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Evaluation of Anticancer Activity of *Mikania micrantha* Kunth (Asteraceae) Against Ehrlich Ascites Carcinoma in Swiss Albino Mice

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Subject:Pharmacology

Abstract

Context: Leaves of *Mikania micrantha* Kunth. (Asteraceae) are traditionally used by the people of North Cachar Hill region of Assam, India for the treatment of many ailments including cancer.
Objective: The present study explores the anticancer potentiality of *M. micrantha* based on the evidences from the ethnomedicianl practice of the plant. In this work *in vivo* model was used to evaluate the anticancer activity of the n-butanolic extract obtained from leaves of *M. micrantha*.
Materials and methods: The n-butanolic extract of *M. micrantha* (BEMM) containing flavonoids were selected for anticancer activity against EAC cell line in Swiss albino mice. The BEMM was assessed by evaluating tumor volume, viable and nonviable tumor cell count, tumor weight and hematological parameters of EAC bearing animals. The n-butanolic extracts were used at the doses of 250, 500 and 1000 mg/kg/day p.o (per oral).
Results*:* Administration of BEMM 500 and 1000 mg/kg/day p.o significantly decrease the tumor volume (3.23 ± 0.20ml and 4.02 ± 0.36ml), increased the life span (58.81% and 54.37%) and significantly decreased tumor mass (1.92 ± 0.067g) as compared with control. Hematological profiles were found to be nearly normal level in extract treated mice compared with tumor bearing control mice. Histopathology of liver of treated mice also shows normal array of hepatic cords radiating from central vein and smaller sinusoids for 500 and 1000 mg/kg/ day p.o extract treated mice.
Discussion and Conclusion: The results demonstrated that the extract possessing dose dependent anticancer activity attributed to the presence of polyphenolic groups.

Keywords: *BEMM, EAC cell line, $LD_{50,}$ Flavonoids*.

Introduction

The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures known as phytochemicals. These phytochemicals, often secondary metabolites present in smaller quantities in higher plants, include the alkaloids, steroids, flavonoids, terpenoids, tannins, and many others. Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been proved scientifically [Kuper *et al*., 1999]. Cancer is one of the most dreaded diseases of the 20thcentury and spreading further continuously with increasing incidence in 21st century. Cancer is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion, and distant metastases. Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into the world medicine [Dashora *et al*., 2012]. Cancer is caused by internal factors (tobacco, chemicals, radiations and infectious organisms) and external factors (mutation, hormones, and immune conditions) and can be treated with surgery, radiation, chemotherapy, hormone therapy, and biological therapy. Herbal drug formulations for the prevention and treatment of cancer appeared to play a significant role in the last three decades and the interest on natural sources of potential chemotherapeutic agent is continuing. The discovery of effective herbs and

elucidation of their underlying mechanisms could lead to the development of an alternative and complementary method for cancer prevention and/ or treatment [Nelson *et al.*, 2005].

Mikania micrantha Kunth. (Asteraceae) is a perennial climber indigenous to tropical regions especially in India, Srilanka, Thailand, China, Bangladesh, Malaysia, and Myanmar. In India, it is widely distributed and is commonly known as 'repujibuddu' and 'repujiloth' [Pattanayak *et al*., 2008]. Decoction of the leaves part has been traditionally used by the tribal of Cachar Hill of Assam, India, for the treatment of tumor. Despite of their wide spread in nature no scientific assessment for anticancer effect has been conducted. Considering their increasing recognition and consumption, the present study was under taken to evaluate the anticancer potential of these plant extracts. This present study was carried out to evaluate the *in vivo* anticancer activity of BEMM against Ehrlich Ascites carcinoma (EAC) cells in mice [Kunwar *et al*., 2005].

Material and methods

Collection and Identification of plant material

The leaves of *M. micrantha* Kunth. (Asteraceae) were collected (during March 2012) from SIRD (State Institute of Rural Development, Kahikuchi, Guwahati, India.) and identified by Dr. G.C Sarma (Department of Botany, Gauhati University, India). The voucher specimen (No: 9177) were deposited in the Departmental herbarium for future reference [Jagtap *et al*., 2006].

Animals

The study was carried out using Swiss albino mice weighing 20 ± 5 g. They were obtained from the animal house of GIPS. The mice were grouped, housed in polyacrylic cages and maintained under standard laboratory conditions (temperature 25 ± 2^{o} C) with light/ dark cycle (12/12 h).They were allowed free access to standard dry pellet diet and water *ad libitum*. The animals were acclimatized to laboratory conditions for 10 days before commencement of the experiment. All experimental studies were done after getting permission from the Institutional Animal Ethics Committee, GIPS, Guwahati, India (**GIPS/IAEC/07)**.

Preparation and Extraction of Plant material

The leaves (500 g) were coarsely powdered and subjected to successive solvent extraction by petroleum ether, ethyl acetate, dichloromethane, n-butanol and methanol. The preliminary qualitative phytochemical analysis was carried out for the presence of flavonoids, tannins, sterols, triterpenoids, alkaloids, glycoside and saponin [Mallavadhani *et al*., 2006]. The preliminary phytochemical analysis (data presented) and anticancer studies were carried out (data not presented) by taking different fractions. The n-butanolic fraction containing flavonoids has shown the significant preliminary anticancer activity and selected for further pharmacological screening [Sharma & Verma, 2009].

Acute Toxicity Study

The acute toxicity study was carried out as per the OECD guidelines 425. Initially BEMM was administered orally at a limit dose of 2000 mg/kg to a single female rat. The rat was observed closely for the first 4 hr and then periodically up to 24 hr for any toxic symptoms and mortality. After 24 hr same dose was administered to four more female rats [Saha *et al*., 2011].

Tumor Cell

The EAC induced mice were originally obtained from Department of Biotechnology NEHU (North East Hill University). The EAC cells were maintained in Swiss albino mice by intraperitoneal (i.p.) transplantation of 1x10^{6} cells / mouse after every 10 days [Litchfield *et al*., 1999].

Effect of BEMM on survival time of EAC bearing mice

Swiss albino mice were divided into six groups (n=6), they were fed with food and water *ad libitum*. All the animals in each groups received EAC Cells 1x10^{6} cells/mouse (0.1 ml of EAC cell/10 g body weight i.p.). This was taken as day 0.

Group I:- Group received 0.9% normal saline orally (Normal).
Group II:- EAC control group (1 x 10^{6} cells) received 0.9% normal saline orally (Control).
Group III:- EAC (1 x 10^{6} cells) treated with standard methotrexate 2.5 mg/kg/day p.o (Standard).
Group IV:- EAC (1 x 10^{6} cells) treated with 250 mg/kg/day p.o of *M. micrantha* extract (BUT 250).

Group V:- EAC (1 x 10^6 cells) treated with 500 mg/kg/day p.o of *M. micrantha* extract (BUT 500).
Group VI:- EAC (1 x 10^6 cells) treated with 1000 mg/kg/day p.o of *M. micrantha* extract (BUT 1000).
All treatments were given for 9 days. The body weight and mean survival time (MST) of each group consisting of 6 mice was noted. The antitumor efficacy of *M. micrantha* was compared to that of methotrexate [Muhit *et al*., 2010] and [Nelson *et al*., 2004]. The percentage increase life span (% ILS) of each mouse was calculated by 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.

Effect of BEMM on Viable and Non viable cell count of EAC bearing mice

The viability and non viability of the cells were checked for the above groups by tryphan blue assay. The cells were stained with tryphan blue (0.4 % in normal saline) dye. Upon staining, the viable cells did not take the stain while the non viable cells were stained blue and counted by using Invitrogen Auto cell counter [Gopal and Shenoy, 2003] and [Sur *et al*., 1994].

Effect of BEMM on Hematological parameter of EAC bearing 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 by using an automatic analyzer. Half of the animals from each group were sacrificed and checked for tumor volume [Oberling and Guerin, 1994].

Statistical Analysis

Statistical significance (*p*) was calculated by one-way ANOVA between the treated groups and the EAC control group followed by Dunnett's *post hoc* test of significance where, $p<0.05$, $p<0.01$ and $p<0.0001$ considered being significant, very significant and highly significant, respectively. All data are expressed as mean ± S.E.M (n = 6 mice per group) [Hogland *et al*., 2007].

Histopathological study

A part of the dissected liver from sacrificed animals of all the groups were cleared off of the surrounding tissues and kept in 10% buffered neutral formalin, dehydrated in alcohol, and then embedded in paraffin. The paraffin blocks were sectioned at a size of 5-µm and stained with haematoxylin and eosin dye and observed under light microscope for the array of hepatic cords radiating from the central vein and size of sinusoids [Rosenberg *et al*., 2002] and [Yan LL *et al*., 2009].

Results & discussion

Results

Preliminary phytochemical analysis of various extracts of *M. micrantha* Kunth. (Asteraceae) demonstrated strong positive test for flavonoids, saponin glycosides, tannins and steroids (Table B 1). BEMM did not show any toxic reactions and mortality up to a dose of 2000 mg/kg. So Lethal Dose (LD_{50}) of BEMM should be more than 2000 mg/kg. For the current research work BUT 250, BUT 500 and BUT 1000 were taken as treatment dose. Antitumor activity of BEMM against EAC tumor bearing mice were assessed by tumor volume, tumor weight, cell count (viable and non viable), MST and percentage increase in life span. The tumor volume, tumor weight and viable cell count were found to be significantly increased and non viable cell count was significantly decreased in control animals when compared with normal animals (Fig A 1, 2 &3). Administration of BEMM at the doses of BUT 500 and BUT 1000 significantly decreased the tumor volume and viable cell count. Non viable cell count was significantly higher in BEMM treated animals when compared with control animals. Further the mean survival time (MST) was increased to 32.21 (%ILS= 58.81%) for BUT 500 and 31.37 ((%ILS= 54.37%) for BUT 1000 on oral administration of BEMM (Table B 2).

There was increased level of WBC and decreased level of hemoglobin (Hb) and RBC in EAC control group compared to normal group. After treatment with BEMM at the doses of BUT 500 and BUT 1000 in EAC bearing mice significantly increased the RBC count and Hb content ($p<0.05$ and $p<0.001$) and significantly reduced the WBC count as compared with the

EAC control group (Fig A 4, 5 and 6). In EAC treated mice liver section showed dilation and congestion in the central and portal veins of liver with respect to control mice (Fig A 7 b). The microscopic examination of liver reveled thickening in hepatic capsule with inflammatory and pigmented cells as well as diffuse kuffer cells. The treatment with BEMM has reduced most of the pathological alteration observed in EAC control group. The liver section showed few inflammatory cells infiltration in the hepatic parenchymal associated with slight congestion in the central vein (Fig A 7. d, e and f). The treatment group of BUT 500 and BUT 1000 shows normal array of hepatic cord radiating from central vein and smaller sinusoids.

Discussion

Synthetic anticancer drugs cause nonspecific killing of cells, where as natural products provides protective and therapeutic actions to all cells with low cytotoxicity and are beneficial in producing nutrient repletion to compromised people. Therefore, there is a need for new templates to use in the design of potential chemotherapeutic agents. Many of the plant derived anticancer drugs such as vinblastine, vincristine, taxol and camptothesin have greatly contributed to the efficacy of cancer chemotherapy. However these plant derived anticancer drugs have side effects and toxicity. Hence there is a great potential for the discovery of newer anticancer drugs from the untapped reservoir of the plant kingdom [Fotsis *et al*., 1997].

The present study was undertaken to evaluate the anticancer activity of BEMM at the doses BUT 250, BUT 500 and BUT 1000 in EAC tumor bearing mice. In EAC tumor bearing mice a regular rapid increase in ascites tumor volume was noted. Ascites fluid is the direct nutritional source for tumor cells and a rapid increase in ascites fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells. Treatment with BEMM decreases the tumor volume, tumor weight, viable tumor cell count and increased life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals. It may be concluded that BEMM increases the life span of EAC bearing mice by decreasing the nutritional fluid volume and arresting tumor growth. The anticancer activity of the BEMM shows good results at BUT 500 & BUT 1000 dose which includes significantly decreased tumor volume and viable cell count and non viable cell count was significantly higher in the above given doses of BEMM treated animals when compared with EAC control animals. [Hamid *et al*., 2011]

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 an strong indication of significant antitumor property of the n-butanolic fraction of the plant [Nisa *et al*., 2011].

Usually in cancer chemotherapy the major problem encountered are myelosuppression and anaemia due to reduction in RBC or HB content. Treatment with *M. micrantha* brought back the HB content; RBC and WBC count more or less to normal levels. This indicates that BEMM possesses protective action on the hemopoietic system.

In the present work the preliminary phytochemical study of n-Butanolic extract indicated the presence of flavonoid, saponin glycoside and tannins. Flavonoids such as quercetin, kaemferol and their glycosides have been shown to possess antimutagenic and antimalignant effect. Furthermore, flavonoids have a chemopreventive role in cancer through their effect on signal transduction in cell proliferation and angiogenesis [Hayo jeong *et al*., 2009].

The mentioned results were further supported by the histopathological examination of mice bearing EAC and/or EAC mice administered with BEMM. There was a diminishing in pathological structure, to a great degree, towards normal intact histological structure. The significant improvement may be due to the presence of active principle in the extracts which were cytotoxic towards tumor cells [Fenninger *et al*., 1994].

Effects of n-Butanolic extract on viability of cells. (Fig. **A 1**) Viable cells; (Fig**. A 2**) Non-viable cells. Each point represents the mean ± S.E.M (n=06 mice per groups).

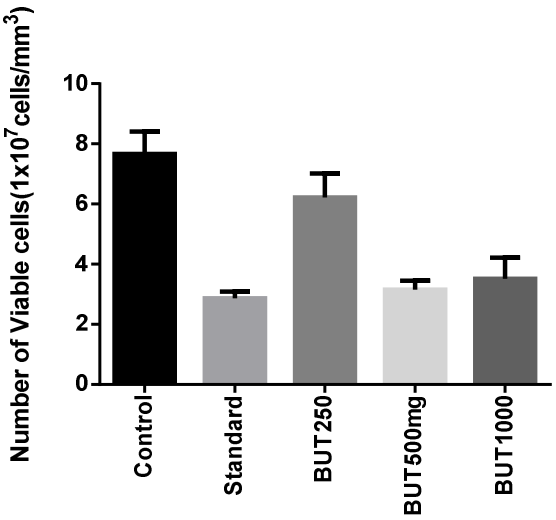

Fig.A 1. P value of control= p^{***} <0.0001, Standard = p^{***}<0.0001, BUT 250 = p^{*}<0.05, BUT 500 = p^{***}<0.0001, BUT1000 = p^{**}<0.001.
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

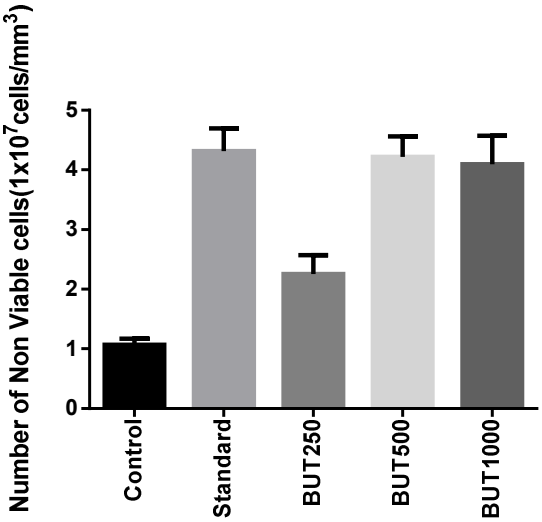

Fig A 2. P value of control = p<0.001, Standard = p^{*}<0.05, BUT 250 = p^{*}<0.05, BUT 500 = p^{***}<0.0001, BUT1000 = p^{***}<0.0001
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

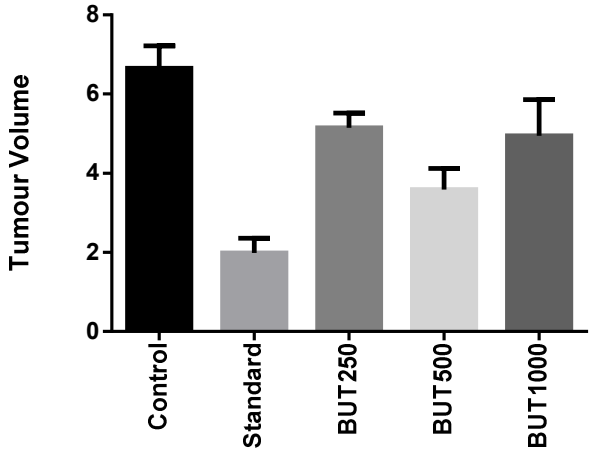

Fig. A 3. Effects of n-Butanolic extracts on tumor volume

P value of control = p^{***}<0.0001, Standard = p^{**}<0.01, BUT 250 = p^{*}<0.05, BUT 500 = p^{***}<0.0001, BUT1000 = p^{**}<0.01
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

Effects of n-Butanolic extract on blood parameters. (**Fig. A 4**) RBC count; (**Fig. A 5**) WBC count; (**Fig. A 6**) Hemoglobin level. Each point represents the mean ± S.E.M (n=06 mice per groups).

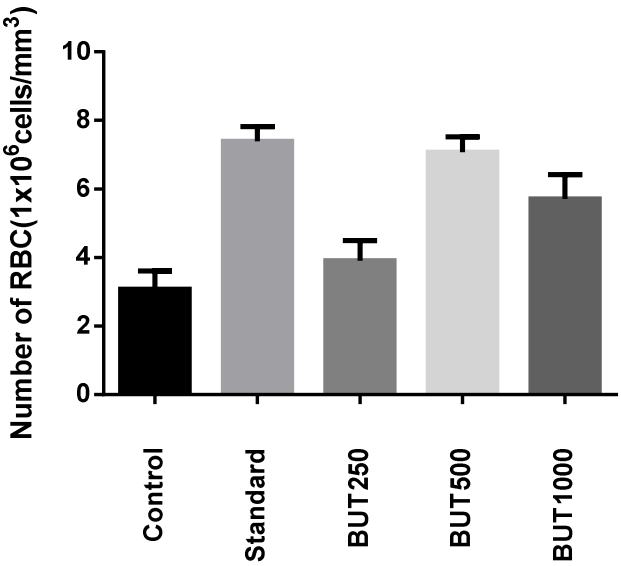

Fig A 4. P value of control = p^{*}<0.05, Standard = p^{**}<0.001, BUT 250 = p^{*}<0.05, BUT 500 = p^{***}<0.0001, BUT 1000 = p^{**}<0.001
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

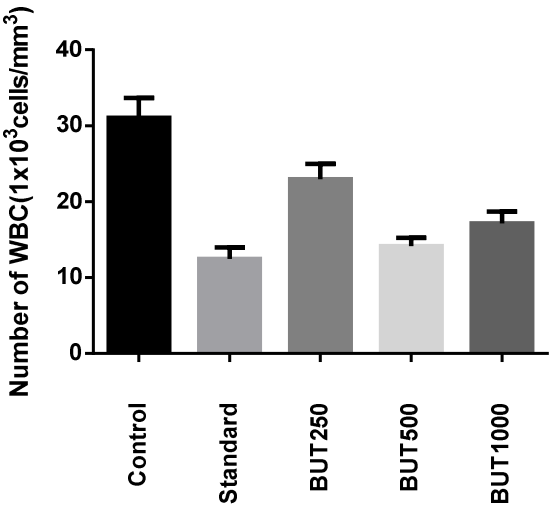

Fig . A 5. P value of control = p^{**}<0.001, Standard = p^{*}<0.05, BUT 250 = p^{***}<0.0001, BUT 500 = p^{***}<0.0001, BUT1000 = p^{**}<0.001
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

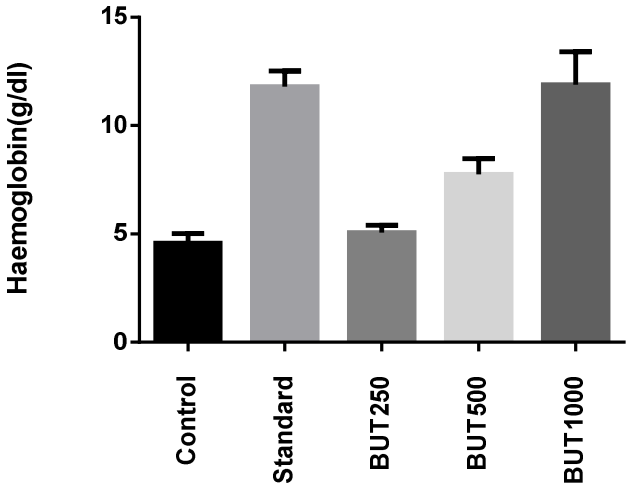

Fig.A 6. P value of control = p^{*}<0.05, Standard = p^{***}<0.0001, BUT 250 = p^{*}<0.05, BUT 500 = p^{***}<0.0001, BUT 1000 p^{**}<0.001.
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

Fig. A 7.Histopathological Interpretation

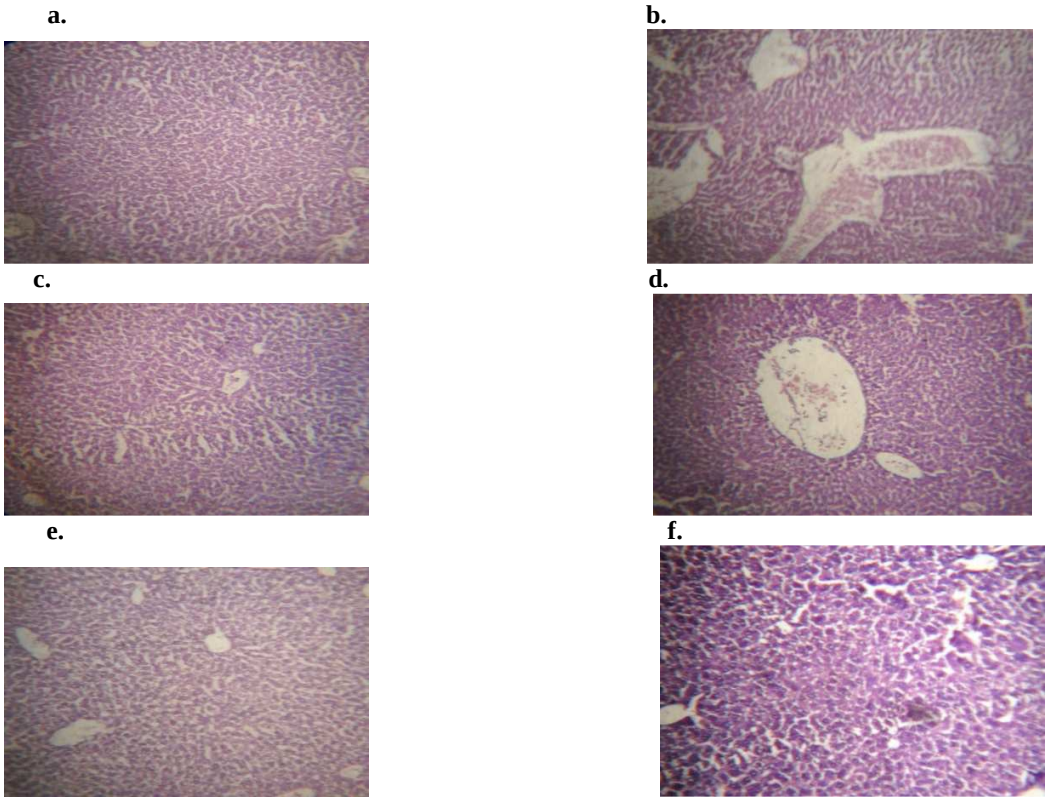

Figure A 7.a.Control: Normal nucleus, hepatocytes are radiating outward from a central vein in the centre. b. EAC Control: Cellular inflammatory infiltration, nuclear hypertrophy, debris in the central vein, hemorrhages and wide sinusoids c. Standard: Cytoplasmic degeneration have been reduced, mild cellular inflammatory infiltration and nuclei of hepatic cells are better. d. BUT 250: Cellular inflammatory infiltration, moderate nuclear hypertrophy, little debris in the central vein and wide sinusoids. e. BUT500: Cytoplasmic degeneration has nearly reduced mild cellular inflammatory infiltration and normal array of hepatic cords radiating from the central vein. f. BUT 1000: Relatively small sinusoids, cellular hemorrhage is not very prominent, no debris in the central vein and mild cellular inflammatory infiltration.

Table B 1. Phytochemical Screening of various fractions

Sr.No	Test	Pet.ether	Eth.Acetate	DCM	n-Butanol	Methanol
1	Alkaloid	-	-	-	-	-
2	Flavonoids	-	-	-	+	-
3	Saponin	-	+	+	+	+
4	Steroid	-	+	-	-	-
5	Tannin	+	-	-	-	+
6.	Mucilage	-	+	-	-	-

Table B 2. Effect of *M. micrantha* extract on tumor weight, MST and life span of EAC bearing mice.

Sr .No	Treatment	Tumor weight(g)	MST (days)	% increase in life span
1.	EAC Control	6.79±0.0172	20.32 ±0.0240*	
2.	Methotrexate 3.5mg/kg	0.85±0.0242**	42.48 ± 0.0163**	109.05
3.	Butanolic 250 mg/kg	2.48±0.034***	28.62 ± 0.0344*	40.84
4.	Butanolic 500 mg/kg	1.99±0.0725*	32.21 ± 0.0233***	58.81
5.	Butanolic 1000 mg/kg	1.92±0.0678**	31.37 ± 0.0165**	54.37

Each point represents the mean ± S.E.M (n=06 mice per groups). *p<0.05, **p<0.01and ***p<0.0001 when treated is compared with control.
p^{*} = Significant, p^{**} = Very significant, p^{***}= Highly significant

Conclusion

The present study revealed that BUT 250, BUT 500 and BUT 1000 significantly increased the life span of the mice when compared to the EAC control. The ultimate criteria for judging the potency of any anticancer drug are prolongation of life span and decrease in WBC. The butanolic extracts delayed the cell division, thereby suggesting the reduction in EAC volume and increased survival time in mice. Butanolic extract at the above given doses significantly improved the MST in tumor bearing mice. No visible sign of toxicity and changes in vital functions were observed in any of treated animals. The prolongation of life span is a reliable criterion for judging efficacy of anticancer drugs and the extract of this plant were able to meet this criterion. The cytotoxicity and anticancer activity of n-butanolic extract is probably due to the presence of these flavonoids. The present research work has a bright prospect in isolation of the active constituent from the extract which can be proved to be a potent anticancer agent.

The present research work aimed to find some new solution against one of the most disappointing health situation in today's world i.e. cancer. The further successful nurturing of this project may provide some relive to the millions of people those who were suffering from this disease.

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