Dr. Yanis Musdja, M.Sc. <yanis.musdja@uinjkt.ac.id>

[AJPCR] Article Review Request

4 pesan

editor ajpcr <ajpcr@innovareacademics.in> 28 Agustus 2019 13.18
Kepada: Muhammad Yanis Musdja <yanis.musdja@uinjkt.ac.id>

Muhammad Yanis Musdja:

I believe that you would serve as an excellent reviewer of the manuscript, "ANTITUMOR ACTIVITY OF CRATEAVA MAGNA IN TRANSPLANTABLE TUMOR MODELS IN MICE," which has been submitted to Asian Journal of Pharmaceutical and Clinical Research. The submission's abstract is inserted below, and I hope that you will consider undertaking this important task for us.

Please log into the journal web site by 2019-09-11 to indicate whether you will undertake the review or not, as well as to access the submission and to record your review and recommendation.

The review itself is due 2019-09-18.

Submission URL: https://innovareacademics.in/journals/index.php/ajpcr/reviewer/submission?submissionId=35479&reviewId=65589&key=AwuihpZn

Thank you for considering this request.

editor ajpcr
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"ANTITUMOR ACTIVITY OF CRATEAVA MAGNA IN TRANSPLANTABLE TUMOR MODELS IN MICE"

Abstract

Objective: The present study aims to evaluate the anticancer and immunomodulatory activity of both the alcoholic and aqueous extracts of *Crateava magna* (ALCM and AQCM) in solid tumor model and ascites tumor model in mice.

Methods: The study was divided into in vitro and in vivo sections.The*in-vitro* anti-oxidant activity of both the extracts was evaluated bywell-established anti-oxidant assays such as DPPH radical scavenging, reducing power, ABTS radical scavenging. Both the extracts were subjected for *in-vitro* preliminary cytotoxicity screening by brine shrimp lethality assay and Trypan blue exclusion assay. The in-vivo antitumor activity of ALCM and AQCM was assessed at the doses of 100mg/kg and 200mg/kg body weight respectively by DLA induced solid tumor model and EAC induced ascites tumor model in mice. Solid tumor volume, tumor weight, and %inhibition of the tumor weight in treated and untreated groups were evaluated.In addition to the investigation of antitumor activity, the alcoholic and aqueous extract of *Crateava magna* leaves, were also evaluated for their possible immunomodulatory activity.

Results: The studiesshowed that the alcoholic extract of *Crateava magna* (ALCM) demonstrated more significant antioxidant activity which further showed more immunomodulatory activity when compared to aqueous extract of *Crateava magna*(AQCM).

Conclusion: The study proves that the plant extract is of higher therapeutic efficacy in cancer. However the extracts require more exploration toward its usefulness in drug discovery.

Dr. Yanis Musdja, M.Sc. <yanis.musdja@uinjkt.ac.id> 28 Agustus 2019 23.47
Kepada: editor ajpcr <ajpcr@innovareacademics.in>

Dear editor ajpcr

I hereby declare that I accept a request from the Editor of AJPCR to conduct a review of the paper with the title:

"ANTITUMOR ACTIVITY OF CRATEAVA MAGNA IN TRANSPLANTABLE TUMOR MODELS IN MICE"

I will send the results of my revision before 2019-09-11

Thank you very much for your kindness and the trust you gave me

Best Regards

Dr. Muhammad Yanis Musdja
[Kutipan teks disembunyikan]

Dr. Yanis Musdja, M.Sc. <yanis.musdja@uinjkt.ac.id> 3 September 2019 17.50
Kepada: editor ajpcr <ajpcr@innovareacademics.in>

Dear Editor AJPCR

I have already sent the results of my revision to the title:

"ANTITUMOR ACTIVITY OF CRATEAVA MAGNA IN TRANSPLANTABLE TUMOR MODELS IN MICE"

Based on the conditions requested, proof of shipment as attached

Thank you for your attention and cooperation

Best Regards
Dr. Muhammad Yanis Musdja

Pada tanggal Rab, 28 Agu 2019 pukul 13.19 editor ajpcr <ajpcr@innovareacademics.in> menulis:
[Kutipan teks disembunyikan]

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ANTITUMOR ACTIVITY OF *CRATEAVA MAGNA* IN TRANSPLANTABLE TUMOR MODELS IN MICE

ABSTRACT:

Objective: The present study aims to evaluate the anticancer and immunomodulatory activity of both the alcoholic and aqueous extracts of *Crateava magna* (ALCM and AQCM) in solid tumor model and ascites tumor model in mice.

Methods: The study was divided into in vitro and in vivo sections.The*in-vitro* anti-oxidant activity of both the extracts was evaluated bywell-established anti-oxidant assays such as DPPH radical scavenging, reducing power, ABTS radical scavenging. Both the extracts were subjected for *in-vitro* preliminary cytotoxicity screening by brine shrimp lethality assay and Trypan blue exclusion assay. The in-vivo antitumor activity of ALCM and AQCM was assessed at the doses of 100mg/kg and 200mg/kg body weight respectively by DLA induced solid tumor model and EAC induced ascites tumor model in mice. Solid tumor volume, tumor weight, and %inhibition of the tumor weight in treated and untreated groups were evaluated.In addition to the investigation of antitumor activity, the alcoholic and aqueous extract of *Crateava magna* leaves, were also evaluated for their possible immunomodulatory activity.

Results: The studiesshowed that the alcoholic extract of *Crateava magna* (ALCM) demonstrated more significant antioxidant activity which further showed more immunomodulatory activity when compared to aqueous extract of *Crateava magna*(AQCM).

Conclusion: The study proves that the plant extract is of higher therapeutic efficacy in cancer. However the extracts require more exploration toward its usefulness in drug discovery.

Keywords:*Crateava magna*, Anti-tumour, Antioxidant, Immunomodulatory.

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Commented [U2]: It is best to write the full name first and then the abbreviation 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

Commented [U3]: It is best to write your full name first, and then the abbreviation, Dalton's lymphoma ascites (DLA)

Commented [U4]: It is best to write the full name first, then the abbreviation, Ehrlich's ascites carcinoma (EAC)

INTRODUCTION:

Cancer is a disease ranked as second leading cause of morbidity next to the cardiovascular diseases[1]. In spite of extensive global research aimed at amending the miserable outcomes of cancer, no clear decline in the overall mortality rate has been observed in the past 30 years. From the ancient period plants were use in the management of cancer[2]. Over 60% of the presently used anti-cancer agents are derived in one or the other from the natural sources like plants, marine organisms and microorganisms[3]).13% of all human deaths occur due to cancer. The American cancer society projects that around 7.6 million people die every year due to cancer[4]. Some of the new chemotherapeutic agents which are currently available for use in a clinical setting are plant based components which includes vinca alkaloids, taxanediterpenoids, epipodophyllotoxin ligands, camptothecin and derivatives of quinolone alkaloid.

Crateava magna (CM) belonging to the family Capparidaceae is commonlyused in the treatment of urinary disorders that reoccur due to progress of antibiotic resistance caused by the infecting organism. In traditional medicine, Leaves have lithotriptic, diuretic, demulcent and tonic properties[5] .Externally, the paste or its leaves is applied in cervical adenitis, abscess and edematous wounds. The same paste is salutary in rheumatic joint for relief of pain. In folk medicine, its stem pith in the tribal people of Kandhamal district of Orissa known as Eastern Ghats of India that the bark is used for lactation after child birth, treatment of urinary disorders, bladder stones, fever, nausea and gastrointestinal irritation. Earlier research done on the plant reports the *in-vivo* sedative and cytotoxic activities of methanolic extract.

The alcoholic extract decreased the locomotors activity of mice in Elevated plus maze, open field and hole cross tests. The alcoholic extract exhibited dose dependent cytotoxicity in brine shrimp lethality assay with an IC_{50} of 59.67µg/ml as compared to 0.45µg/ml exhibited by standard vincristine sulphate[6]evaluated the bark of the plant for its antipyretic activity on TAB-VACCINE induced pyrexia in rabbits. The alcoholic extract of aerial parts of *C.magna*formed a significant decrease in the bodytemperature in hyperthermic rats. Remya *et al* (2009) investigated the entire plant for the treatment of benign prostatic hyperplasia. Alam *et al* (2006) evaluated the crude extract of leaves of *C.magna* for its anti-nociceptive activity in mice. The 'acetic acid' analgesic test method in mice was used to assess the antinociceptive effect. Crude ethanolic extracts of *Crataeva magna* (250–500 mg/kg PO) showed dose-dependent, antinociceptive effect against chemically induced nociceptive pain stimuli in mice. The in-vivo and in-vitro antitumor activity of leaves of *Crateava magna* has not been reported so far. Hence the current study emphases the antitumor activity of ethanolic and aqueous extract of the *Crateava magna.*

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MATERIALS AND METHOD:

Plant material and extraction:

The plant material **(**Fresh leaves of *Crateava Magna*) was procured from Natural Remedies Pvt. Ltd. Bangalore and was authenticated by Dr.M.N.Nagandini, Department of Pharmacognosy, JSS College of Pharmacy, Mysore, India. The coarse powder was extracted with 95% ethanol in the Soxhlet extractor. The residue was dried over water bath to obtain the alcoholic extract of *Crateava magna* (ALCM).The aqueous extract was prepared by cold maceration method. After completion of

extraction the marc was filtered through muslin cloth followed by filter paper and concentrated and dried on water bath to obtain the aqueous extract of *Crateava magna* (AQCM).

Cell lines:

Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines were procured from Amala Cancer Research Center, Thrissur, India.

IN-VITRO ANTIOXIDANT ACTIVITY :

DPPH free radical scavenging assay:

2ml of 0.1mM of DPPH was added to 2ml methanol. The absorbance was taken immediately, at a wavelength of 517nm, which serves as control.2ml of DPPH was added to 2ml of test extracts (ALCM and AQCM) and shaken well. The test samples were incubated for 30min The absorbance was recorded against methanol as blank at a wavelength of 517nm[7**]**. The % scavenging activity and IC_{50} was determined[8].

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}$$

***ABTS- Decolorizing assay*:**

Different concentrations of test extracts (ALCM and AQCM) (50,100,150,200,250 and 300µg/ml) was reacted with 5 ml of fresh $ABTS^{\bullet+}$solution. Absorbance was measured against blank after 15 minutes of incubation in the dark at 734 nm.L-Ascorbic acid was used as a Standard.The % scavenging activity was determined and IC_{50} was calculated[9**]**.

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}$$

Reducing power assay:

1 ml of different concentrations of sample(20,40,60,80 and 100µg/ml) were assorted with 2.5 ml of PBS (0.2 M,pH 7.6) and 2.5 ml of potassium ferricyanide (1%W/V). The combined solution was incubated at 50°C for 30 minutes. 2.5 ml of trichloroaceticacid(10%W/V)was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes.2.5ml of the upper layer of the solution was mixed with 2.5ml of distilled water and 0.5ml of FeCl3(0.1%W/V), the absorbance was measured against blank at 700nm[10**]**.

IN VITRO CYTOTOXIC ACTIVITY:-

Trypan blue exclusion assay:

Ehrlich ascites carcinoma (EAC) and Dalton lymphoma ascites (DLA) cells were aspirated from the peritoneal cavity of tumor-bearing mice. Two to three times the cells were washed with PBS and one million cells were incubated with different concentration of ALCM and AQCM (20,40,60,80,100µg/ml)[11**]**.Cyclophosphamide (50µg/ml) alone in a volume of 0.1ml was used as a control, in 1 ml of PBS for 3 h at 37°C in sterile test tubes. 100 µL of Trypan blue dye (0.4% in PBS) was added after incubation and the total number of dead (stained) and viable (unstained) cells were counted using a haemocytometer[12]:

$$\% \text{ Cytotoxicity} = \frac{\text{No of Stained cells} * 100}{\text{Total no of cells}}$$

Brine Shrimp Lethality Assay:

The test extracts of ALCM and AQCM were prepared in sea water. 10 napullies were transfer into sample vials and were treated with different concentration of test compounds. (100, 200, 300,400,500) micro molar in 5ml of sea water for 24 hours.A drop of suspension was added to each vial. Vials were maintained under illumination. After 24 hr, number of surviving nauplii was counted using 3x magnifying glass, and the percentage cytotoxicity and IC_{50} values were determined[13**]**.

% lethality = (Dead nauplii- Total nauplii) * 100
Total nauplii

Animals:

All the animal experiment and handling of animals were approved by the Institutional Animal Ethics Committee, JSS College of Pharmacy, Mysore, India (IAEC approval number 134/2013).Swiss albino mice of eight to ten weeks old weighing between 25-30g were selected for study. Animals were acclimatized to the experimental room for one week prior to the experiment. Animals were maintained under controlled conditions of temperature (27 ± 2º C) and were caged in sterile polypropylene cages containing sterile paddy husk as bedding material with maximum of six animals in each cage. The mice were fed on standard diet and water *ad libitum*.

***In vivo* antitumor activity of ALCM and AQCM against EAC inoculated Ascites tumor model:**
EAC cells were procured from the peritoneal cavity of an EAC bearing mouse, after 15 days of tumor transplantation. The ascitic fluid was withdrawn and suitably diluted in phosphate buffer saline to obtain a stock cell concentration of 10^7 cells per ml. For ascitic tumor 2.5×10^6 EAC cells (0.25 ml of stock suspension) was injected intraperitonially to each mouse[15]. Treatment was started after 24 h tumor inoculation, continued for 15 days and various parameter were evaluated[14**]**:

% Increase in body weight as compared to day "0"weight:
The animals were weighed on the day of inoculation and once in 3 days thereafterin the post inoculation period. The %increase in body weight was calculated as follows[16**]**:
% increase in weight = (animal weight on respective day)-1X 100
Weight of animal on day 0

Mean survival time (MST) and Increase in life span [%ILS]:
Total number of days an animal survived from the day of tumor inoculation was counted. Subsequently the mean survival time was calculated. The %ILS was calculated as:-
% ILS = [(MST Test – MST Conentration)] X 100
MST Concentration

Hematological parameters:
In order to assess the effect of treatment on the hematological status of animals, blood was collected from retro orbital plexus of the animals in to sodium citrate treated micro centrifuge tubes and following parameters were monitored[17**]**.

Determination of total WBC count:
Blood was drawn up to 0.5 mark of WBC pipette and excess of blood from the tip was wiped off. The collected blood was diluted with WBC diluting fluid(1% acetic acid,1 ml Glacial acetic acid was added to 99ml distilled water) up to mark 11 and was mixed thoroughly for 1 minute. The fluid was

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charged on the counting chamber of Hemocytometer. Total number of ells in all the four corner squares of WBC counting area was counted using 10 x objective. Them the total WBC count was calculated from the number of cells/mm using the formula:

Total WBC count = Total no. of cells in the WBC counting area× 50

Determination of Total WBC count:

Blood was drawn up to 0.5 mark of RBC pipette and the excess of blood was wiped off from the tip. Then the pipette was filled to 101 marks with RBC diluting fluid and mixed thoroughly for 1 minute. The fluid was charged on the counting chamber of Hemocytometer. Total number of cells in RBC counting area was counted using 45 x objective. The average number of cells/mm^3 was calculated using the formula:

Total RBC count=Total no. of cells in the RBC counting area×1000

Where, 1000 is the volume of RBC counting area.

Estimation of serum biomarkers:

Estimation of Serum glutamic oxaloacetictransaminase(SGOT):

Four volumes of Reagent-1 (TRIS,L-Aspartate,MDH and LDH) were mixed with one volume of Reagent-2 (Alpha-ketoglutarate,NADH). These two reagents were mixed and incubated for 1 minute at 37˚C.Abosarbance was measured after 1 minute using mercksemiautoanalyser[18**]**.*Estimation of Serum glutamate pyruvate transaminase (SGPT):*

The 1000µl of working solution consisting of Trisbuffer,L-alanine,LDH,α-ketoglutarate and NADH and 10µl of sample solution were mixed and incubated for 1 minute. Absorbance was read after 1 minute using mercksemiautoanalyser.

Estimation of Alkaline phosphatase (ALP):

Four volumes of Reagent-1 (Diethanolamine,Magnesiumcholide) was mixed with one volume of Reagent-2 (p-Nitrophenyl phosphate). The 500µl of working solution and 10µl of sample solution were incubated for 50 seconds and absorbance was read using semiautoanalyser.

Estimation of Lactate Dehydrogenase:

Four volumes of Reagent-1 (Imidazole pyruvate-65mmol/L) was mixed with one volume of Reagent-1(NADH-0.18mmol/L) and was incubated. 3.0µL of working sample and 50 µL of sample solution was incubated. Then the initial absorbance of the sample was measured. The absorbance was read at 1 minute interval thereafter for 3 minutes. The difference between the absorbance and the average absorbance difference per minute was calculated (ΔA/min)

Estimation of liver endogenous antioxidant enzymes:

3ml of hydrogen peroxide phosphate buffer solution was added to 50 µl of tissue homogenate and absorbance was measured at 240nm[19**]**.

Estimation of Glutathione-S-Transferase (GSH):

500µL of TCA solution was added to 500µl of tissue homogenate and then it was centrifuged.500µl of supernatant was incubated with 3ml of PBS and 500µL DTNB for 10 min at room temperature.Absorbance was read at 412 nm against blank and glutathione concentration was calculated[20**]**.

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Commented [U9]: It is best to write the full name first and then the abbreviation, 5'-Dithiobis- (2-nitrobenzoic acid) (DTNB),

Estimation of Superoxide dismutase(SOD):
1850µL of carbonate buffer 0.1 M was added to 50µl of tissue homogenate.100 µl of Adrenaline was added directly in cuvette after putting in UV cuvette holder, Absorbance was read at 480nm (A0-A60) (Kinetic method)

DNA Fragmentation assay:
A DNA ladder represents a band of DNA molecule of varying lengths sperated in agarose gel by electrophoresis. Agarosewas added to electrophoresis buffer to prepare gel. The solution was placed in microwave oven until the agarose particles were liquefied. The molten agarose was cooled and Ethidium bromide was added and the gel was prepared. Fixed number of cells were treated with different concentration fractions, vechicle control and standard for 48 hourss. Cells were then collected , centrifuged and lysis buffer was added to the cell pellet. Cell pellet in lysis buffer was incubated for 1 hour with RNAse solution for 1 hour. The processed cells were cooled and loaded into wells of agarose gel followed by electrophoresis. Images were taken using gel documentation system[21**]**.

***In vivo* antitumor activity of ALCM and AQCM against DLA inoculated solid tumor model:**
After 15 days of tumor transplantation the DLA cells were aspirated from the peritoneal cavity of DLA bearing mouse. Tumor viability was determined by Trypan blue exclusion test and total number of viable cells were counted using Haemocytometer. The Ascitic fluid was appropriately diluted in saline to get a concentration of 10^7 cells/ml of tumor cell suspension. Around 0.1 ml of this solution was injected subcutaneously to the right hind limb of mice to obtain a solid tumor. After 24 h tumor inoculation the treatment was started and was continued for 15 days, tumor volume, tumor weight were assessed[22**]**.

Tumor volume:
Vernier caliper was used to measure the radii of developing tumor at 5 days interval for 1 month and tumor volume was calculated using the formula:
$V = 4/3\ \pi\ dc^2$
Where c & d represent the major and minor radii .

***Tumor weight*:**
Animals were sacrificed at the end of the fourth week under anesthesia using diethyl ether; tumor was extirpated and weighed .The percentage inhibition was calculated by the formula:
$\%\ \text{Inhibition} = (1-D/C) \times 100$
Where, C is the average tumor weight of control group, D is that of treated group.

In-vivo immunomodulatory activity:-
Carbon clearance test:
Swiss albino mice of 25-30g of body weight were used in experiment. Animals were randomized into the following six groups comprising of 6 animals each.Animals were given the test extracts for two days. The mice were administered with carbon ink suspension through tail vein after 48 hours of intraperitonial injection. Blood samples were collected from the retro-orbital vein using glass capillaries at 5min, 15min[23**]**. Collected blood sampleswere mixed with 0.1% sodium carbonate

solutionfor the lysis of erythrocytes. Absorbance was measured using the spectrophotometer at 675 nm, the phagocytic activity was expressed by the phagocytic index (K)[24**]**.

$$K = \frac{(\ln OD_1 - \ln OD_2)}{(t_2 - t_1)}$$

Where,

OD1 and OD2 are optical densities at time t1 and t2.

The clearance rate is expressed as the half-life period of carbon ink in the blood.

$t\ ½ = 0.693/K$

***Delayed type hypersensitivity*:**

Daily treatment of test extracts were given to the mice 5 days prior to the challenge. Control received vehicle on each day. On day 0, all animals were immunized with 20µl of Sheep red blood cell (SRBC) solution($5x10^9$ SRBC/ml) injected subcutaneously into their right hind footpad. After 5 days of treatment, the thickness of each animal's footpad was measured just before the challenge using a digital plethysmometer. The animals were then challenged by injecting the 20µl of SRBC solution into the left hind footpad (deemed time 0). Foot thickness was re measured after 24 hr[(25)].

The difference between the thickness of left foot just before and after challenge (in mm) was taken as the measure of DTH.

$$\text{DTH Response} = \left[\frac{\text{Srbc treated paw - saline treated paw}}{\text{Srbc treated paw}} \right] \times 100$$

STATISTICAL ANALYSIS:

All experiments were done in triplicate and values were reported as Mean ± SEM. Data was analyzed by one way ANOVA followed post Tukey's multiple comparison. The level of significance was set up to $p<0.05$.

RESULTS :

DPPH –free radical scavenging assay:

Both extracts of ALCM and AQCM exhibited dose dependent free radical scavenging activity. The IC_{50} of AQCM and ALCM was found to be 89.75± 0.001µg/ml and 55.97±0.002 µg/ml respectively when compared to ascorbic acid with an IC_{50} of 3.6±0.001µg/ml which are shown in figure 1.

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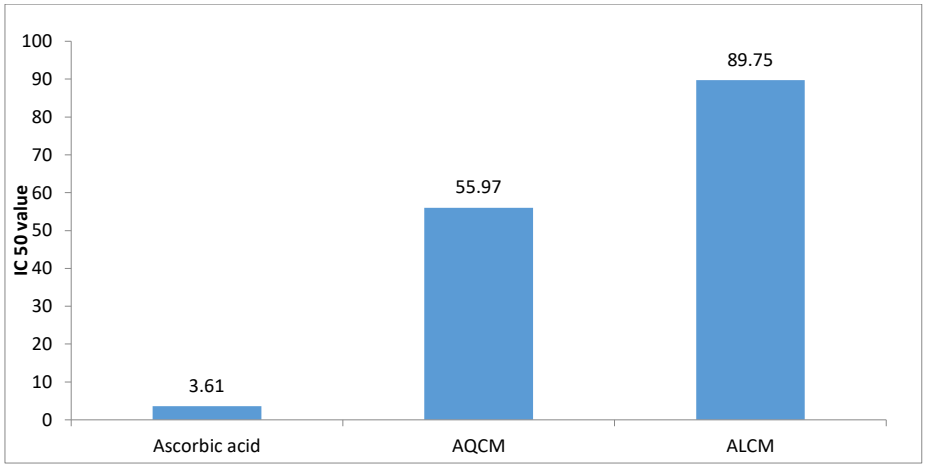


Fig.1: ABTS radical scavenging activity of ALCM and AQCM with respect to standard.All values are represented as Mean ± SEM, n=3.

ABTS- Decolorising assay:

Both the extracts ALCM and AQCM exhibited dose dependent free radical scavenging activity. The IC_{50} of AQCM and ALCM was found to be 268.70± 0.5 µg/ml and 270.07± 0.38µg/ml respectively when compared to ascorbic acid with an IC_{50} of 22.16± 0.4µg/ml as shown in figure 2.

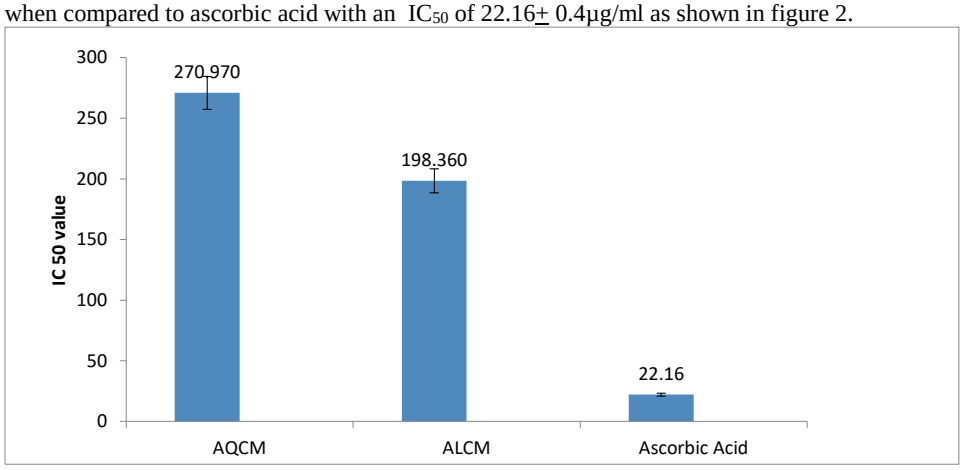


Fig. 2: ABTS radical scavenging IC_{50} of ALCM and AQCM with respect to standard. All values are represented as Mean ± SEM, n=3.

Reducing power assay:

Reducing power assay of ALCM and AQCM is shown in figure 3:

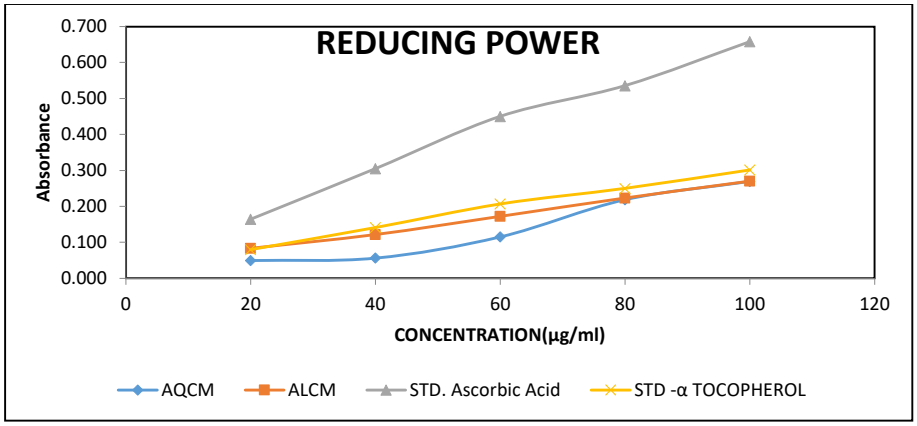


Fig.3 Reducing power activity of ALCM and AQCM with respect to standard. All the values are represent Mean ± SEM, n=3

Trypan blue exclusion assay:

In vitro cytotoxicity of extracts ALCM and AQCM on EAC cells was determined by trypan blue exclusion assay. Both the extracts showed dose dependent cytotoxicity with an IC_{50} of 298± 0.50 and 356± 0.62µg/ml respectively. The percentage cytotoxicity of ALCM and AQCM on EAC cells by trypan blue exclusion assay is shown in figure 4.

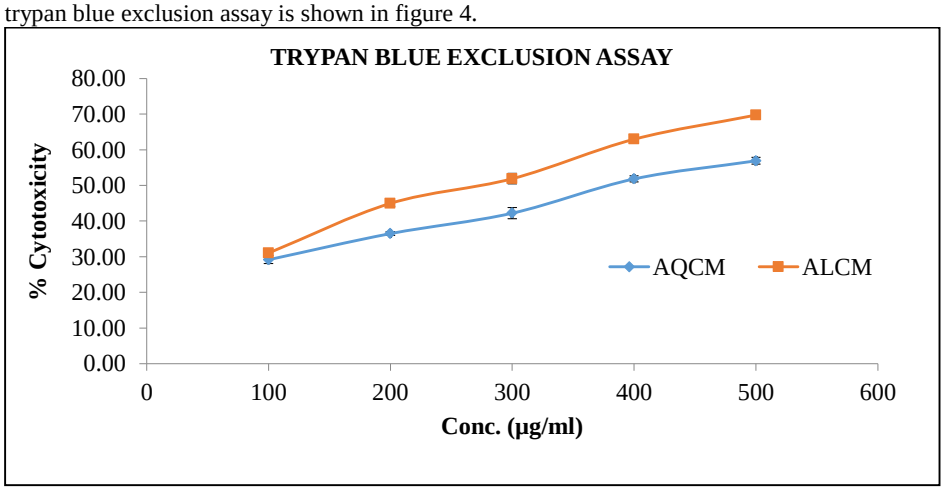


Fig.4Percentage cytotoxicity of ALCM and AQCM on EAC cells by trypan blue exclusion assay.All values are expresses as Mean ± SEM, n=3.

Brine shrimp lethality assay:

Brine shrim lethality assay was carried out on both the extracts. ALCM and AQCM showed dose dependent cytotoxicity with an IC_{50} of 88.74± 0.62 and 119.68±0.82µg/ml respectively. The percentage lethality of ALCM and AQCM by Brine shrimp lethality assay is shown in figure 5.

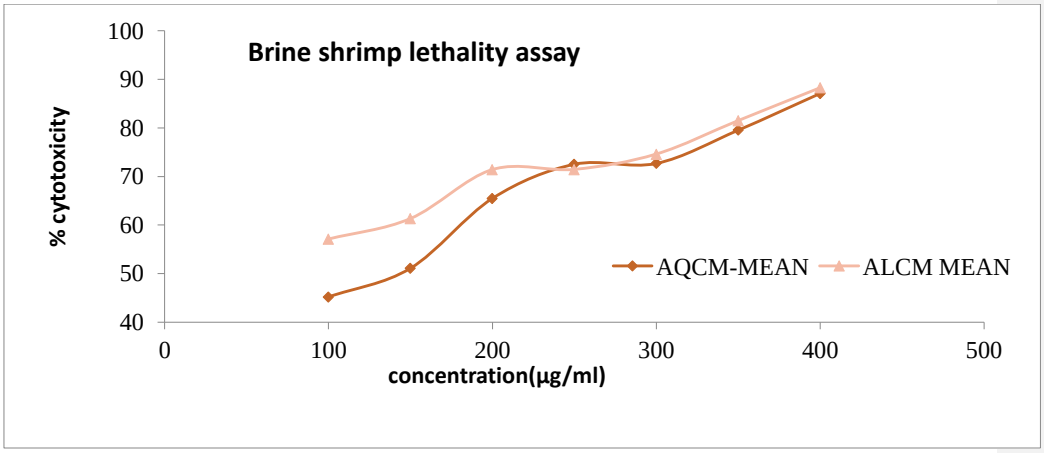
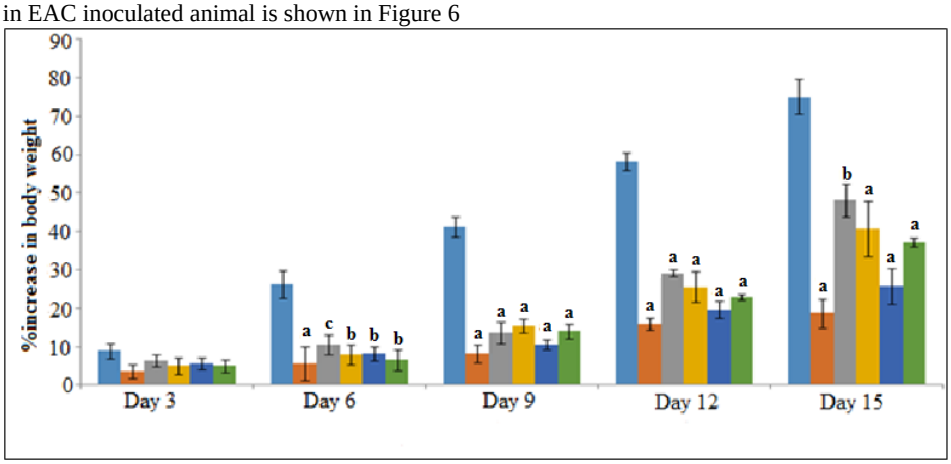


Fig.5. Percentage lethality of ALCM and AQCM by Brine shrimp lethality assay.All values are expressed as Mean ± SEM, n=3.

Effect of AQCM and ALCM on body weight changes:

Substantial increase in body weight was seen in EAC inoculated control animal with a maximum increase of (75.0± 4.5%) on day 15 compared to day 0. The Cyclophosphomide treatment significantly reduced bodyweight (18.67± 3.6%) compared to control. ALCM treatment at 200 mg/kg significantly decreased the tumor induced percentage increase in the body weight (37.19± 1.1%) and the efficacy was compared with standard. The effect of ALCM and AQCM on body weight changes in EAC inoculated animal is shown in Figure 6



Where **ontrol (CMC 0.25%)** **andard (cycle 10mg/kg)** **QCM (100mg/kg)** **QCM (200ng/kg)** **LCM(100mg/kg)** **LCM (200mg/kg)**

Fig. 6: Effect of ALCM and AQCM on body weight changes in EAC inoculated mice. All values represent Mean ± SEM, n=6. Where [a]*p*< 0.05 when compared to normal, [b]*p*< 0.05 when

compared to control. Data was analyzed by one way ANOVA followed post Tukey's multiple comparison.

Effect of ALCM and AQCM on Mean survival time and % increase in life span of EAC inoculated mice:

Mean survival time of EAC inoculated mice was 15.167±0.7 days. Standard Cyclophosphomide treatment at 10 mg/kg significantly improved the MST to 22.83±1.22 days when compared to control. ALCM at 200mg/kg increased the (MST) to 20.00±0.816 days respectively when compared to control. The % increase in life span (%ILS) of mice treated with ALCM at 200 mg/kg was 31.87%.

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Effect of ALCM and AQCM on hematological parameters in EAC inoculated mice:

To assess the influence of ALCM and AQCM treatment on hematological parameters, the total RCBs, WBCs and hemoglobin content of all the treatment groups were checked on 15th day of tumor inoculation.

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Effect on total RBC:

A significant decrease in total RBC count was seen in EAC inoculated control animal (2.84±0.05 cells/mm^3) when compared with the normal mice (5.53±0.34cells/mm^3). Treatment with Cyclophosphamide 10mg/kg significantly reversed this reduction to (5.16±0.09 cells/mm^3) as compared to control; ALCM at 100mg/kg doses increased the total RBC count to 3.51±0.81 and 4.08±0.19 cells/mm^{3}when compared to control animal.

Total WBC:

A significant rise in total WBC count was seen in EAC inoculated control animal (20.63±0.91cells/mm^3) when compared to normal animal (7.01± 0.53 cells/mm^3). Standard Cyclophosphamide treatment at a dose of 10mg/kg resulted in the reduction in the WBC count to (6.05± 0.86 cells/mm^3) when compared with control. ALCM at dose 100mg/kg and 200mg/kg significantly reversed the elevated WBC to 11.67± 0.69 cells/mm^3 and 13.72±0.63 cells/mm^3 when compared to control.

Effect of ALCM and AQCM on serum enzyme levels:

To assess the influence of ALCM and AQCM treatment on serum enzyme levels, the ALP,SGOT and SGPT of all the treatment groups were checked on 15th day of tumor inoculation.

Effect on serum glutamate oxaloacetictransaminase(SGOT):

A significant increase in serum SGOT level was seen in EAC inoculated control animal (74.967± 2.5mg/kg) when compared to normal animal (39.98±3.305mg/kg). Cyclophosphamide at 10mg/kg reversed tumor induced elevation in SGOT level(53.63±2.565mg/kg) when compared with control. ALCM and AQCM at dose of 200mg/kg decrease the elevated SGOT level to 60.083±2.735mg/kg and 61.867±3.635mg/kg compared to control.

***Effect on Serum glutamate pyruvate transaminase(SGPT)*:**

A significant increase in serum SGPT level was seen in EAC inoculated control animal (62.30±3.195mg/kg) when compared to normal animal(19.82±1.275mg/kg). Cyclophosphamide at 10mg/kg reversed tumor induced elevation in SGPT level (35.750±3.605mg/kg) when compared with control. ALCM and AQCM at both doses significantly decreased the elevated SGPT level to 41.700±3.44 5mg/kg and 44.467±1.765mg/kg compared to control.

Effect on Alkaline phosphatase(ALP):
A significant increase in serum ALP level was observed in EAC inoculated control animal (69.83±1.145mg/kg) when compared to normal animal (22.69±1.605mg/kg). Standard Cyclophosphamide treatment at 10mg/kg reversed tumor induced elevation in ALP level(30.10±0.845mg/kg) when compared with control. ALCM and AQCM at dose of 200mg/kg significantly decreased the elevated ALP level to 39.100±0.845mg/kg and 43.11±2.065mg/kg and the reduction in ALP level was comparable to standard.

Effect on serum lactate dehydrogenase(LDH):
A significant increase in serum LDH level was seen in EAC inoculated control animal (238.00±12.35mg/kg) when compared with control. ALCM at 200mg/kg dose reversed tumor induced elevation of LDH to 140.32±2.055mg/kg.

Evaluation of liver endogenous antioxidant enzymes:
To assess the influence of ALCM and AQCM treatment on endogenous liver anti-oxidant levels, of all the treatment groups were checked on 15th day of tumor inoculation.

Effect of Glutathione-S-Transferase(GSH):
A significant decrease of GSH concentration was observed in liver homogenate of ECA inoculated control mice 0.850± 0.15mg/kg when compared to normal animal 2.950±0.35mg/kg. cyclophosphamide at a dose of 10mg/kg significantly reversed the tumor induced decrease in GSH concentration 1.78±0.15mg/kg when compared with control. ALCM at 200mg/kg significantly reversed GSH concentration to 1.574±0.2305mg/kg and the efficacy was compared with standard group.

Effect of superoxide dismutase(SOD):
A significant decrease in SOD concentration was observed in liver homogenate of EAC inoculated control mice 2.28±0.25mg/kg when compared to normal animal 4.54±0.25mg/kg .Standard Cyclophosphamide treatment at 10mg/kg dose significantly reversed tumor induced decrease in SOD concentration 3.92±0.155mg/kg when compared with control. ALCM at 200mg/kg reversed GSH concentration to 3.09±0.2 5mg/kg significantly in treated animals the efficacy was compared with standard group.

Influence of extracts on DNA fragmentation assay:
The effect of extracts on DNA fragmentation assay is shown in figure 7:

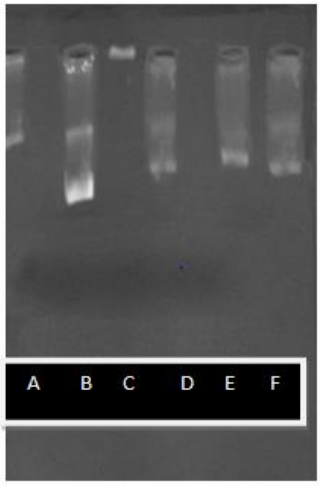

Fig.7 Effect of extracts on DNA fragmentation assay. Where C-control,B-standard, A-ALCM(100mg/kg), D-ALCM(200mg/kg),E-AQCM(100mg/kg), F-AQCM-(200mg/kg).

In-Vivo anticancer activity of ALCM and AQCM on DLA inoculated solid tumor model:
The weight of solid tumor in control animal was found to be 6.85±0.226gm by the end of fourth week. Cyclophosphamide at 10mg/kg dose showed significant reduction in the solid tumor weight by 85.76±1.02 gm when compared to control. ALCM and AQCM at the dose of 200mg/kg caused significant reduction in the solid tumor weight when compared with control. ALCM at a dose of 200mg/kg was more effective in reducing the tumor weight by 54.02±6.3% the extent of tumor growth inhibition was not comparable to standard.

Carbon-clearance test:
There was increase in the clearance of carbon particles from blood after administration of ALCM at 100mg/kg and 200mg/kg p.o and is specified by a significant rise in phagocytic index, when compared to control group. The effect of ALCM and AQCM on the phagocytic index is shown in figure 8

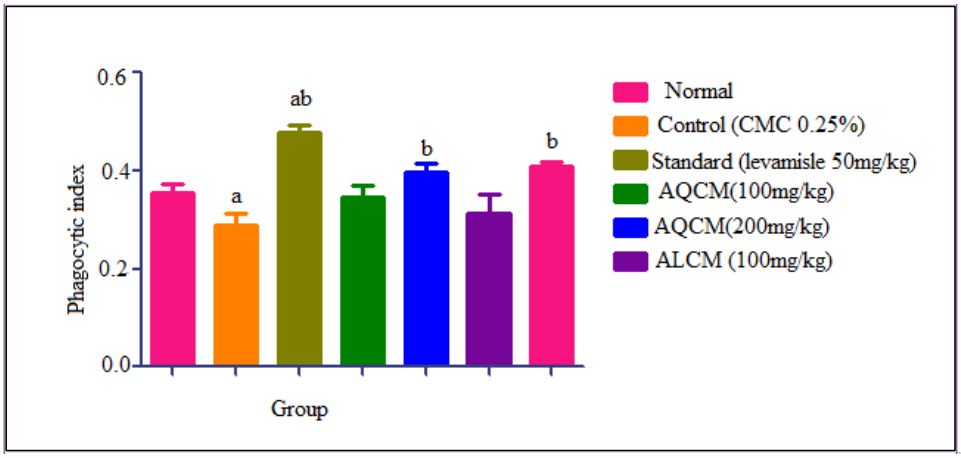

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Fig.8. Effect of ALCM and AQCM on the phagocytic index.All values are represented as Mean ± SEM, n=6.Where [a] *p*< 0.05 when compared to normal, [b] *p*< 0.05 when compared to control. The data was analyzed by one way ANOVA followed by post hoc Tukey's multiple comparison test.

Delayed Type hypersensitivity:

Administration of both ALCM 100mg/kg and 200 mg/kg p.o and AQCM 100mg/kg and 200 mg/kg p.o showed dose dependent increase in the paw volume in mice when compared with control group. ALCM 200mg/kg and AQCM 200 mg/kg showed maximum of 54.88±2.40% and of ALCM and AQCM on the paw volume in mice is shown in figure 9. 39.92±4.01% increase in paw volume which was significant when compared to control. Theeffect of ALCM and AQCM on the paw volume in mice is shown in figure 9.

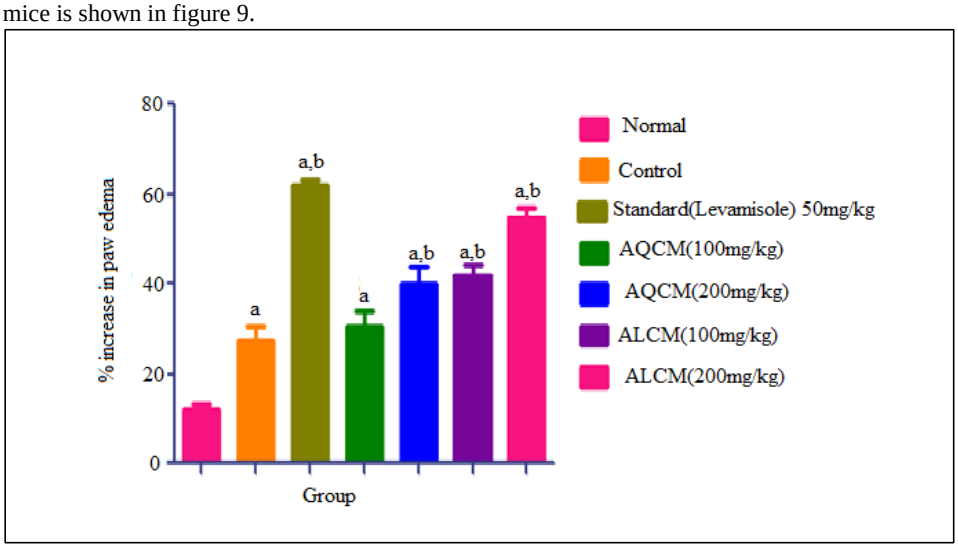

Fig. 9: Effect of ALCM and AQCM on the paw volume in mice. All values represents Mean ± SEM, n=6 Where [a] $p< 0.05$ when compared to normal, [b] $p< 0.05$ when compared to control.The data was analyzed by one way ANOVA followed by post hoc Tukey's multiple comparison test.

DISCUSSION:

The current study was designed to explore the possible antitumor activity of *Crateava magna* in transplantable tumor models. The study involves DLA induced and EAC induced ascites solid tumor model. On the basis of encouraging results both the extracts *in-vitro* antioxidant assays and preliminary cytotoxic assay, were proceeded further for investigating the antitumor potential in transplantable tumor models. Ascites tumor model is a type of rapidly growing carcinoma which is inoculated in the outbred mice by serialintraperitoneal transplantation. Ascites fluid was aspirated from the tumor bearing mice and injected to the normal mice. On the 15th day of tumor inoculation, substantial rise in the body weight was seen in the tumor control animals. Maximum increment in the body weight was 75.0g in mice bearing tumor. Treatment by ALCM and AQCM at the dose of 200mg/kg substantially decreased the body weight to 37.1g and 40.6g. It is quite evident that ALCM demonstrated more significant activity when compared to AQCM. Mean survival time was also enhanced by ALCM treatment at dose of 200mg/kg. The life span was increased up to 31.87% by ALCM at the dose of 200mg/kg. AQCM 200mg/kg also enhanced the survival time up to 27.47%. Increase in life span by 25% or more when compared to control is considered as an effective anticancer response. Both the extracts showed the enhancement in the life span, more than that of control. The influence of tumor growth on hematological and biochemical parameters is well established hence to assess the influence of ALCM and AQCM on the hematologicalparameters, the total RBC count, WBC count, hemoglobin content of all the treated groups were checked on 15th day of tumor inoculation. In tumor control animals there was a significant rise in the WBC count. Treatment with ALCM and AQCM at 200mg/kg dose reversed the enhanced WBC count in treated group which was more significant to that of control. Standard cyclophosphamideat 10mg/kg dose decreased the cell count than the normal reflecting mylosupression. There was a decrease in RBC count in the control group which was restored by the treatment of ALCM and AQCM at 200mg/kg dose. Ascites tumor bearing mice reduced the hemoglobin content which is a hallmark in iron deficiency. The treatment with ALCM and AQCM at 200mg/kg reversed the tumor induced alternation in hematological parameters such as elevation in the content of hemoglobin and total RBC count to assess the effect of ALCM and AQCM on the serum biomarkers .

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CONCLUSION:

The study has shown that ALCM extract exhibited more antioxidant potential by well established methods. Both the extracts of *Crtaeava magna* were assessed for their *in vivo* anti-tumor activity in both EAC and DLA (Dalton's lymphoma ascites) transplantable tumor bearing animals. Both the extracts reflected their cytotoxic potential as evident in *in vitro* cytotoxicity assays like brine shrimp lethality assay and trypan blue cytotoxicity assay. ALCM exhibited more potent cytotoxicity as compared to AQCM.

In EAC model, ALCM showed reduction in the increased body weight, as well as increment in the survival time which indicates inhibition in the tumor progression. The alcoholic extract at the dose of 200mg/kg b.w reversed the haematologicalparameters and restored the endogenous antioxidant and serum enzymes, which was significant with that of control.

In DLA model, ALCM at the doses 100mg/kg and 200 mg/kg decreased growth of solid tumor as evidenced by the reduction of solid tumor weight and volume when compared with control.

Both the extracts were also evaluated for their immunomodulatory activity *in-vivo.* ALCM and AQCM were screened for immunomodulatory models like carbon clearance test and delayed type hypersensitivity. ALCM exhibited low phagocytic index compared to standard which indicates its ability to enhance the carbon clearance rate. SRBC-induced delayed-type hypersensitivity was used to assess the effect of the extract on cell-mediated immunity. ALCM exhibited much reduction in the paw edema as compared to control. Therefore, increase in DTH reaction to cell dependent antigen showed the inhibitory effect of alcoholic extracts of *Crateavamagna* .

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Conflict of interest:
Authors declares no conflict of interest

REFERENCES:

1.Chanda D, Bhushan S, Guru SK, Shanker K, Wani ZA, Rah BA, et al. Anticancer activity, toxicity and pharmacokinetic profile of an indanone derivative. Eur J Pharm Sci Off J Eur Fed Pharm Sci. 2012 Dec;47(5):988–95.
2. Mayakrishnan V, Kannappan P, Shanmugasundaram K, Abdullah N. Anticancer activity of Cyathula prostrata (Linn) Blume against Dalton's lymphomae in mice model. Pak J Pharm Sci. 2014;27(6):1911–7.
3. Dahiru D, Obidoa O. Evaluation of the antioxidant effects of Ziziphus mauritiana Lam. Leaf extracts against chronic ethanol-induced hepatotoxicity in rat liver. Afr J Tradit Complement Altern Med AJTCAM. 2007 Oct 27;5(1):39–45.
4. Meera R, Chidambaranathan N. Anti cancer Activity of Ethanolic Extract of Crataeva magna Lour (DC) against Ehrlich Ascitic Carcinoma Cell Lines in Mice. J Pharm Sci. 2017;9:5.
5.siddha global [Internet]. [cited 2019 Jul 25]. Available from: http://siddhaglobal.blogspot.com//2010_03_01_archive.html.
6. Nasrin M, Dash PR, Ali MS. In Vitro antibacterial and in Vivo cytotoxic activities of Grewia paniculata. Avicenna J Phytomedicine. 2015;5(2):98–104.
7. Mandal P, Misra TK, Ghosal M. Free-radical scavenging activity and phytochemical analysis in the leaf and stem of Drymaria diandra Blume. Int J Integr Biol. 2009 Jan 1;7.
8. Sreejayan N, Rao MN. Free radical scavenging activity of curcuminoids. Arzneimittelforschung. 1996 Feb;46(2):169–71.
9. Oszmiański J, Wojdyło A. Comparative study of phenolic content and antioxidant activity of strawberry puree, clear, and cloudy juices. Eur Food Res Technol. 2009 Feb 1;228(4):623–31.
10. Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet. 1986;44(6):307–15.
11. Fadeyi SA, Fadeyi OO, Adejumo AA, Okoro C, Myles EL. In vitro anticancer screening of 24 locally used Nigerian medicinal plants. BMC Complement Altern Med. 2013 Apr 8;13(1):79.
12. Weisenthal LM, Marsden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing in vitro chemosensitivity of human tumors. Cancer Res. 1983 Feb;43(2):749–57.
13. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982 May;45(5):31–4.

14. Manjula SN, Kenganora M, Parihar VK, Kumar S, Nayak PG, Kumar N, et al. Antitumor and antioxidant activity of Polyalthia longifolia stem bark ethanol extract. Pharm Biol. 2010 Jun;48(6):690–6.
15. Kumar N, Dhamija I, Raj PV, Jayashree BS, Parihar V, Manjula SN, et al. Preliminary investigation of cytotoxic potential of 2-quinolone derivatives using in vitro and in vivo (solid tumor and liquid tumor) models of cancer. Arab J Chem. 2014;7(4):409–17.
16. Eckhardt AE, Malone BN, Goldstein IJ. Inhibition of Ehrlich ascites tumor cell growth by Griffonia simplicifolia I lectin in vivo. Cancer Res. 1982 Aug;42(8):2977–9.
17. Mukherjee A, Dutta S, Sanyal U. Evaluation of dimethoxydop-NU as a novel anti-tumor agent. J Exp Clin Cancer Res CR. 2007 Dec;26(4):489–97.
18. Kumar RS, Rajkapoor B, Perumal P. Antitumor and cytotoxic activities of methanol extract of Indigofera linnaei Ali. Asian Pac J Cancer Prev APJCP. 2011;12(3):613–8.
19. Thefeld W, Hoffmeister H, Busch E-W, Koller PU, Vollmar J. Referenzwerte für die Bestimmungen der Transaminasen GOT und GPT sowie der alkalischen Phosphatase im Serum mit optimierten Standardmethoden. DMW - Dtsch Med Wochenschr. 1974 Feb;99(8):343–51.
20. Chakraborty SP, Das S, PANCHANAN, Pramanik, Roy S. Vancomycin Resistant Staphylococcus Aureus Induced Oxidative Stress In Liver And Kidney : Protective Role Of Nanoconjugated Vancomycin. In 2011.
21. Voytas D. Agarose Gel Electrophoresis. Curr Protoc Mol Biol. 2000;51(1):2.5A.1-2.5A.9.
22. Jayaseelan RS, Vijayan FP, Mathesvaran M. Cytotoxic And Antitumor Activity Of Methonolic Extracts Desmodium Triangulare (Retz) Merr. Root. 4(3):3.
23. Kori Ml, Gaur K, Dixit Vk. Investigation Of Immunomodulatory Potential Of Cleome Gynandra Linn. Asian J Pharm Clin Res. 2009;2(1):5.
24. Tripathi S, Maurya A, Kahrana M, Kaul A, Sahu DrR. Immunomodulatory property of ethanolic extract of Trigonella Foenum- Graeceum leaves on mice. Pharm Lett 2012 4 2708-713. 2012 Apr 1;4:708–13.
25. Lamont AG, Bruce MG, Watret KC, Ferguson A. Suppression of an established DTH response to ovalbumin in mice by feeding antigen after immunization. Immunology. 1988 May;64(1):135–9.

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