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Evaluation and in Vivo Anti-Cancer Property of Methanol Extract of Acrostichum Aureum L and Insilco Toxicity Prediction

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KEYWORDS

Dalton's Lymphoma Ascites, tumour progression, MEAA treated tumour bearing animals.

ABSTRACT:

This study is to evaluate the in vivo anti-cancer property of methanol extract of Acrostichum aureum L. against Dalton's Lymphoma Ascites (DLA) murine models. Is done by Preparation of methanol extract of Acrostichum aureum L. leaves using cold extraction method and it is Determined by in vivo anti-tumour activity of methanol extract of Acrostichum aureum (MEAA) by administering MEAA in DLA-induced ascites-bearing animals, to know the progression of the tumour the Evaluation of tumour progression in MEAA treated ascites tumour-bearing animals is carried out and haematological parameters of MEAA treated ascites tumour-bearing animals shows maximum effectiveness , Finally evaluation is done by MEAA treated tumour bearing animals. Analgesic and antioxidant activity of extracts from this species. Ethanolic extracts of A. aureum showed anti-fertility activity in rats as per the report of Dhar et al., in 1992. The cytotoxic effect of water and methanol extracts from a Bangladeshi specimen of A.aureum on gastric, colon and breast cancer cells were studied by Uddin et al., 2010. The anti-inflammatory activity of ethanolic (95%) crude extract of the roots of A. aureum was reported in varied carrageenan-induced inflammation rat models by Hossain et al., in 2011. Ethanol extract of A. aureum possesses analgesic activity and in vitro antioxidant activity which was found to be significantly effective in scavenging DPPH (EC50 =41.95µg/ml).

Introduction:

Cancer is a generic form for distinct diseases that can affect any part of the body. It is characterized by the uncontrolled growth of abnormal cells which can invade the adjoining organs of the body. Cancer affects people of all ages, even foetuses (WHO, 2009). However, it is seen that the risk of cancer incidence increases with age. Cancer starts when a single cell tends to proliferate abnormally. This mutated cell divides to form proliferating cells, which divides to form four abnormal cells and so on (Geoffrey MC, 1993). These cells pile up to a non-structured mass called tumour. Often, the term tumour is used interchangeably with neoplasm.

Malignant tumours attain the property of metastasis, which is defined as the formation of new tumours (secondary and tertiary tumour nests) in tissues and organs away from the primary site of tumour origin (Ahmad, 2017).

Genetic factors

Alteration of certain important genes results in production of faulty proteins which may directly or indirectly lead to cancer. Mutations in three broad classes of genes – proto-oncogenes, tumour-suppressor genes and caretaker genes – play key roles in cancer induction (Lodish et al., 2008).

1. Proto-oncogenes: These are the genes which promote cell proliferation and stop cell death. Defects in their regulation result in cancer.
2. Tumour suppressor genes: These genes are protective in nature and normally limit the development and growth of tumours; when a tumour suppressor gene is mutated, it may fail to prevent a cancer from growing.
3. Caretaker genes: These are the genes which produce DNA damage repair enzymes. These genes maintain integrity of the genome and the fidelity of information transfer from one generation of cells to the next. Loss of function of mismatch repair genes could make a cell error prone and lead to cancer.

Journal of Chemical Health Risks

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Dalton's Lymphoma Ascites (DLA) murine models of study

Animal models in general, and especially mouse models, are irreplaceable tools for the study of carcinogenesis and the availability of rodent models have enabled rational screening of drugs. Hematological malignancies have been extensively studied in mouse models and broad range of lymphoid neoplasms has been reported in laboratory mice. Lymphoma is the broadest category of a family of related blood cancer, involving a group of cells, called the lymphocytes, which in turn make up the lymphatic system.

Anticancer property of a number of drugs and their subsequent mechanism of action and the pathways implicated has been elucidated using murine model. Dalton's lymphoma is a transplantable T-cell lymphoma of spontaneous origin in thymus of murine host and has emerged as an interesting model for cancer research.

Dalton's Lymphoma Ascities tumorigenesis model in mice provides a convenient model system to study such effects within a short time (Shankar et al., 2000). Following transplantation of DLA cells in to the abdominal cavity of healthy recipient mice, tumorigenesis begins immediately and aggressively (Goldie and Felix, 1951). Recipient or transformed mice usually survive up to 3weeks (Koiri et al., 2006).

Dalton's Lymphoma can be easily maintained in laboratory in ascites by serial transplantation in mice by intra peritoneal injection of $5x10^5$ cells / mouse (Koiri et al., 2009). A range of parameters can be used to evaluate drug effect on tumors in this model. Tumor volume and changes in body weight are simple and easily reproducible parameters. Morphologic changes and alterations in tumor immunogenicity or invasiveness are other markers of response. Specific assays have been developed for the measurement of treatment effects on tumors.

Active components of a large number of natural plant products have been studied with reproducible biologic end point. Anti-tumor activity of ethanolic extract of active fraction of Emilia sonchifolia (Shylesh et al., 2005), Aegle marmelos (Chockalingam et al., 2012), Crocin from Kasmiri saffron Crocus sativus (Bakshi et al., 2009) have been demonstrated against Dalton's Lymphoma in mice. In cancer drug development, the animal model is selected to demonstrate the cytotoxic effect of the drug or biological agent on the tumor passage in this model system

Dalton's Lymphoma has emerged as an important murine model for understanding the progression and development of cancers and is playing an important role in drug discovery and development.

Golden Leather Fern (Acrostichum)

Acrostichum is commonly known as golden leather fern, swamp fern and mangrove fern. These ferns mainly grow in wet localities, swamps and shady places with salinity. Acrostichum aureum, A. speciosum and A.danaeifolium are some among the species belonging to the genus Acrostichum. A. danaeifolium is similar to A.aureum (Adam and Tomilson, 1979; Tomilson, 1986).

Journal of Chemical Health Risks

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JCHR (2023) 13(4s), 214-225 | ISSN:2251-6727

Pharmacological properties of Acrostichum aureum

1. Antioxidant property: Among 15 species of ferns the leaves, of A. aureum gets the eighth position of the moderate category for total phenolic contents and antioxidant activities (Lai and Linn, 2011). The IC50 value for the sufficient free radical scavenging activities of the ethanol leaf extract of A. aureum is 42µg/ml while that of ascorbic acid was 16µg/ml (Khan et al., 2013).
2. Cytotoxicity: The ethyl extract of A. aureum showed cytotoxic activity against HeLa human cervical cancer cells with an IC50 value of 6.3µg/ml (Dai et al., 2005). The methanol leaf extract of A. aureum exhibited cytotoxicity with IC50 value of 1.0mg/ml against AGS gastric cancer cells (Uddin et al., 2011).
3. Analgesic property: According to dose dependent manner the ethanol extract of A. aureum at 250 and 500mg/kg exhibited sufficient analgesic activity in the results of an acetic acid induced writhing in mice (Khan et al., 2013).
4. Anti-inflammatory activity: The ethanolic root extract of A. aureum exhibited anti-inflammatory activity at 400mg/kg in the results of Carrageenan induced oedema test (Hussain et al., 2011).

Materials and Methods:

Plant material

Acrostichum aureum L. (Golden Leather Fern) was the plant material for the study (Figure 1). A. aureum leaves were collected from Poovar, Thiruvananthapuram and authenticated by the Department of Botany, University of Kerala, Karyavattom, Thiruvananthapuram. The collected leaves were washed thoroughly with running tap water leaves were chopped, oven dried (40°C-45°C) and powdered. Powder was stored in air tight Containers. This powder was used for subsequent extraction and analysis.

Preparation of experimental animals

Male BALB/C mice (6-8) weeks old, weighing 22-25 grams were obtained from animal house breeding section, Regional Cancer Center, Thiruvananthapuram, Kerala, India (Figure 2a). The animals were maintained in a sterile environment at constant temperature (24 ± 2^0C), relative humidity (50%) and 12 hours' light / dark cycle (Figure 2c). All the animals were fed with normal mice chow and water. Animals experiments were done after getting approval from Institutional Animal Ethical Committee (IAEC), Regional Cancer Center, Thiruvananthapuram.

3.3. Maintenance of Dalton's Lymphoma Ascites (DLA) cell line

Dalton's Lymphoma Ascites (DLA) cells were maintained at the animal house facility of Regional Cancer Centre, Thiruvananthapuram and propagated in the peritoneal cavity of Balb/c mice (Figure 2b). Freshly aspirated cells from the peritoneal cavity of were washed in 1xPBS (pH=7.4) to remove dead cells and other debris under sterile conditions. These cells were then used freshly for experiments.

3.4. Preparation of plant extract

The air dried and powdered Acrostichum aureum was subjected to the process of cold extraction using methanol (Figure 3). 10 g of powdered A. aureum was weighed and dissolved in 100 ml of methanol and was subjected to overnight magnetic stirring at 1,000 rpm at 37°C. It was then centrifuged for 12,000 rpm at 4°C for 10 minutes. The supernatant was collected and the pellet was re-suspended in 100 ml of methanol.

Figure1: Leaf of Acrostichum aureum

Journal of Chemical Health Risks

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JCHR (2023) 13(4s), 214-225 | ISSN:2251-6727

Figure 1: Plant material, *Acrostichum aureum* L. (a) Habit (b) A twig

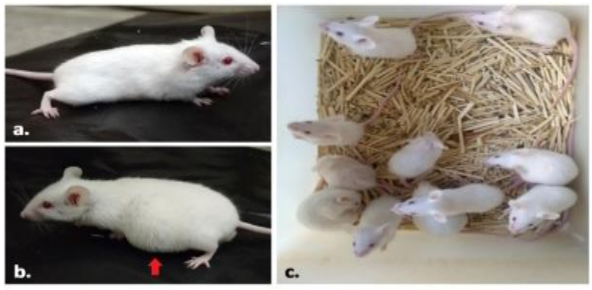

Figure 2: Animals used for in vivo experiments: Balb/c Mice
(a) Normal Balb/c mice
(b) Balb/c mice with DLA cells maintained in the peritoneal cavity; ⬆ represents abdominal distension as a result of formation and accumulation of ascitic fluid
(c) Maintenance of experimental animals

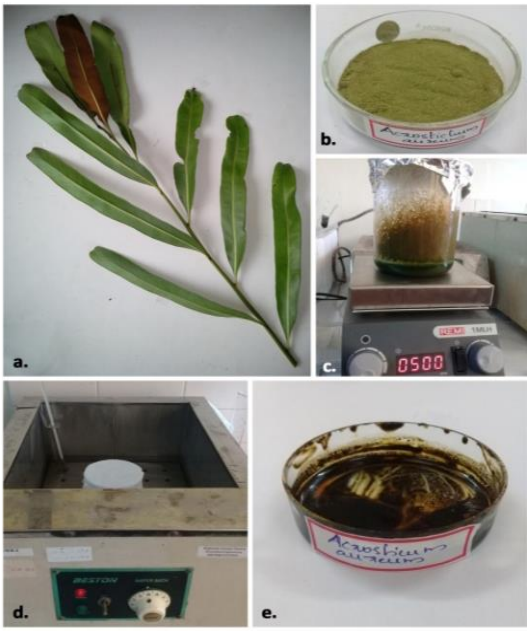

Figure 3: Preparation of plant extract
(a) Plant material, *Acrostichum aureum* L.
(b) Powdered leaves of the plant material
(c) Magnetic stirring of powdered plant material with methanol
(d) Evaporation of supernatant in water bath
(e) Methanol extract of *Acrostichum aureum* (MEAA) collected in a petri dish

The re-suspended pellet was again subjected to overnight magnetic stirring and centrifugation. The pellet was then discarded and the supernatants obtained were pooled. The supernatant was then filtered using Whatman's No.1 filter paper and concentrated by evaporating in water bath.

The methanol extract of Acrostichum aureum (MEAA) was then weighed and the yield was determined.

Preparation of drug

The extract was re-suspended in 1xPBS for in-vitro experiments. For administration in animal, MEAA was re-suspended in 1% gum acacia at a concentration of 2.5mg/ml.

Determination of effect of MEAA in ascetic tumour in mice

Male BALB/c mice procured from the animal house breeding section, Regional Cancer Center, Thiruvananthapuram were divided into four groups (6 animals/ group) viz. Group 1, Group 2, Group 3 and Group 4. Freshly aspirated and washed Dalton's Lymphoma Ascites (DLA) cells were used to induce ascitic tumour in experimental animals. Animals belonging to Groups2, 3 and 4 were induced ascites tumor by injecting 1 x 10^6DLA cells/animal to the peritoneal cavity. Group 1 served as the normal control and Group 2 served as the tumour control. Animals belonging to Group 3 were treated with the standard drug, cyclophosphamide (CTX) at a concentration of 10mg/kg body weight for ten consecutive days (1 injection per day) starting from one hour after DLA injection. Animals belonging to Group 4 were treated with MEAA at a concentration of 10mg/kg body weight for ten consecutive days (1 injection per day) starting from one hour after DLA injection. Thus the experimental groups could be summarized as follows:

Group 1: Normal Control

Journal of Chemical Health Risks

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JCHR (2023) 13(4s), 214-225 | ISSN:2251-6727

Group 2: Tumour Control
Group 3: Tumour +CTX
Group 4: Tumour +MEAA
Blood was collected from the animals on four time intervals in EDTA coated tubes through tail vein bleeding and blood parameters were evaluated. Body weights of the animals were taken on every third day of DLA injection till the 15th day. The animals were sacrificed on 15th day and the total ascitic fluid, blood (by cardiac puncture) and liver were obtained from the sacrificed mice.

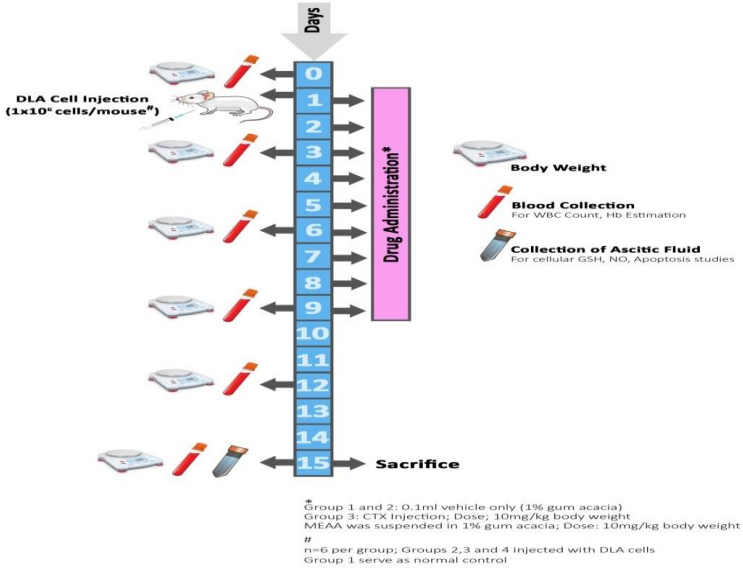

Figure 4: Pictorial Representation of DLA method.

DLA cells in the total ascitic fluid was used to calculate total protein content, cellular glutathione and nitric oxide content. Liver was used for histopathological evaluation of ascites induced organ damage.

Determination of effect of MEAA in blood Haemoglobin

20 µl of the blood samples collected through tail-vein bleeding in EDTA coated Eppendorf tubeswere mixed with 5ml of Drabkins reagents (1:250 ratio)and then incubated for 5 minutes in room temperature. The absorbance was then measured 540 nm against reagent blank. Standard haemoglobin solution and Drabkins reagent in the ratio 1:250 is used as standard. Hb count was calculated by using the following formula:

Hb(gm/dl) = (OD of sample/OD of standard) x concentration of standard x 0.25ml

Determination of effect of MEAA in total WBC Count

10µlof blood samples collected through tail-vein bleeding in EDTA coated Eppendorf tubeswere mixed with 190 µl of Turk's fluid. The above mixture was incubated at room temperature for 2-3 minutes. Cells were gently mixed and loaded in haemocytometer. Cell numbers in four large corner squares were counted.After cell counting, the total leukocytes count was calculated by using the formula:

Total leucocyte count/mm^2 = (Number of cells counted x Dilution factor x Depth factor) / area coated.

Determination of total protein content in ascites-derived DLA cells

Ascitic fluid obtained from mice were repeatedly washed in 1xPBS (pH=7.4) and centrifuged. The pellet was the re-suspended in 1ml 0.1M phosphate buffer (pH 7.5) and sonicated to obtain the cell lysate. The lysate was then centrifuged and the supernatant was used for determination of total protein content using Bradford Reagent. 0.1ml of supernatant was made up to the volume of 1ml with 0.1M phosphate buffer (pH 7.5). 0.1ml of this sample solution was transferred to a well of a 96 well plate, 0.5ml Bradford Reagent was added and thoroughly mixed. Absorbance was recorded at 595nm against reagent blank and the protein concentrations were determined from the standard curve.

Determination of reduced glutathione (GSH) in ascites-derived DLA cells

Journal of Chemical Health Risks

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JCHR (2023) 13(4s), 214-225 | ISSN:2251-6727

Reduced glutathione (GSH) is estimated by methods based on principles of methods of Moron et al., 1976. 0.5 ml of cell lysate was taken in a test tube and 2ml of distilled water was added, mixed well. Then centrifuged for 5 min at 5000rpm. 0.5 ml of supernatant was taken, to which 0.5ml of TCA (5%) was added and then centrifuged for 10 minutes at 10000rpm. 0.5 ml of supernatant was taken to which 2.5ml of phosphate buffer (pH 8) was added. To this 1ml DTNB was added. This solution was inverted for 3 times to mix. The absorbance was read on spectrophotometer at 412nm within 4 min. of preparing the mixtures. Standard graph of reduced glutathione GSH concentrations was plotted. Determination of reduced glutathione GSH concentration in lysate of DLA cells were done from the graph.

Determination of cellular nitric oxide (NO) content in ascites-derived DLA cells

Nitric oxide content was determined based on Green et al., 1982. Cell lysate was used for estimation of NO using Griess reaction. The reaction mixture containing 50 ml sodium nitroprusside (10 mM) and 50 ml serum was incubated at 37ºC for 3 h. Thereafter, 1 ml of the mixture was combined with an equal volume of Griess reagent (0.1% napthylethylenediamine dihydrochloride in water/1% sulphanilamide in 5% phosphoric acid) and incubated for 15 min at room temperature. The pink chromophore generated was loaded into a 96 well plate and was measured at 540 nm using an ELISA plate reader. Sample values were extrapolated from a standard curve generated in parallel with varying levels of sodium nitrite. All values were expressed in µmoles.

Evaluation of cytotoxicity and cell morphology in ascites-derived DLA cells using Giemsa staining

DLA cells obtained from the experimental mice on the 15th day of DLA inoculation were repeatedly washed using 1XPBS. About 100 µl of cell suspension was taken and dropped on a clean glass slide and kept for drying on slide warmer for 20 min. Then the slides are stained by using Giemsa stain for 2 min and washed two times with distilled water and morphology of cells was observed under microscope.

Histopathological evaluation of liver to estimate ascites-induced organ damage

On 15th day, mice from all four groups were sacrificed by cervical dislocation and dissected. Small portion of liver were recovered from each mouse and was fixed in 10% formalin solution. After several steps of dehydration in alcohol, sections of 4µm thickness were prepared and stained with Haematoxylin and Eosin (H&E). Thereafter, histopathological analysis was carried out using EVOS-x1 CORE light microscope. All samples were analysed in blind manner. All the analysis and interpretation was done by a certified histopathologist.

ADME Property and Toxicity Studies:

The SWISSADME was used to determine the properties of compounds, including absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiling. The chemical descriptors, 2D polar surface area (PSA 2D), a key factor in fractional absorption, and the lipophilicity levels in the form of atom-based LogP strongly correlate with ADME properties (AlogP98). Drug absorption is influenced by a number of variables, including skin permeability, intestinal absorption, intestinal absorption of medicines, and P-glycoprotein substrate or inhibitor levels. The blood-brain barrier (logBB), CNS permeability, and the volume of distribution are all factors that affect how medications are distributed (VDss). On the basis of the substrate or inhibitor, metabolism is predicted. Based on the substrate and total clearance model, excretion is expected. Using the pkCSM server at https://biosig.lab.uq.edu.au/pkcsm/prediction the toxicity of medications is predicted. Several variables were computed, and their adherence to the expected ranges was examined.

Results and Discussions:

Air-dried and powdered Acrostichum aureum was subjected to cold extraction using methanol. The yield of the obtained extract (MEAA) was 15%.MEAA was re-suspended in 1% gum acacia at a concentration of 2.5mg/ml for administration in animals.

Effect of MEAA administration in the body weight and tumour load of DLA mice

It was observed that IP administration of MEAA significantly mitigated the increase in body weight and tumour load in DLA mice (Figure 5a and 5b).

The highest body weight was observed on 15th day in tumour control group (30.67±2.49g). Administration of MEAA significantly mitigated the increase in body weight, with the average body weight of Tumour + MEAA group at 15th day being 28.00±0.82g. Standard drug (CTX) showed the highest activity in reducing the increase in body weight, with the average body weight of Tumour + CTX group at 15th day being 25.34±0.94g.

Table 1: Effect of administration of MEAA in the body weight of experimental animals

Group	Body Weight (in grams)					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15

Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(4s), 214-225 | ISSN:2251-6727

Group 1: Normal Control	23.67±1.25	23.67±1.25	24.00±1.40	24.34±1.70	24.34±1.70	24.34±1.70
Group 2: Tumour Control	22.34±2.05	22.34±2.05	24.00±1.63	25.34±2.05	27.67±2.49	30.67±2.49
Group 3: Tumour +CTX	23.34±1.70	23.34±1.70	23.67±1.89	24.34±1.69	25.00±1.41	25.34±0.94
Group 4: Tumour +MEAA	22.67±1.69	23.00±2.16	24.34±2.05	24.67±1.69	26.34±1.25	28.00±0.82

Each value represents the mean ± SD of n = 6.

The ability of MEAA to reduce the progression of ascitic tumour was also evident from the amount of ascitic fluid collected on the day of sacrifice (Day 15). The amount of ascitic fluid collected was the highest in tumour control group (8.57±0.32ml). Administration of MEAA significantly mitigated the accumulation of ascitic fluid in peritoneal cavity, with the average amount of ascitic fluid collected in Tumour + MEAA group at 15th day being 2.36±0.31ml. Standard drug (CTX) showed the highest activity in reducing the accumulation of ascitic fluid in peritoneal cavity, with the average amount of ascitic fluid collected in Tumour + CTX group at 15th day being 1.70±0.14ml.

Table 2: Effect of administration of MEAA in the tumour load of experimental animals

Group	Tumour load (Represented as volume of ascitic fliud in ml)
Tumour Control	8.57±0.32
Tumour +CTX	1.70±0.14
Tumour +MEAA	2.36±0.31

Each value represents the mean ± SD of n =6.

Thus, the in vivo treatment of Dalton's Lymphoma Ascites (DLA)-induced ascitic tumour-bearing Balb/c mice with MEAA administered intraperitoneally was found to be effective in reducing the tumor. The anti-tumoral action of the extract was evident by the reduction in volume of ascites fluid and decrease in the rate of increase of body weight.

Effect of MEAA administration in the total WBC count and haemoglobin content of DLA mice

The effect of MEAA on total haemoglobin content and WBC count are shown in Table3 and Table 4 respectively.

Table 3: Effect of administration of MEAA in the hemoglobin (g/dl) level of experimental animals during ascites tumour progression

Group	Haemoglobin levels (g/dl)					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Group 1: Normal Control	15.56±0.84	15.61±0.91	15.58±0.97	15.61±0.95	15.60±1.02	15.58±1.05
Group 2: Tumour Control	15.36±1.06	14.85±1.03	14.38±1.13	13.65±1.16	12.93±1.07	12.59±0.97
Group 3: Tumour +CTX	15.57±0.74	15.31±0.78	15.16±0.81	15.06±0.86	14.98±0.84	14.96±0.83
Group 4: Tumour +MEAA	15.61±1.23	14.96±1.17	14.59±1.19	14.36±1.22	14.06±1.24	13.87±1.18

Each value represents the mean ± SD of n = 6.

Journal of Chemical Health Risks

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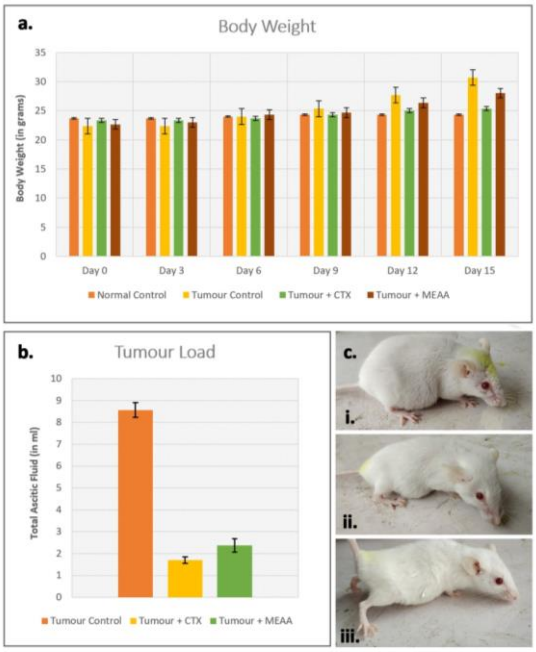

Figure 5: Effect of MEAA administration in tumour progression.
(a) Graph showing the effect of MEAA in the body weights of experimental animals.
(b) Graph showing the effect of MEAA in the tumour load of experimental animals on day 15.
(c) Photographs of animals representing experimental groups on day 15
(i) Group 2: Tumour Control; (ii) Group 3: Tumour + CTX; (iii) Group 4: Tumour + MEAA

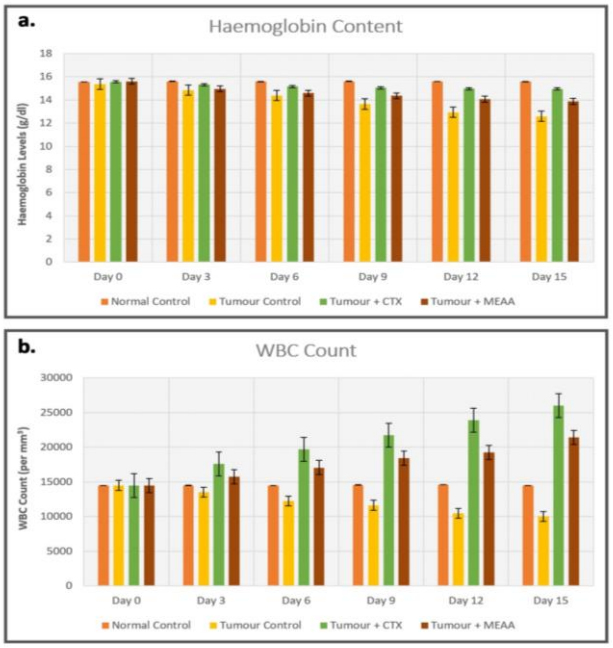

Figure 6: Effect of MEAA administration in major hematological parameters
(a) Effect of MEAA administration in haemoglobin content
(b) Effect of MEAA administration in WBC Count

MEAA administration significantly increased the hemoglobin level (Day 15: 13.87±1.18 g/dl) compared to tumor bearing control animals (Day 15: 12.59±0.97 g/dl).Treatment with MEAA significantly increased the total WBC count (Day 15: 21395.33 ± 955.16 cells/mm^3) compared to tumor bearing control animals (Day 15: 10040.00 ± 359.10 cells/mm^3).

Table 4: Effect of administration of MEAA in the total WBC count of experimental animals during ascites tumour progression

Group	WBC count (cells per mm^3)					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Group 1: Normal Control	14469.00 ± 90.82	14500.00 ± 123.73	14476.33 ± 89.12	14560.67 ± 110.16	14599.33 ± 281.76	14473.00 ± 87.47
Group 2: Tumour Control	14509.33 ± 47.23	13500.33 ± 45.82	12242.00 ± 469.61	11643.00 ± 280.34	10473.00 ± 356.98	10040.00 ± 359.10
Group 3: Tumour +CTX	14398.50 ± 119.04	17616.00 ± 334.09	19947.50 ± 374.37	21692.50 ± 280.18	24146.50 ± 663.78	26114.00 ± 290.44
Group 4: Tumour +MEAA	14461.00 ± 72.39	15729.00 ± 247.67	17056.00 ± 299.11	18439.00 ± 388.19	19271.00 ± 287.53	21395.33 ± 955.16

Each value represents the mean ± SD of n = 3.

Chemotherapy is a common treatment optionfor cancer. The major shortcomings of cancer chemotherapy are myelosuppression and anemia. Anemia is mainly due to a decline in haemoglobin content (Mika and Guruvayoorappan, 2013). Further, chemotherapeutics drugs are seen to be causing a decline in the WBC content thereby deteriorating the hematological parameters of patients. Thus, the ability to increase WBC content is considered as a reliable criterion for judging the value of any anti-cancer drug (Clarkson and Burchenal, 1965).

It was seen that treatment MEAA restored the hemoglobin content as well as WBC count. This indicates that the extract possesses protective action on hemopoietic system. The results signify that MEAA can shrink ascitic tumor volume, and improve the hematological parameters of the tumor bearing mice.

Effect of MEAA administration in cellular GSH and NO levels ofDLA mice

The effect of MEAA on the cellular GSH and NO levels of DLA cells obtained on day 15 from the ascitic fluid of experimental mice is shown in Table 5. The GSH content in DLA cells was found to be 21.38±1.32 nmol/mg protein in tumour control. MEAA treatment significantly reduced cellular GSH levels in experimental mice (15.99±1.18nmol/mg protein).

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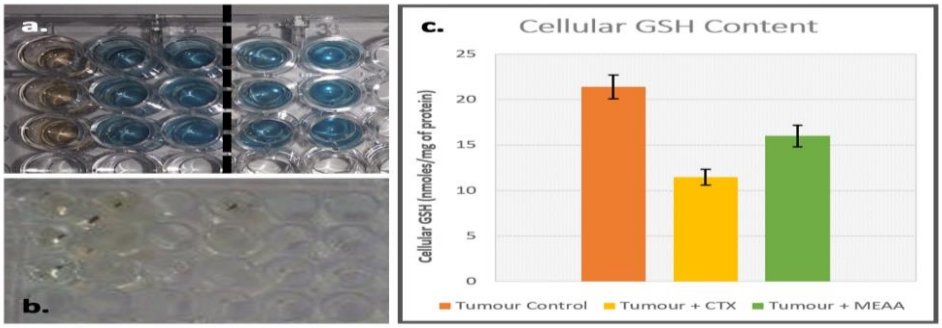

Figure 7: Effect of MEAA administration in cellular GSH content of DLA cells
(a) Reaction mixture in a 96 well plate for the estimation of protein content in DLA cell lysate
(b) Reaction mixture in a 96 well plate for the estimation GSH content in DLA cell lysate
(c) Graphs representing the cellular GSH content in the DLA cells obtained from the ascitic fluid of experimental animals

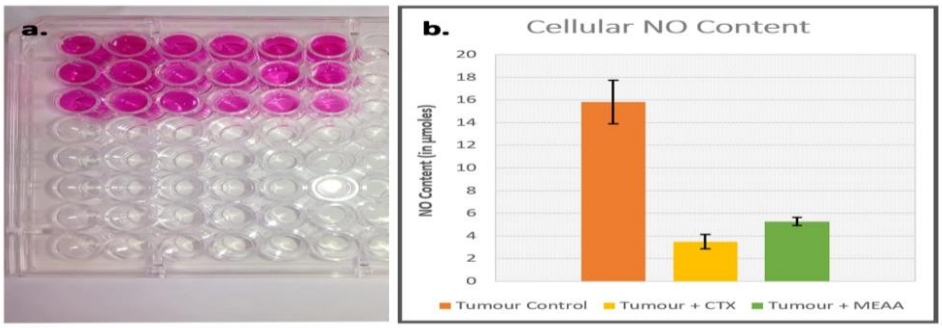

Figure 8: Effect of MEAA administration in cellular nitric oxide content of DLA cells
(a) Reaction mixture in a 96 well plate for the estimation of nitric oxide content in DLA cell lysate
(b) Graph representing the cellular nitric oxide content in the DLA cells obtained from the ascitic fluid of experimental animals

This was comparable to the GSH levels obtained on treatment with standard drug (CTX), with the cellular GSH level in Tumour + CTX group being 11.46±0.86nmol/mg protein.

The nitric oxide levels in DLA cells were also found to be reduced inMEAA-treated tumour bearing animals.The NO content in DLA cells was found to be 15.81±1.92µM in tumour control. MEAA treatment significantly reduced cellular NO levels in experimental mice (5.27±0.35µM). This was comparable to the GSH levels obtained on treatment with standard drug (CTX), with the cellular GSH level in Tumour + CTX group being 3.49±0.65 µM.

Table 5: Effect of administration of MEAA in the biochemical parameters of DLA cells obtained from experimental animals

Group	Cellular GSH Content (nmol/mg protein)	Cellular Nitric Oxide Content (µM)
Tumour Control	21.38±1.32	15.81±1.92
Tumour +CTX	11.46±0.86	3.49±0.65
Tumour +MEAA	15.99±1.18	5.27±0.35

Each value represents the mean ± SD of n = 3.

GSH is a major thiol required for the proliferation and metabolism of tumor cells. GSHis necessary for cell survival as it involved in protection against free radicals and its reduction increases the cellular susceptibility to apoptosis (Morales et al., 1998). High intracellular GSH levels have been linked to apoptosis resistance, while GSH depletion has been shown to induce apoptosis (Friesen et al., 2004; Cazanave et al., 2007). The ability of MEAA to deplete the amount of GSH can be linked to its ability to induce apoptosis.

Nitric oxide (NO) is a free radical and has the ability to modify the redox environment of vascular cells. It has contrasting, dose-dependent effects on the intracellular redox environment. Low levels of NO reduce the presence of intracellular reactive oxygen species while high levels of NO through the formation of peroxynitrite ($ONOO^{-}$)may promote oxidative damage (Mika and

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JCHR (2023) 13(4s), 214-225 | ISSN:2251-6727

Guruvayoorappan, 2013). The ability of MEAA to scavenge nitric oxide free radicals can be linked to its ability to effectively defend oxidative damage in cells.

Evaluation of cytotoxicity in ascites-derived DLA cells using giemsa staining

DLA cells obtained from the experimental mice on the 15th day were fixed and evaluated by Giemsa staining to estimate the cytotoxicity.

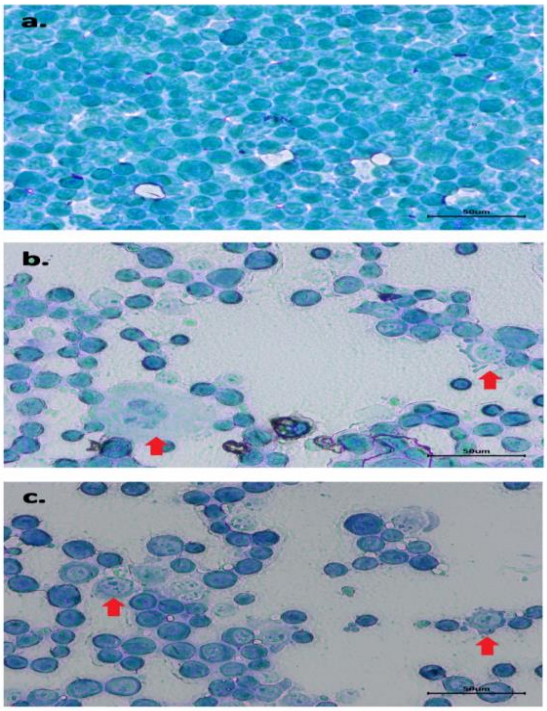

Figure 9: Evaluation of cytotoxicity of MEAA in DLA cells using Giemsa staining. A few among of the cells undergoing apoptosis have been represented by ⬆
(a) Giemsa staining of DLA cells obtained from tumour-bearing control animals
(b) Giemsa staining of DLA cells obtained from CTX-administered tumour-bearing animals
(c) Giemsa staining of DLA cells obtained from MEAA-administered tumour-bearing animals

DLA cells belonging to the tumour control group principally consisted of clustered round shaped cells, with densely stained nucleus suggesting the lack of apoptotic cells. DLA cells obtained from MEAA treated mice were characterized by a large fraction of apoptotic cells. Prominent hallmarks of apoptosis, such as membrane blebbing and nuclear disintegration were evident in MEAA-treated cells. This suggests that MEAA ameliorates ascites tumour progression in experimental animals by enhancing apoptotic cell death.

Histopathological evaluation of liver to estimate ascites-induced organ damage

The histopathological examination of representative liver sections of all four groups of animals were collected at the end of experimental period, during sacrifice (Day 15). The haematological findings were confirmed by evidences of histopathological examination. Tumour control showed destructed lobular architecture with centrilobular necrosis surrounded by inflammatory infiltrated and interface hepatitis. Individual hepatocytes showed bi-nucleation. Sinusoids showed mild dialation. The sections from the liver of Tumour + CTX group showed altered lobular architecture with interface hepatitis. Individual

hepatocytes showed cytoplasmic vacuolation and binucleation. MEAA-treated group showed normal lobular architecture and individual hepatocytes showed no significant pathology.

Conclusion:

Cancer is the second leading cause of death in the world, affecting people of all ages. Though, research is carried out around the globe to alleviate this malady, a promising drug which could selectively halt the proliferation of cancerous cells, without harming normal cells has still not been developed.

Journal of Chemical Health Risks

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Ascites is the abnormal buildup of fluid in the peritoneal cavity and is frequently associated with cancer. Anticancer property of a number of drugs and their subsequent mechanism of action has been elucidated using murine models. Dalton's Lymphoma Ascities (DLA) tumorigenesis model in mice is a convenient model system to study the effect of drugs against ascitic tumours within a short time. The ability of MEAA to ameliorate ascitic tumours in the peritoneal cavity of Balb/c mice is evaluated in this study.

Male BALB/c mice procured from the animal house breeding section, Regional Cancer Center, Thiruvananthapuram were divided into four groups (6 animals/ group) viz. Group 1, Group 2, Group 3 and Group 4. Freshly aspirated and washed Dalton's Lymphoma Ascites (DLA) cells were used to induce ascitic tumour in experimental animals. Animals belonging to Groups 2, 3 and 4 were induced ascites tumor by injecting 1 x 106 DLA cells animal to the peritoneal cavity. Group 1 served as the normal control and Group 2 served as the tumour control. Animals belonging to Group 3 were treated with the standard drug, cyclophosphamide (CTX) at a concentration of 10mg/kg body weight for ten consecutive days (1 injection per day) starting from one hour after DLA injection. Animals belonging to Group 4 were treated with MEAA at a concentration of 10mg/kg body weight for ten consecutive days (1 injection per day) starting from one hour after DLA injection.

Blood was collected from the animals on four time intervals in EDTA coated tubes through tail vein bleeding and blood parameters were evaluated. Body weights of the animals were taken on every third day of DLA injection till the 15th day. The animals were sacrificed on 15th day and the total ascitic fluid, blood (by cardiac puncture) and liver were obtained from the sacrificed mice. DLA cells in the total ascitic fluid was used to calculate total protein content, cellular glutathione and nitric oxide content. Liver was used for histopathological evaluation of ascites induced organ damage.

It was seen that MEAA significantly reduced the progression of ascitic tumour in experimental mice. It was evident from the decrease in body weight and a reduction in tumour load (volume of ascitic fluid collected on day 15) of MEAA-treated tumour bearing animals. Administration of MEAA significantly mitigated the accumulation of ascitic fluid in peritoneal cavity, with the average amount of ascitic fluid collected in Tumour + MEAA group at 15th day being 2.36±0.31ml, as compared to tumour control (8.57±0.32ml).

MEAA administration also improved the haematological parameters of tumour bearing animals. MEAA administration significantly increased the hemoglobin level (Day 15: 13.87±1.18 g/dl) compared to tumor bearing control animals (Day 15: 12.59±0.97 g/dl). Treatment with MEAA also increased the total WBC count (Day 15: 21395.33 ± 955.16 cells/mm3) compared to tumor bearing control animals (Day 15: 10040.00 ± 359.10 cells/mm3) significantly.This indicates that the extract possesses protective action on hemopoietic system.

DLA cells isolated from the ascitic fluid of experimental animals collected on the day of sacrifice (Day 15) were lysed and the biochemical parameters viz., cellular GSH and NO content were determined. The GSH content in DLA cells was found to be 21.38±1.32 nmol/mg protein in tumour control. MEAA treatment significantly reduced cellular GSH levels in experimental mice (15.99±1.18nmol/mg protein).The nitric oxide levels in DLA cells were also found to be reduced in MEAA-treated tumour bearing animals. The NO content in DLA cells was found to be 15.81±1.92 µM in tumour control. MEAA treatment significantly reduced cellular NO levels in experimental mice (5.27±0.35 µM).GSH depletion in MEAA treated DLA cells can be linked to apoptosis induction and the ability of MEAA to scavenge nitric oxide free radicals can be linked to its ability to effectively defend oxidative damage in cells.

DLA cells obtained from the experimental mice on the 15th day were fixed and evaluated by Giemsa staining to estimate the cytotoxicity. DLA cells belonging to the tumour control group principally consisted of clustered round shaped cells, with densely stained nucleus suggesting the lack of apoptotic cells. DLA cells obtained from MEAA treated mice were characterized by a large fraction of apoptotic cells. Prominent hallmarks of apoptosis, such as membrane blebbing and nuclear disintegration were evident in MEAA-treated cells. This suggests that MEAA ameliorates ascites tumour progression in experimental animals by enhancing apoptotic cell death.

MEAA treatment ensured minimum damage to liver, as suggested by histopathological analysis. Liver from tumour control showed destructed lobular architecture with centrilobular necrosis surrounded by inflammatory infiltrated and interface hepatitis. Individual hepatocytes showed bi-nucleation. Sinusoids showed mild dilation. Liver from MEAA-treated group showed normal lobular architecture and individual hepatocytes showed no significant pathology.

Methanol extract of A. aureum (MEAA) was thus found out as a potent anticancer agent which can inhibit the proliferation of DLA cells in vivo. MEAA can shrink ascitic tumor volume by enhancing tumour cell apoptosis, and improve the hematological parameters of the tumor bearing mice.

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