International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Evaluation of Anticancer Potentials of Oecophylla Smaragdina Extracts on Murine Ascites Dalton's Lymphoma from Meghalaya

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Abstract
Weaver ants, Oecophylla smaragdina are very fascinating ants with a unique behaviour of nest building. They use their abdominal secretion (formic acid) to avoid infection in their nest. These ants have shown utility as natural pest control agents, a source of food and in traditional medicinal practices. However, no studies have been undertaken to assess its anticancer potential. The outcome of the present study shows that the weaver ants have anticancer potential. Aqueous, ethanol and methanol extracts of adults, pupae and larvae of these ants were prepared and evaluation of anticancer properties was carried out on Dalton's lymphoma bearing mice. It was observed that the ethanol extract of adult ants at 25 mg/kg body weight has more promising anticancer potentials. The anticancer properties were based on the findings of an increase in the life span of the tumour-bearing hosts and the development of apoptotic features in Dalton's lymphoma cells similar to that treated with cisplatin. Histopathological effects on liver and kidney were comparatively less toxic on treatment with the weaver want extract than cisplatin. Thus, it is suggested that these ants have anticancer properties. However, the bioactive component(s) and molecular mechanisms behind its effect need to be explored.

Keywords: Weaver ants, Anti-cancer activity, Dalton's Lymphoma

Introduction

Weaver ants, Oecophylla smaragdina are arboreal ants having unique characteristics for nest building using the leaves of the host tree. Depending on the population and the nest size, the number of nests is increased [1]. Weaver ants are used as a source of food and medicine to stimulate gastric juice secretion and also as an aphrodisiac [2]. These ants are also being used as medicine for the treatment of fever, flu and headache [3]. It is also believed that these weaver ants play an important role in the detoxification of blood, arresting haemorrhage, restoration of the uterus, stimulating pulse and heartbeat, and relief of asthmatic symptoms and dizziness [2].

Many new therapeutic compounds have been discovered in nature which include plants and microorganisms possessing diverse chemical properties including anti-cancer potential [4] (Butler 2004). Animals and animal-derived products have also been used for treating various ailments in traditional

therapeutic practices [5] (Lev 2003). Even in Indian traditional healing practices, asthma, cough, tuberculosis and common colds are treated using animal or animal-derived products, out of which 22% of animals being used are reported to be invertebrates [6] (Mahawar and Jaroli 2008).

North-East India comprises of large number of ethnic groups inhabiting these regions and are rich in traditional knowledge and practicing traditional healing since the olden days [7]. The Nyishi and the Galo of Arunachal Pradesh use 81 species of edible and therapeutic insects and 36 vertebrate species for treating various ailments and diseases [8, 9]. In Karbi Anglong District of Assam, 48 different animal species were recorded to be used for ethno-medical purposes[10]. In the Indigenous Khasi tribes of Sohiong village of Meghalaya 13 animal groups were recorded to be used for the treatment of different ailments like anaemia, cancer, diarrhoea, cough, etc., [11].

According to the World Health Organization (2022), cancer is one of the diseases accounting for nearly 10 million deaths worldwide. The understanding of the causes and treatment of cancer is of utmost importance to human beings. Chemotherapy is widely used solo or in combination with other methods for treating various cancers. Several chemotherapeutic drugs like cisplatin, chlorambucil, cyclophosphamide, 5-fluoro-uracil, etc. are being used for the same [12]. Even though chemotherapy is one of the effective methods for cancer treatment, it often leads to various side effects like hair loss, infertility, lymphedema, anaemia, loss of appetite, vomiting, etc., leading many researchers to find alternative forms of medicines or drugs for cancer treatment from natural resources [13]. Several arthropods such as scorpions, bees, wasps, spiders, ants and caterpillar toxins or venoms possess several active molecules capable of interfering with human cellular physiology and may also possess potential anti-tumour agents [14]. For example like the venom from fire ants, Selonopsis invicta was reported to contain the primary alkaloid Selonopsin A which promotes antiangiogenic activity [15].

Weaver ants known for so many medicinal properties in various traditional healing practices, have not been documented nor assessed for their anticancer activities. Hence, this study was undertaken to examine the anticancer properties of different extracts of the castes of O. smaragdina and this is the first study for anticancer activities using these weaver ants from this region. Furthermore, cisplatin was used as a reference drug. After the screening of the extracts, the most potent extract was used further to analyse the fluorescence-based apoptotic study. It revealed that the ethanol extract of adult ants has better anticancer potentials.

Materials and method

Sample collection and extract preparation

The nest of weaver ant, O. smaragdina was collected from Umran-Dairy village, Ri-Bhoi district, Meghalaya and brought to the laboratory, freeze killed at 4°C and segregation of the different castes (the worker ants, pupae and larvae) was done. A 100g of each caste was taken, and crushed using mortar and pestle and extraction was carried out using Soxhlet apparatus in different solvents like aqueous, ethanol and methanol (1:2 each). The solvent was evaporated at 30°C, and then the crude extracts collected were stored at -20°C until used.

Animal maintenance and tumour model

Inbred Swiss Albino mice maintained in standard laboratory conditions were used as an animal model. Mice in the age group of about 10-12 weeks weighing 25-30 g were used for the experiments.

Ascites Dalton's Lymphoma (DL) is being maintained in vivo in mice by serial intraperitonial (i.p.) transplantation (1×10^6 tumour cells per animal). Mice transplanted with DL cells usually survive for about 18-20 days. The use of animals and the experimental plan was approved by the Institutional Ethical Committee, North-Eastern Hill University, Shillong vide certificate No IEC/MS/Misc./21 dated 3rd December 2020.

Screening of dose and extract for anticancer potential

The Dalton's Lymphoma tumour transplanted mice were divided into four groups with 10 mice in each group for each extract (aqueous, ethanol and methanol extract) and a control group as given in Table 1. Each group except controls were treated intraperitoneally (i.p.) with 10, 25, 50 and 100 mg/kg body weight of the crude extracts i.e., the 1st dose on the 5th day and the 2nd dose on the 7th day. The grouping was established to evaluate the different extracts (aqueous, ethanol and methanol extract) from the three castes of the weaver ants i.e., adult/worker, pupae and larvae separately. The survival details as an increase in lifespan (%ILS) of the mice in each group were calculated.

Table 1: Groupings for the preliminary screening of potential extract

	(Aqueous Extract) mg/kg body weight	(Ethanol Extract) mg/kg body weight	(Methanol Extract) mg/kg body weight
Control (Untreated mice)	-	-	-
Group I	10	10	10
Group II	25	25	25
Group III	50	50	50
Group IV	100	100	100

Increase in lifespan (%ILS) was also calculated using the formula given below:

$$ILS\% = \frac{MST\ of\ Treated - MST\ of\ Control}{MST\ of\ Control} \times 100$$

Where,

Mean Survival time (MST) = $\Sigma \frac{Mean\ No.of\ mice\ survived\ of\ each\ group(days)}{Total\ No.\ of\ mice\ in\ each\ group}$

The best dose and extract giving high ILS% were selected for further cell viability and apoptotic studies.

Cell viability

Trypan blue exclusion test on DL cells was carried out as per standard protocol [16], DL cells were collected from mice treated with 25mg/kg body weight ethanol extract of adult worker ants, Oecophylla smaragdina (EEOS) at time intervals of 24h, 48h, 72h and 96h. Similarly, DL cells were collected from mice treated with cisplatin (8mg/kg body weight) at different time intervals. The cell viability was determined as per the following formula:

$$\%\ Dead\ Cells = \frac{No.of\ Dead\ Cells}{No.of\ viable + No.of\ Dead\ Cells} \times 100$$

Fluorescence-based apoptosis study

Further, various apoptotic features were analysed in the ethanol extract and cisplatin-treated DL cells were collected for viability study using the standard protocol by Squier and Cohen, (1948) [17]. For the

fluorescence-based apoptotic study stains acridine orange (AO) and ethidium bromide (EtBr) were used. Apoptotic features were carefully analysed under a fluorescence microscope, and photographed and the apoptotic index was calculated.

$$\%\ Apoptotic\ Cells = \frac{No.of\ Apoptotic\ Cells}{Total\ No.of\ Cells} \times 100$$

Histopathological Study

Tissues such as liver and kidney from control, extract treated and cisplatin treated group were taken on the 11th day post treatment and fixed in 10% formalin fixative. The tissues were processed in ascending grades of alcohol (30, 50, 70, 90 and 100%). Then, clearing was done twice in xylene before embedding. Embedding of the tissues in paraffin wax was done at 50-60°C and cooled at room temperature. Thin tissue sections were cut at a thickness of 5µm with a microtome (Weswox) and mounted on clean glass slides smeared with a drop of albumin. Hydration was done by passing through a decreasing concentration of alcohol grades (100, 90, 70, 50 and 30%) followed by staining with hematoxylin. Excess stain was removed in running water followed by dehydration in alcohol grades and staining with eosin. Xylene was used as clearing agent and the tissues were mounted using DPX. The prepared histological sections of the tissues were thoroughly examined under a light microscope (Leica) for changes in cellular organization.

Statistical analysis

Analysis of variance (ANOVA) and Student's T-test were used for statistical analysis.

Results

Evaluation of antitumour activity of aqueous, ethanolic and methanolic extracts

The preliminary evaluation for the anticancer study carried out to find the best survivability pattern of the tumour-bearing mice treated with different extracts revealed that 25 mg/kg body weight of the aqueous extract of worker ants increased the lifespan of tumour-bearing mice to about 23% (Table 2). Similarly, ethanol extract of the worker ants at a dose of 25mg/kg body weight resulted in increasing the lifespan of tumour-bearing mice to about 73.3% (Table 3). Treatment with methanol extract from adult workers showed about a 34.7% increase in lifespan at a dose of 25mg/kg body weight (Table 4). The result revealed that a low dose of 25mg/kg body weight of all adult worker ant extract showed a significant increase in lifespan in tumour-bearing mice, and among them, the ethanol extract exhibited a better increase in lifespan. However, other higher doses such as 50 and 100 mg/kg body weight may have been lethal resulting in the early death of the mice under different treatment conditions. Thus, the screening of the doses and the extracts of the ants showed that the dose of 25mg/kg body weight of ethanol extract of the adult worker ants (EEOS) has better therapeutic efficacy than the others. A comparison of the efficacies of antitumor activities of various extracts has been shown in Fig. 1.

Table 2: Screening of antitumour activity of aqueous extracts

Treatment group	Dose (mg/kg body weight)	Life span (days) (MST± SD)	% ILS	Survival on day 30 Survival/total
Control	-	19 ± 0.96	-	0/10
Adult	10mg	20 ± 0.96	8.0	0/10
	25mg	23 ± 1.41*	22.7	1/10
	50mg	19.5 ± 1.29	4.0	0/10

	100mg	17.5 ± 0.58	-6.7	0/10
Pupae	10mg	20.25 ± 0.50	6.7	0/10
	25mg	21.75 ± 1.26	16.0	0/10
	50mg	19 ± 1.83	1.3	0/10
	100mg	17.5 ± 0.58	-6.7	0/10
Larvae	10mg	20.25 ± 0.50	8.0	0/10
	25mg	19.75 ± 0.96	5.3	0/10
	50mg	18.05 ± 1.29	-1.3	0/10
	100mg	17.75 ± 0.96	-5.3	0/10

Table 3: Screening of antitumour activity of ethanol extracts.

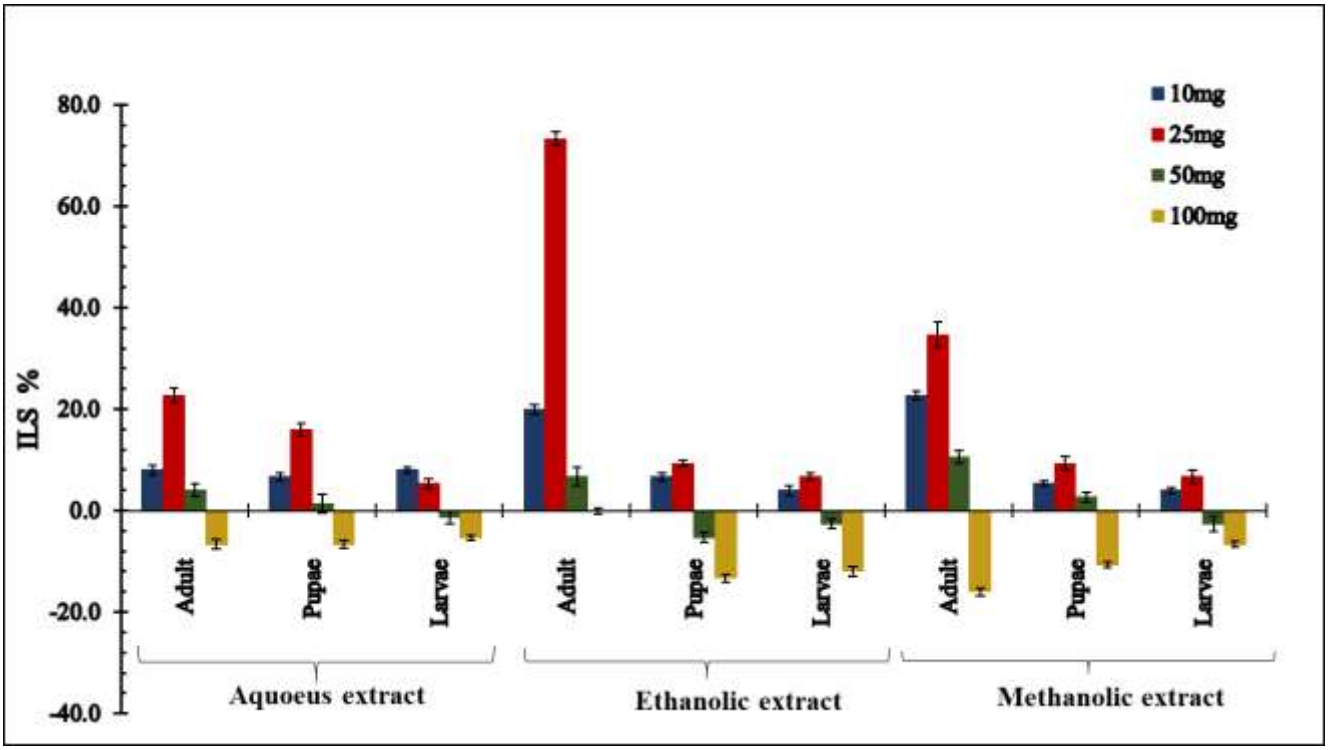
Treatment group	**Dose (mg/kg body weight)**	**Life span (days) (MST± SD)**	**% ILS**	**Survival on day 30 Survival/total**
Control	-	19 ± 0.96	-	0/10
Adult	10mg	22.5 ± 0.58	20.0	1/10
	25mg	32.5 ± 1.29*	73.3	4/10
	50mg	20 ± 1.83	6.7	0/10
	100mg	18.75 ± 1.71	-1.3	0/10
Pupae	10mg	20 ± 0.82	6.7	0/10
	25mg	20.5 ± 0.58	9.3	0/10
	50mg	17.75 ± 0.96	-5.3	0/10
	100mg	16.25 ± 0.96	-13.3	0/10
Larvae	10mg	19.5 ± 1.00	4.0	0/10
	25mg	20 ± 0.82	6.7	0/10
	50mg	18.25 ± 0.96	-2.7	0/10
	100mg	16.5 ± 1.73	-12.0	0/10

Table 4: Screening of antitumour activity of methanol extracts

Treatment group	Dose (mg/kg body weight)	Life span (days) (MST± SD)	% ILS	Survival on day 30 Survival/total
Control	-	19 ± 0.96	-	0/10
Adult	10mg	23 ± 0.82*	22.7	0/10
	25mg	25.25 ± 2.50*	34.7	2/10
	50mg	20.75 ± 1.26	10.7	0/10
	100mg	15.75 ± 0.96	-16.0	0/10
Pupae	10mg	19.75 ± 0.50	5.3	0/10
	25mg	20.5 ± 1.29	9.3	0/10
	50mg	19.25 ± 0.96	2.7	0/10

	100mg	16.75 ± 1.50	-10.7	0/10
Larvae	10mg	19.5 ± 0.58	4.0	0/10
	25mg	20 ± 1.41	6.7	0/10
	50mg	18.3 ± 1.5	-2.7	0/10
	100mg	17.5 ± 1.29	-6.7	0/10

Figure 1: Survival patterns of tumour-bearing mice under different treatment conditions. The ethanol extract of adult ant at a dose of 25 mg/kg body weight showed better ILS i.e. 73%

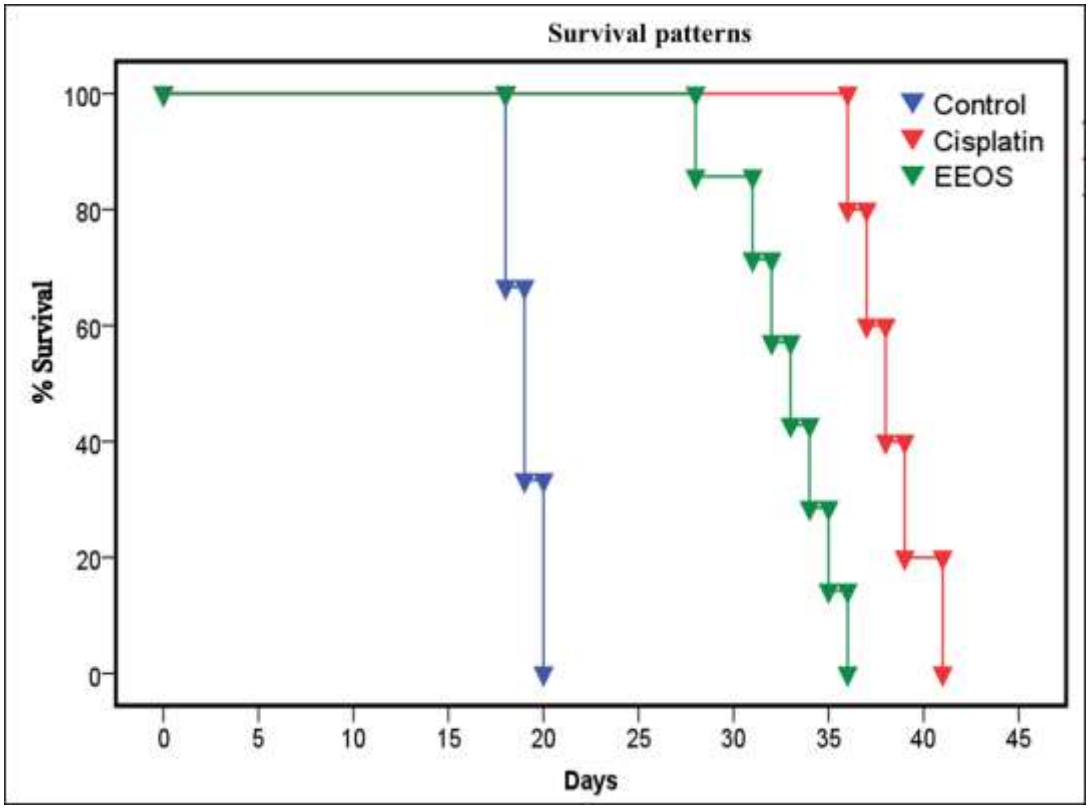


Further scrutiny of the anticancer activity of ethanolic extract of adult ants, Oecophylla smaragdina (EEOS) was carried out in comparison with a common cancer chemotherapeutic drug, cisplatin. It was observed that EEOS at 25 mg/kg body weight is effective against murine ascites Dalton's lymphoma and increased the lifespan to approximately 33 days i.e. 73% as compared to control. Tumour-bearing mice treated with cisplatin showed more increase in lifespan of about 39 days (104% ILS) as compared to the control (Table 5). The comparative survival plot was constructed using Kaplan-Meyer's survival plot to show the average life span of the tumour-bearing mice (untreated) control, cisplatin-treated tumour-bearing mice and EEOS-treated tumour-bearing mice (Fig. 2).

Table 5: Mean survival time (MST) and % ILS of tumour-bearing mice at different treatment conditions.

Treatment groups	Days of treatment	Route of treatment	Survival Days (MST ± SD)	% ILS
Group I (Control)	-	Intraperitoneal (i.p.)	19 ± 0.96	-
Group II (Cisplatin)	7th day		39 ± 2.22	104
Group III (EEOS)	5th & 7th day		33 ± 1.29	73

Figure 2: Kaplan-Meier survival plot- survival pattern of tumour-bearing mice under different treatment conditions



Cells viability study

The trypan blue exclusion test has been used to check the viability of the cells in the preliminary investigation of potential anticancer activities of various extracts or drugs. The study revealed that the viability of DL cells remain unchanged in control group (Fig. 3a). However, gradual decrease in viable DL cells and an increases in the number of dead DL cells was observed in a time-dependent manner from 24 hours to 96 hours after treatment with cisplatin (Fig. 3b-e) and EEOS (Fig. 3f-i). The highest record of dead DL cells was observed at 96 hours post-treatment in both these treatment groups (Fig. 4).

Figure 3: Trypan blue exclusion test of DL cells. a) Control, b-e) Cisplatin-treated group, f-i) EEOS- treated group. Both cisplatin and EEOS treated groups showed significant increase of dead DL cells

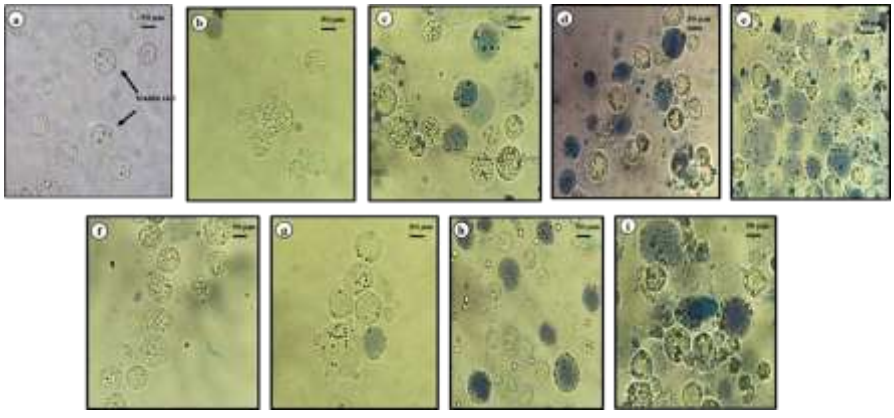
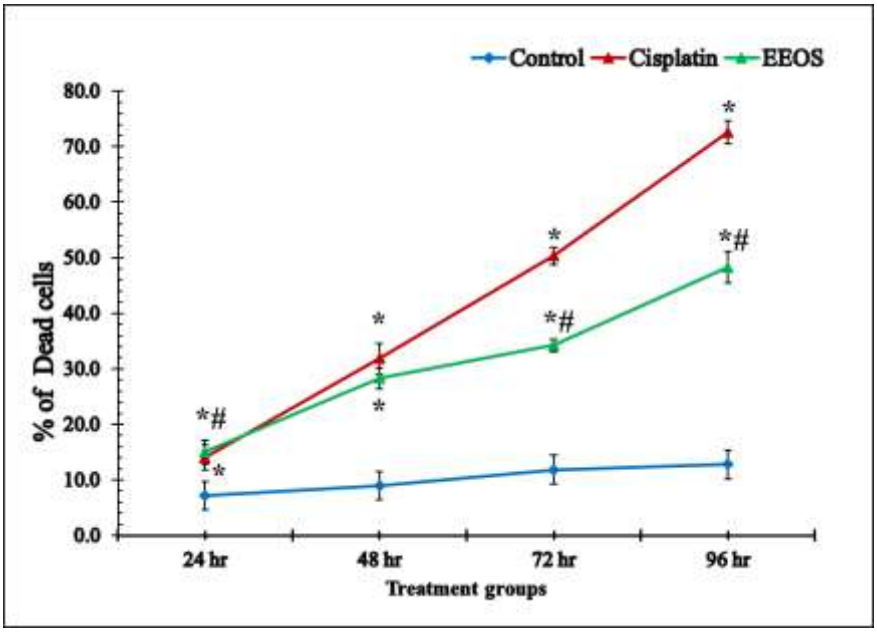


Figure 4: Graphical presentation of patterns of percent dead cells in different treatment groups after the Trypan blue exclusion test. The number of dead DL cells increased in cisplatin and EEOS- (ethanol extract of adult Oecophylla smaragdina) treated groups. (Significance *p≤ 0.05, n=3 as compared to the control; #p≤ 0.05, as compared to cisplatin)



Fluorescence-based apoptosis study

The study revealed that DL cells stained with acridine orange (AO) and ethidium bromide (EtBr) gave out green fluorescence colour in viable or live DL cells (Fig. 5a) as in control whereas dead or dying DL cells gave out yellow to orange fluorescence colour. The DL cells in the cisplatin-treated group showed high apoptotic features at 96 hours post-treatment (Fig.5b). The apoptotic features such as membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation were observed in both the treatment groups (Fig. 5). Similarly, the cells in the EEOS-treated group also showed a gradual increase in the number of apoptotic features at 24-96 hour post-treatment (Fig. c-f). The number of apoptotic cells was analysed under ten different observation fields in each experimental group and the approximate apoptotic index of DL cells under different treatment conditions was calculated (Fig. 6).

Figure 5: Apoptotic features in Dalton's lymphoma (DL) cells stained with acridine orange and ethidium bromide. (a) Control DL cells; (b) 96 hours cisplatin; (c-f) EEOS treated (24, 48, 72 and 96 hours). Prominent apoptotic features such as membrane blebbing, nuclear fragmentation, chromatin condensation and cell shrinkage were noted. The scale bar of each picture is 50 µm

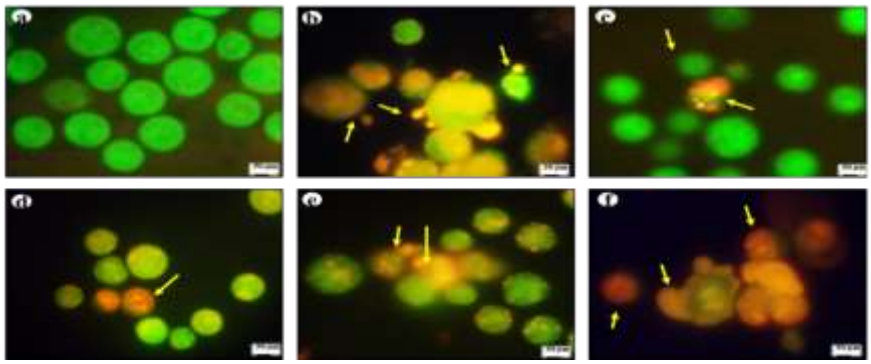
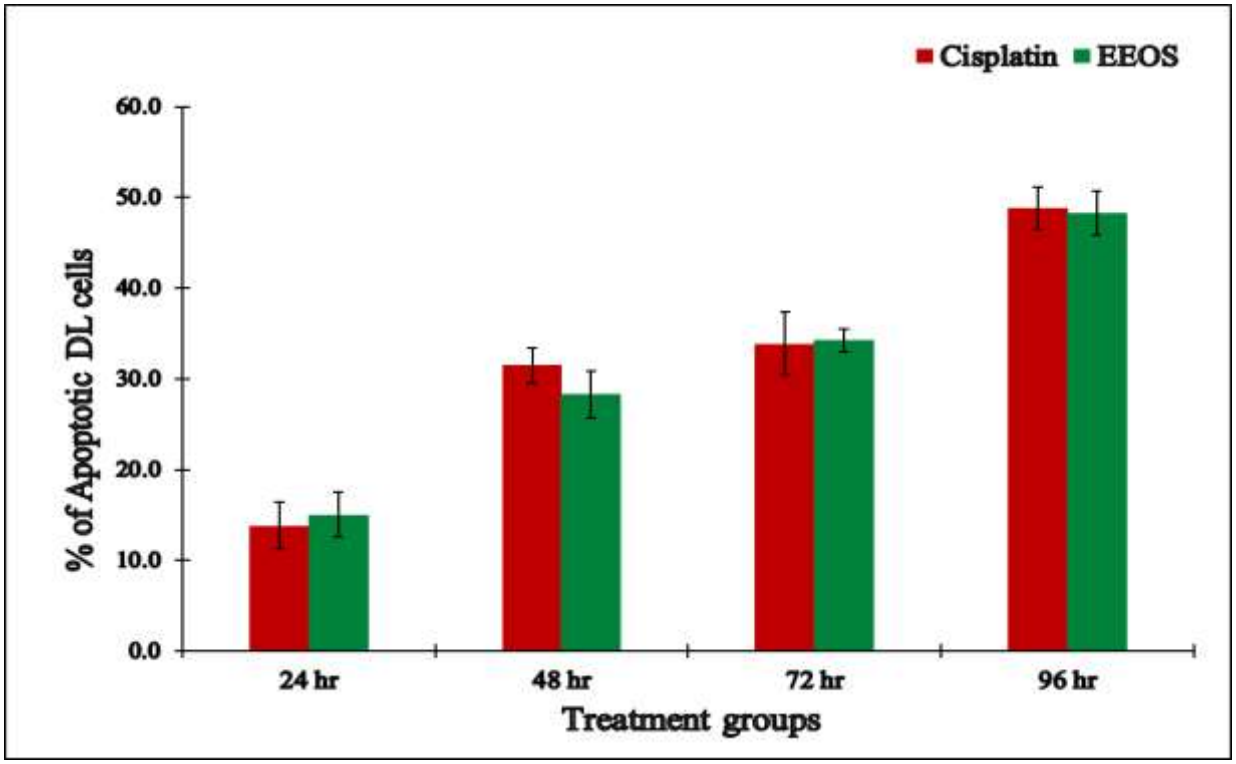


Figure 6: Apoptotic index (% control) of DL cells from mice under different treatment conditions. Results are expressed as mean ± S.D.



Histopathological study

Histopathology of the liver from control tumour-bearing mice showed normal distribution of the hepatocytes and proper architecture of central and portal veins (Figure 7a). Cisplatin treatment caused damage and congestion in the central and portal vein with haemorrhage inside, and the size of the hepatocytes becomes small with the dispersed arrangement (Figure 7b). However, mice treated with ethanol extract of adult Oecophylla smaragdina (EEOS) displayed fewer damages in the portal vein and haemorrhage in the central vein, and hepatocytes were larger with a normal distribution as compared to cisplatin treatment (Figure 7c). Histopathological features of the kidney of the control tumour-bearing mice showed intact epithelial cells and normal renal glomeruli and tubules (Figure7d). However, cisplatin treatment caused damages in renal cells, tubular damage disruption, Bowman's capsule disruption and severe glomerular disruption indicating severe toxicity in the mice (Figure 7e). EEOS-treated mice showed comparatively less damaging features as compared to that in cisplatin treatment (Figure 7f). This study elaborates that treatment with cisplatin has higher pathological toxicity as compared to treatment with weaver ant extract (EEOS) (Table 6).

Figure 7: Histopathological features of liver (a-c) and kidney (d-f) from control, cisplatin treated and EEOS-treated mice showing normal architecture in control (a & d), however severe pathological changes observed in cisplatin treated (b & e), and less pathological effects shoed on treatment with EEOS (c & f).

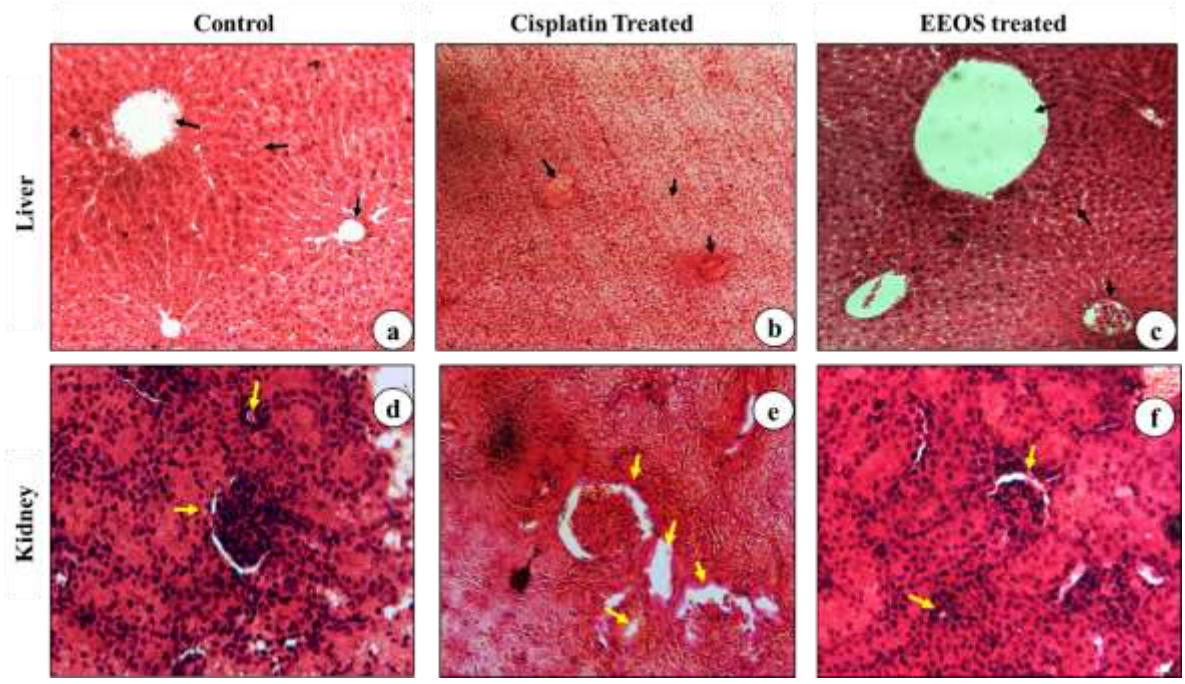


Table 6: Histopathological damages grading in the liver and kidney of tumour-bearing mice under different treatment conditions.

Tissues	Pathological damages	Control	Cisplatin	EEOS
Liver	Congestion in a central vein	-	++++	+
	Hepatocytes damage	-	++++	++
	Haemorrhage in the central vein	-	++++	++
	Haemorrhage in the portal vein	-	++++	+
Kidney	Tubular disruption	-	++	++
	Glomerular shrinkage	-	+++	-
	Glomerular disruption	-	++++	+
	Bowman's capsule disruption	-	+++	+

(-) Nil, (+) Low/Minimal (< 10 %), (++) Mild/moderate (< 30 %), (+++) high (< 50%), (++++) very high/severe (> 50%)

Discussion

Weaver ants have been very important and resourceful to people worldwide. A study conducted in Thailand and Vietnam showed that weaver ants could be used as natural pesticides as they are cost-effective, environment-friendly and crop-friendly insects [18]. These weaver ants build their nest on the trees, as their population grow they increase their nest size (400 cm^3 to more than 10,000 cm^3) by adding additional layers of leaves and gluing them together using their larval silk, hence, generating more room

[1]. Many traditional practitioners have been using weaver ants for treating various ailments and investigations on biochemical and pharmacological are conducted to prove the medicinal importance of the ants [19].

The preliminary evaluation for the anticancer study carried out on tumour-bearing mice treated with different extracts of the weaver ant, Oecophylla smaragdina is evident that pupae and the larvae extracts in all doses from all the solvents did not show any anticancer activities. The use of a low dose of ethanolic extract at 25mg/kg body weight gave better results, However, other higher doses such as 50 and 100 mg/kg body weight may have been lethal resulting in the early death of the mice under different treatment conditions. However, the dose at 25 mg/kg body weight of adult/worker weaver ants on all solvents showed promising results with the aqueous extract giving %ILS of 23% (Table 2), the ethanol extract giving %ILS of 73.3 % (Table 3) and methanol extract giving %ILS of 34% (Table 4 and Fig. 1). Cisplatin is a common anticancer drug used by many researchers as a control drug for cancer studies [20]. Even though its effectiveness against tumours or cancer is very high, various side effects are experienced by patients undergoing chemotherapy [21]. From our study, it was observed that tumour-bearing mice treated with cisplatin (8mg/kg body weight) showed an increase in lifespan of about 104% as compared to the control. A similar study has also reported that treatment with 8mg/kg body weight of cisplatin in tumour-bearing mice increases the lifespan to about 127.37% [22]. Treatment with 25 mg/kg body weight of ethanol extract of adult Oecophylla smaragdina (EEOS) showed promising results against murine ascites Dalton's lymphoma and increased the lifespan to approximately 33 days i.e. 73% as compared to control. This explains the effectiveness of the dose minimizing the toxic effect and causing the tumour cells to undergo apoptosis (Table 5). The comparative graphical elaboration made using Kaplan-Meyer's survival plot further elaborates survival patterns of the tumour-bearing mice (untreated) control with no increase in lifespan, but, cisplatin-treated tumour-bearing mice and EEOS-treated tumour-bearing mice showed significant increase in their lifespan (Fig. 2).

The trypan blue exclusion test enables to determine the viable population of the cell. Staining with trypan blue dye shows that the viable DL cells remained unstained, however, the dead DL cells absorbed the stain and hence look like blue-dyed cells. The study showed a gradual increase in the number of dead cells from 24 to 96 hours after treatment with EEOS and cisplatin (Fig. 3 and Fig. 4). Further, fluorescence based techniques allow researcher to monitor cellular changes like viability or apoptosis in response to drug treatment. Many anticancer drugs eliminate cancer cells by inducing apoptosis and the ability to induce apoptosis on cancer/tumour cells exhibits the strength of the drug [23]. From the study it is revealed that a gradual increase in apoptotic DL cells was observed from 24 to 96 hours in the cisplatin-treated group. Similarly, the EEOS-treated group also showed a gradual increase in the number of apoptotic DL cells at 24 h, 48 h, 72 h and 96 h with features such as membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation (Fig. 5). From both cisplatin and EEOS treated group, DL cells exhibit a gradual increase apoptotic features (Fig. 6).

Histopathological examination is one of the method which elucidate the pathological effects caused by the treatment [24]. Cisplatin is known to accumulate more in liver and kidney causing significant side effect [25]. From the study it has be observed that treatment with cisplatin results in severe histopathological abnormality like dilation and shrinkage of the hepatocytes and congestion of both central and portal veins due to haemorrhage in the liver. However, less hepatotoxicity was observed when treated with the extract (EEOS) (Fig.7) (Table 7). Kidney being one of the organ to eliminate waste is also reported to be affected by several anticancer drugs leading to renal complications [26]. Severe disruption of the glomerulus and

the Bowman's capsule was observed from the study due to cisplatin. Similar features were also observed when treated with the ant extract, however it is comparatively less toxic and showed fewer abnormality than cisplatin. Thus concluding the effectiveness of the ant extract being lesser toxic than cisplatin (Table 7).

Thus, this may be significant to conclude that EEOS treatment also induced the tumour cells to undergo apoptosis, increases the lifespan of the tumour bearing mice and has lesser side effects as compare to cisplatin. Raza et al. (2022) reported that weaver ants are a good source of antioxidants and also contain large amounts of flavonoids and phenolic compounds which are important bioactive molecules having potential anticancer agents [27]. Other studies also have reported the presence of antioxidants and phenolic contents in the weaver ant extracts, apart from that, the presence of volatile compounds like acids, alcohols, alkanes, aldehydes, ketones, terpenes, fatty acid esters [28]. Their study could be significant concerning our study supporting the weaver ant crude extract does possess potential anticancer agents.

Conclusion

Animal or animal-based products could also be focused on new drug discovery and development for future use. Therefore, our study aims to give a new lead from the region towards anticancer study and treatment using weaver ants. From the above study, it is evident that ethanol extract of adult Oecophylla smaragdina (EEOS) has better therapeutic potential against murine ascites Dalton's lymphoma. Further toxicological and bioactive compound-related studies could be undertaken in the near future.

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