical screening of different fruits extract of *Flacourtia jangomas* showed the presence of phytocostituents like, flavonoids, steroids, glycosides and tannins (Table-1). The *n*-butanolic extract of the plant revealed the presence of only flavonoids, which is the major active bioconstituents and hence the extract was selected for further study.

Spectral analysis of isolated compound from *n*-butanolic extract of fruits of *Flacourtia jangomas* Lour. The chemical structure of isolated compound was characterized by its physical parameters and spectral analysis including UV, IR, mass, ^{1}H- and ^{13}C NMR. The UV absorption spectrum of isolated compound (*n*-butanolic extracts of *Flacourtia jangomas*) was obtained as a light yellow colour crystal which is soluble in methanol, DMSO and ethanol. The compound showed strong absorption at 376 nm in its spectrum, which implied the presence of phenolic aromatic rings. The *n*-butanolic solution of compound exhibited typical UV absorption characteristics after the addition of various shifting reagents, respectively. The UV spectrum showed absorption bands reagents shifts of the compound to be a 7-substituted derivatives. The absence of free 7-hydroxyl group in the compound was observed in lack of shift of band II in the presence of sodium acetate (Table-2).

TABLE-1
RESULTS OF PRELIMINARY PHYTOCHEMICAL SCREENING

Name of the test	Ethyl acetate extract	*n*-Butanol extract	DCM extract
Glycoside	+	–	+
Alkaloid	–	–	–
Flavonoid	–	+	–
Protein	–	–	+
Amino acid	–	–	–
Tannin	+	–	
Steroid	+	–	+
Phenolics	+	+	+
Terpinoids	–	–	–

IR spectrum of the compound confirmed the presence of -OH group (3253 cm^{-1}), conjugated carbonyl group (1661 cm^{-1}) and aromatic C-C (1605 cm^{-1}) (Fig. 1). ESI-MS spectrum (positive ion mode) showed the presence of base peak at *m/z* 286.07 (M^{+}) indicating the molecular weight of compound 286 (Fig. 2). The ^{1}H NMR spectrum of the compound is represented in Fig. 3. In this spectra, it was exhibited that the formation of singlet peak at 2.50 ppm indicate the presence of 1-H at C-6 position. The singlet peak at δ 3.53 ppm indicates the presence of 1-H at C-8 position. The presence of lone pair of electron on the oxygen atom of -OH ion makes -OH proton deshielded as a result the OH peak appeared at down field. The double peak at 6.19 ppm is due to the C-OH proton at C-3 position (J = 1.03MHz) again two doublet at 6.42 ppm and 6.90 ppm are due to presence of -OH at C-5 and C-7 position (J = 1.06 and 1.04, respectively). The multiplet at 7.56 ppm is due to 2′ 1H of phenyl ring substituted at 2 position of the coumarin moiety (J = 1.00 MHz). The doublet at 7.68 ppm is due to one proton present in the substituted phenyl ring at C-5′ position. Singlet at 9.32 ppm is due to the 1H at C-6′ position. The singlet peak

TABLE-2
UV SPECTRA DATA INTERPRETATION OF ISOLATED COMPOUND FROM *n*-BUTANOLIC EXTRACT OF FRUITS OF *Flacourtia jangomas* Lour.

S. No.	Preparation	λ_{max} (nm) of compound **1**	Spectral effect	Structural diagnosis
1	Methanol solution of compound	376, 246	–	Flavone-3-ol
2	Methanol solution of compound + 3 drops of sodium methoxide solution	411	35 nm shift in band I	4'-C=O
3	Methanol solution of compound + 6 drops of aluminium chloride	396,246	20 nm Shift in band I	5-OH Free
4	Methanol solution of compound + 6 drops of aluminium chloride and 3 drops of hydrochloric acid	401, 246	25 nm shift in band I	Presence of di-OH in B ring
5	Methanol solution of compound + powdered NaOAc	246	Lack of shift in band II	Absence of free 7-hydroxyl group

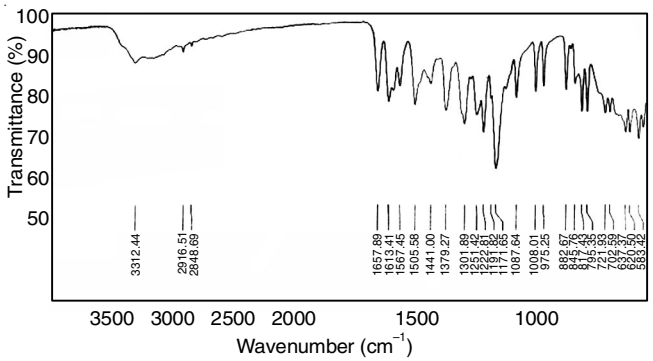

Fig. 1. IR spectrum of compound isolated from *n*-butanolic extract of roots of *Flacourtia jangomas* Lour.

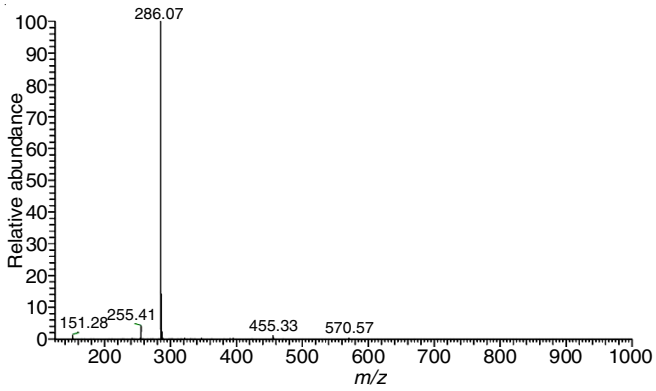

Fig. 2. MASS spectra of compound isolated from *n*-butanolic extract of roots of *Flacourtia jangomas* Lour.

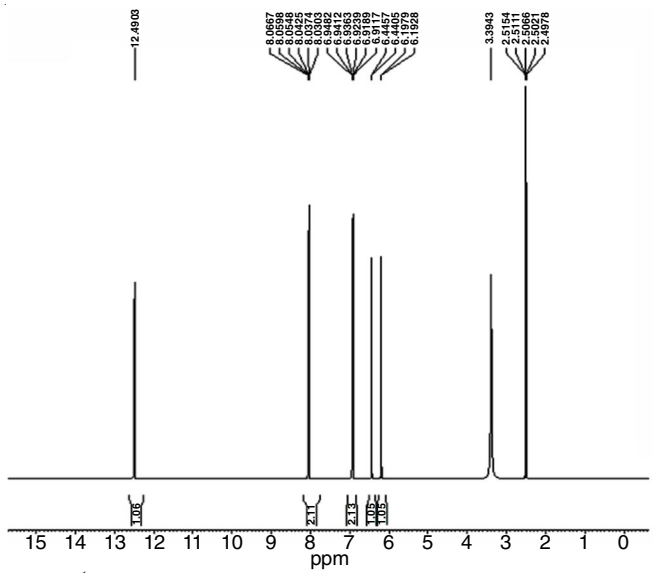

Fig. 3. ^{1}H NMR of compound isolated from *n*-butanolic extract of roots of *Flacourtia jangomas* Lour

at 10.81 and 12.48 ppm are due to presence of C-OH proton at C-3′ and C-4′ position.

^{13}C NMR spectrum of the compound revealed the presence of 15 carbons (Fig. 4). A signal at δ 175.9 in ^{13}C NMR spectrum indicates the presence of conjugated carbonyl group in the compound. The prominent peaks at δ 93.33 and 98.15 ppm correspond to the carbon atom at position 2 and 6 respectively. The peak at δ 102.98 corresponds for C-(C=O) at positon 4, the peak at δ 135.68, 145 and 146.76 corresponds to the carbon atom (C-OH) at position 3, 5 and 7, respectively. The prominent

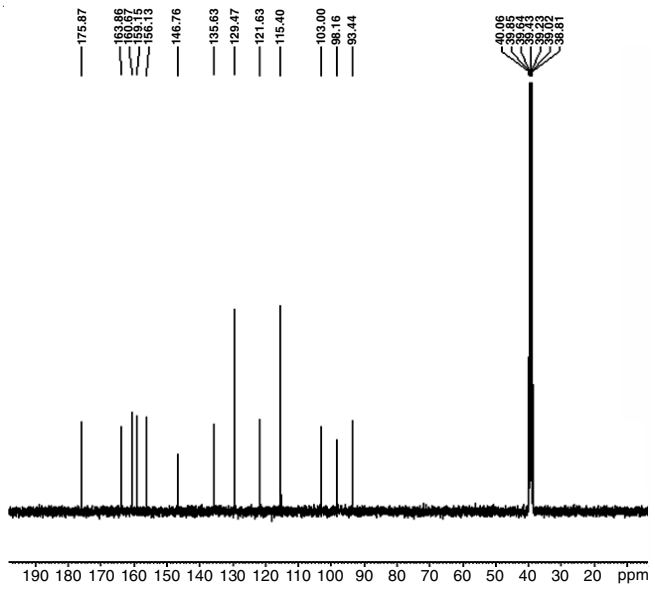

Fig. 4. ^{13}C NMR of compound isolated from *n*-butanolic extract of roots of *Flacourtia jangomas* Lour

peak at 115.03 corresponds to the carbon atom at position **8**. The peak at δ 115.57 and 119.97 ppm are due to the fused carbon between two rings of chromen nucleus at position **9** and **10**. The prominent peak at δ 163.82 and 175.78 ppm is due to the C atom (C-OH) at position **3′** and **4′** of substituted phenyl ring. Peak at δ 121.94 ppm depicted the C atom at **1′** position. Finally peak at δ 174.64, 156.11 and 160.57 ppm is due to the carbon atom (CH) at position **2′**, **5′**, **6′**, respectively.

On the basis of physical properties and spectroscopic data, structure of compound was identified as 3,5,7-trihydroxy-2-(4 hydroxyphenyl)-4*H*-chromen-4-one (**1**).

Acute dose toxicity study: The *n*-butanolic extract did not show any toxic reactions and mortality up to a dose of 2000 mg/kg for the extract and 2.5 mg/kg for the isolated compound. No changes in food consumption, water intake or behaviour (tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma) were observed in the mice after dose administration. Hence, dichloromethane at different concentrations *viz*., 100, 500 and 1000 mg/kg were taken as treatment dose for this study.

Anticancer activity of *n*-butanolic extract of *Flacourtia jangomas*: Administration of *n*-butanolic extract at the doses of 500 mg/kg, 1000 mg/kg and isolated compound at 2.5 mg/kg significantly decreased the tumor isolated compound on oral administration. After treatment with *n*-butanolic extract at the doses, 500 mg/kg, 1000 mg/kg and isolated compound at 2.5 mg/kg in Dalton-bearing mice significantly increased the RBC count and H_b content ($p < 0.05$) and significantly reduced the WBC count as compared with the Dalton control group (Table-3).

The anticancer activity of *Flacourtia jangomas* at the concentration of *n*-butanolic extract of *Flacourtia jangomas* 500 and 1000 mg/kg was found to be equipotent with the standard drug methotrexate at 2.5 mg/kg concentration.

In Dalton tumor bearing mice a regular rapid increase in ascites tumor volume was noted. Treatment with *n*-butanolic extract of *Flacourtia jangomas* decreases the tumor volume, tumor weight, viable tumor cell count and increased life span in a dose dependent manner of tumor bearing mice (Table-4). The

TABLE-3
EFFECT OF *n*-BUTANOLIC EXTRACT OF *Flacourtia jangomas* ON HAEMATOLOGICAL PARAMETERS OF DALTON TREATED MICE

S. No.	Treatment	RBC count (1×10^6 cells/mm^3)	WBC count (1×10^3 cells/mm^3)	Viable cells (1×10^7 cells/mm^3)	Non-viable cells (1×10^7 cells/mm^3)	Haemoglobin (g/dl)	Tumour volume (mL)
1	Dalton Control	2.23 ± 0.032**	30.67 ± 1.267	7.82 ± 0.032*	1.88 ± 0.054*	3.82 ± 1.265*	7.43 ± 0.054
2	Methotrexate 2.5mg/kg	8.56 ± 0.078*	10.98 ± 1.012**	2.50 ± 0.043**	5.32 ± 0.98	12.27 ± 0.086**	1.67 ± 0.023*
3	NB-FJ 100 mg/kg	3.78 ± 0.092	24.53 ± 0.087	6.84 ± 0.015*	2.31 ± 0.032**	6.32 ± 0.098	5.28 ± 1.25
4	NB-FJ 500 mg/kg	5.65 ± 0.076**	12.18 ± 0.067**	2.22 ± 1.434***	5.28 ± 0.089*	10.17 ± 0.096***	2.72 ± 0.165**
5	NB-FJ 1000 mg/kg	7.42 ± 0.178	14.22 ± 1.53	3.78 ± 0.983	4.78 ± 0.002**	11.47 ± 1.132	3.29 ± 0.027*
6	Compound **1** 2.5 mg/kg	8.23 ± 0.132**	11.92 ± 0.025*	2.75 ± 1.123**	5.93 ± 0.0125*	12.62 ± 0.271**	1.91 ± 0.012**

Values are mean ± SEM of 3 replicates; * Significant, **Very Significant and *** Highly Significant. NB-FJ 100-Butanolic extract of *Flacourtia jangomas* fruit at 100 mg/kg body weight, NB-FJ 500-Butanolic extract of *Flacourtia jangomas* fruit at 500 mg/kg body weight and NB-FJ 1000-Butanolic extract of *Flacourtia jangomas* fruit at 1000 mg/kg body weight.

TABLE-4
EFFECT OF *n*-BUTANOLIC EXTRACT OF *Flacourtia jangomas* ON TUMOR WEIGHT, MST AND LIFE SPAN OF DALTON BEARING MICE

S. No.	Treatment	Tumor weight (g)	MST (days)	Increase in life span (%)
1.	Dalton control	7.79 ± 0.0152	19.32 ± 0.0240*	–
2.	Methotrexate 2.5 mg/kg	0.75 ± 0.0242**	42.48 ± 0.0163**	100.05 ± 0.012*
3.	100 mg/kg (NB-FJ)	2.45 ± 0.034***	26.62 ± 0.0344*	41.84 ± 0.238**
4.	500 mg/kg (NB-FJ)	1.89 ± 0.0725*	32.21 ± 0.0233***	50.81 ± 0.187*
5.	1000 mg/kg (NB-FJ)	1.92 ± 0.0678**	35.37 ± 0.0165**	62.37 ± 0.036***
6.	Isolated compound 2.5 mg/kg	1.03 ± 0.154**	41.76 ± 0.128***	85.26 ± 0.024**

Values are mean ± SEM of 3 replicates; *Significant, ** moderately significant and ***Highly Significant. NB-FJ 100-Butanolic extract of *Flacourtia jangomas* fruit at 100 mg/kg body weight, NB-FJ 500-Butanolic extract of *Flacourtia jangomas* fruit at 500 mg/kg body weight and NB-FJ 1000- Butanolic extract of *Flacourtia jangomas* fruit at 1000 mg/kg body weight.

reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals. It may be concluded that *n*-butanolic extract of *Flacourtia jangomas* increases the life span of Dalton bearing mice by decreasing the nutritional fluid volume and arresting tumor growth. The anticancer activity of *n*-butanolic extract of *F. jangomas* shows good results for NB-FJ 500, NB-FJ 1000 and compound dose which includes significantly decreased tumor volume and viable cell count and non-viable cell count was significantly higher in above given doses of *n*-butanolic extract of *F. jangomas* treated animals when compared with Dalton cell control animals [15].

The decrease in nutritional fluid volume and arresting the tumor growth with increase in the life span of tumor bearing mice after the extract treatment is a strong indication of significant antitumor property of *n*-butanolic fraction of the plant [16]. Usually in cancer chemotherapy, the major problem encountered are myelosuppression and anaemia due to reduction in RBC or Hb content. Treatment with *n*-butanolic extract of *F. jangomas* brought back RBC, WBC and Hb content, count more or less to normal levels. This indicates that *n*-butanolic extract of *Flacourtia jangomas* possesses protective action on the hemopoietic system.

ACKNOWLEDGEMENTS

The authors sincerely thank Girijananda Chowdhury Institute of Pharmaceutical Science, Guwahati, India for providing all the research facilities to carry out this study.

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