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In vivo antioxidant and immunomodulatory effect of the aqueous extract of *Mimela* sp. On cyclophosphamide induced immunocompromised mice

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Abstract
Insect and their products have been used as an integral part of local therapies around the globe since decades. Several studies on insects have found it to be highly nutritious and rich in several bioactive compounds possibly attributing to its anti-oxidative, anti-carcinogenic, anti-inflammatory, anti-microbial, anti-diabetic and immune regulating properties. *Mimela* sp. is a small shiny green beetle highly preferred as food by the people of Arunachal Pradesh and is known to be therapeutically used in other regions. The Immunomodulatory effect of the aqueous extract of *Mimela* sp. was investigated in the present study on albino mice. The female mice were randomly divided into normal control, model control, low dose (500mg/kg), medium dose (1000mg/kg) and high dose (2000mg/kg) group. The mice in the dosage groups were fed with extract via oral gavage once a day for 14 days. The mice body weight, spleen and thymus indexes, RBC, WBC and HGB were measured. The oxidative stress parameter like lipid peroxidation, glutathione level and catalase activity were evaluated. Tissue morphology of spleen and thymus was observed. Delayed type hypersensitivity response, hemagglutination titer cytokines like IL-6 and TNF-α levels were measured. The results show significant increase in immune organ weight, leukocyte count, mice footpad volume, antibody formation and cytokine level. Enzymatic and non- enzymatic antioxidant levels were also increased. Further histomorphological observation showed improvement in numbers of immune cells mainly lymphocytes. Thus, suggesting that the aqueous extract of *Mimela* sp. have antioxidant and immunodulatory potentials.

Keywords: *Mimela* sp., edible insects, antioxidant, immunomodulation, immuno suppression, cyclophosphamide

Introduction
Immune system act as an amazing sentinel of animal body keeping one protected from the diverse mutating pathogenic organisms. It is involved in the etiology as well pathophysiological mechanisms of many diseases and is influenced by many exogenous and endogenous factors such as food, pharmaceuticals, physical and psychological stresses, resulting in either immunosuppression or immunostimulation (Geetha. S. *et al.* 2005) [1]. Normal functioning of this system is crucial for one's good health. There are certain agents possessing activity to normalize or modulate pathophysiological processes called immunomodulatory agents. Modulation of immune response to alleviate diseases has been of interest for many years (Sharma P, 1983) [2]. These immunomodulatorscan provide supportive theory to chemotherapy (Kokata CK, 1998) [3]. Currently available chemotherapeutic drugs and immunomodulators like cyclophosphamide, levamisole, glucans, telerones and L-fucose are known to have adverse effect on human health including immunosuppression. Due to which search for natural immunomodulatorsdo gaining lots of attention for their lesser side effects. Besides, several plants and insect have been used as food and folk remedies by ethnic people since time immemorial for their palatability, effective healing with no adverse ramifications, easy availability and cost effectiveness (Rego TJA, 1995; Tang *et al.*, 2010; Chakravorty *et al.* 2011, 2013; etc) [4, 8, 5, 6]. Insects being the richest in species amongst all the animal taxon have proven to be very important sources of drugs for modern medicine (Ahn *et al.*, 2002) [10].

Chemical screening applied to some insects has confirmed the presence of various types of bioactive substances (Costa-Neto, 2002) [11], such as antioxidant, phenolic, flavonoid compounds etc that are known to ameliorate the damages caused by free radicals. Many pharmaceutical functions of medicinal insects have been found that include anti-bacterial activity, anti-inflammatory activity, immune regulation, anti-sensitivity reactions, anti-oxidant activity and reducing blood sugar (Yamakawa, 1998) [12]. Nowadays, there is a growing interest in identifying and characterizing insects with immunomodulatory effect (Tang *et al*. 2010) [8]. A number of insects used in Chinese traditional medicines and food systems for rejuvenation therapy have shown to modulate immune responses (Zhang and Xu.1990) [13]. The various healthful functions of insects are due to the synergy of its nutritional components.

A number of insects are available and traditionally used as food and medicine to treat variety of disease conditions in Arunachal Pradesh but many of them remained to be explored scientifically. *Mimela* sp. a coleaopteran bug belonging to the family scarabaedae is a green shiny little bug that mostly feed on leaves of fruit plants. It is one of the most preferred and abundantly found edible insect of the state, rich in proteins, essential amino acids, essential fatty acid, vitamins and minerals. The *Mimela* sp. is reported to be consumed in Korea (Kim *et al*., 2008) [14] and Northern Thailand (Leksawasdi, 2010) [15] for their nutritional and therapeutic value. The toxicological study on the aqueous extract of *Mimela* sp. has reported their LD50 value to be greater than 4000mg/kg body wt. of mice (Yashung P *et al*. 2019) [16]. Although studies have been reported on its biology and nutritional composition no work has ever taken to elucidate its therapeutic potential. So the present work to evaluate the antioxidant and immunomodulatory activity of the various doses of aqueous extract of *Mimela* sp. has been taken up to provide scientific basis for the comprehensive utilization of the insect.

Materials and Methods

Sample collection: The adult *Mimela* sp. was handpicked from the Malus domestica and Alnus nepalensis tree, Menchuka, Shi-Yomi district of Arunachal Pradesh in the month of June and July. After collecting they were immediately brought to the laboratory and kept at -20 degree freezer for further analysis. The sample was identified and confirmed by zoological survey of India, Kolkata.

Sample preparation: The samples after being brought to the laboratory were washed properly with distilled water, blotted dry and kept in the incubator at 40-50 °C for 72 hrs. After drying the wings and appendages were removed, the remaining body was grinded into powdered form. About 3mg of insect powder was weighed and dissolved in 100ml of distil water, which were soaked overnight at room temperature. Subsequently, it was filtered using whatman filter paper No.1and the filtrate was freeze dried to obtain powder like insect aqueous extract which were used for dosing experimental animals.

Chemicals and reagents: All the chemicals and reagent used were of analytical grade.

Animal grouping and drugs administration: Healthy female albino mice (18-25g) were procured from the animal care center of dept. of zoology, RGU for the study. The animals were housed under controlled temperature (20-24^{o}C) and 12hrs light/dark cycles with ad libitum access to food and water. All the animals were acclimatized for 7 days to the laboratory conditions prior testing them. The animal experiments were approved by the Institutional animal care and Ethical committee, Rajiv Gandhi University, A.P, India and all the experimental procedures were followed according to the CPCSEA guidelines.

The animals were divided into five groups with 5 animals per group. Group I (Normal control) received normal saline, Group II (Model) were injected with cyclophosphamide followed by normal saline for the remaining days. The rest of the groups were intra gastrically via oral gavage administered with aqueous solution of insect extract (500, 1000 and 2000mg/kg body wt.) considered as low dose (Group III, M-L), medium dose (Group IV, M-M) and high dose (Group V, M-H) groups. The model group and extract treated groups were immunocompromised by injection cyclophosphamide i.e 70mg/kg body wt. for the first 3 days of the experiment. For humoral immune response animals of all groups were challenged with 10% of SRBC, i.p on the 12th day.

Measurement of body weight, organ indexes and histomorphological observations: All the animals were weighed and sacrificed 24hrs after the last dose.The thymus and spleen of the mice were isolated, washed in PBS, excess tissues and fascia were stripped, and then weighed. The thymus and spleen indexes were calculated according to the following equation:

Organ Index= Organ mass/ animal body mass

The thymus and spleen were fixed in bouins fluid for 22hrs, transferred to 70% alcohol with three wash for 15 mins each and then kept at room temperature till further processing. The pathological specimens were routinely sliced (5 µm), embedded in paraffin and stained with H&E. at room temperature. Pathological changes were observed under a Leica light microscope and images were captured.

Assessment of Antioxidant parameter: The spleen of animals of all groups were collected and washed in ice cold phosphate buffered saline (PBS) to remove blood. Spleen tissue were then homogenized in 10% phosphate buffer (Ph 7.4) followed by centrifugation at 12000 g for 20 mins at 4 °C to obtain the supernatant that was used for the estimation of oxidative stress biomarker (Das D et al.,1993) [17].

Assay for MDA: 0.250 ml of 10% homogenate was mixed with 1ml Thiobarbituric acid and 25ul of butylated hydroxyl toluene to which, 3ml of o. phosphoric acid was added. The tubes were then covered with aluminium foil and kept in water bath at 90^{o}c for 45 mins. The boiling tubes were then removed and cooled under running tap water. The resultant precipitate was removed by centrifugation at 3000 rpm for 15 mins. The absorbance was then read at 540 nm against blank. The concentration of the TBARS was determined using a molar extinction coefficient of 1.56×10^5/M/cm (Das D *et al.* 1993) [17].

Assay of Glutathione: The homogenate was mixed with 4% sulfosalicylic acid in the ratio of 1:1 and kept at 4°C for 1hr. After that it was centrifuged at 4000rp for 15 mins at 4°C. Following centrifugation 0.4 ml of supernatant was mixed with 2.2ml of phosphate buffer and 0.4ml of DTNB. The optical density of reaction product was then read immediately at 412 nm and the results were expressed as umol GSH/gm tissue. For blank we took 3ml phosphate buffer (Ellman *et al*., 1959) [31].

Assay for Catalase: About 50 ul of homogenate was taken in a cuvette containing 2.95 ml 0f 19 Mm/l solution of hydrogen peroxide (H2O2) prepared in potassium phosphate buffer. Change in optical density was measured at 240 nm by kinetic mode with 1 min interval for a total of 3 minutes. The units of catalase activity were expressed in nmoles of H2O2 consumed per mg protein (Clairbone A, 1985) [18]. The protein content was estimated by the Lowry method (1951) with Bovine Serum Albumin (BSA) as protein standard.

Blood parameters: Blood samples were collected from antigenically challenged mice after fasting them overnight before necropsy. About 1ml of blood was withdrawn after cardiac puncture into an EDTA coated tube. The blood parameters were then analyzed using the Mythic TM 18 Vet (orphee) an automated hematology analyzer (Germany).

Cell mediated immune response: The cell mediated immune responses were assessed by measuring the change in volume of mice foot pad. On the last day of treatment after measuring the footpad volume of both the legs, SRBC (0.025x 10^9 cell) were injected into the left foot and same volume of saline was injected into the right food. Next day after 24 hrs the increase or decrease in the volume of footpad was measured (Joharapurkar, 2003) [19].

Humoral immunity response: Measurement of antibody titer by hemagglutination reaction was used to evaluate the humoral immune response activity following the method of Bin-Hafeez *et al*. (2001) [33] with some modification. The blood collected after being allowed to settle for few mins were centrifuged at 1000-1500g for 10 mins to separate the serum. The serum of mice was then used for determination of hemagglutination titer. Micro titrer plate with 96 wells used for carrying out titration. Serial two fold dilutions of serum were prepared. To each cup 25 ± l µl of 1% v / v SRBC was added. The plate was then incubated at 37 ºC for 1 h and Observe for agglutination. The antibody titer was expressed in terms of maximum dilution, which gave positive hemagglutination reaction (Hafeez BB, 2001) [33].

Determination of Pro-inflammatory cytokines: The concentrations of TNF- α and IL-6 in the splenic tissue of mice were determined by specific quantitative sandwich ELISA kits for mouse according to the instructional manual of the producers.

Statistical analysis: The statistical analysis was performed by comparing the data of model group with that of control group and that of dose group with model group. Values of body wt, organ indexes and blood parameters are expressed as Mean ± SD while that of footpad volume and HA titer as Mean ± SEM. One-way ANOVA was used for comparison between groups followed by a Tukey's post-hoc test and significance value were expressed by $P<0.05$.

Results

Effects of *Mimela* sp. aqueous extract on oxidative stress markers; MDA, GSH and CAT: The Malondialdehyde (MDA) measured as an index of lipid peroxidation was shown to be significantly higher in model group compared to control group and extract treated groups($P<0.05$). The cyclophosphamide injected extract treated group showed slightly higher MDA content to that of control group.

The Glutathione (GSH) content and Catalase (CAT) activity in cyclophosphamide injected model group were significantly lower than the control groups, M-L and M-M groups. The extract treated groups however showed lesser CAT activity and Glutathione content when compared to control group.(Figure.2)

Effect of *Mimela* sp. on body weight, immune organ weight and histomorphology: There was significant difference in the body weight of model groups ($P<0.05$).All the aqueous extract groups also show significant differences in body weight compared to model group. The spleen and thymus index of control group was significantly higher than that of model control group. Furthermore compared with that of model group, the spleen and thymus index in M-L, M-M and M-H groups were significantly increased ($P<0.05$) as shown in Table.1

As presented in Fig. 1, histo-morphological examination of spleen of control group shows clear demarcation between red pulp and white pulp, also the numbers of splenic corpuscles were normal. Compared to control group the numbers of splenic corpuscles were reduced and their volume was also lower in model group. Whereas, the extract treated groups shows slightly reduced number of splenic corpuscles compared to control group but their volume shows an obvious increase in comparison to model group. Also the boundary between red and white pulp were clear and close to that of control group.

The thymus of model group shows decrease in number of lymphocytes in both cortex and medulla region compared to control group. Compared with that of the model group the lymphocytes number increased in all the three-extract treated group with the highest shown by M-M group followed by M-H then M-L.

Table 1: Effect of Aqueous extract on body weight and organ Indexes

Groups	Body weight (gms)		Spleen Index (mg/g)	Thymus Index (mg/g)
	Initial wt.	Final wt.		
Normal control	24.26±1.30	23.18±2.01	4.302±0.929	1.581±0.40
Model control	22.17[a]±1.51	18.19[a]±0.94	2.123[a]±0.542	0.772[a]±0.06
Low dose (M-L)	23.36 ±1.82	20.51[c]±1.52	3.830[c]±1.112	1.202[c]±0.39
Medium dose (M-M)	23.15±1.53	20.09[d]±1.05	3.896[d]±0.534	1.196[d]±0.25
High dose (M-H)	22.08±1.57	19.59[a]±1.13	3.947[a]±0.792	1.104[a]±0.24

Values are expressed as the Mean± SD of 5 animal S. [a]$P<0.05$ Statistical significance verses Group I; [c]$P<0.05$ Statistical significance against Group II (Model control); [d] $P<0.05$ Statistical significance of Group IV (M-M) against Group II (Model control).

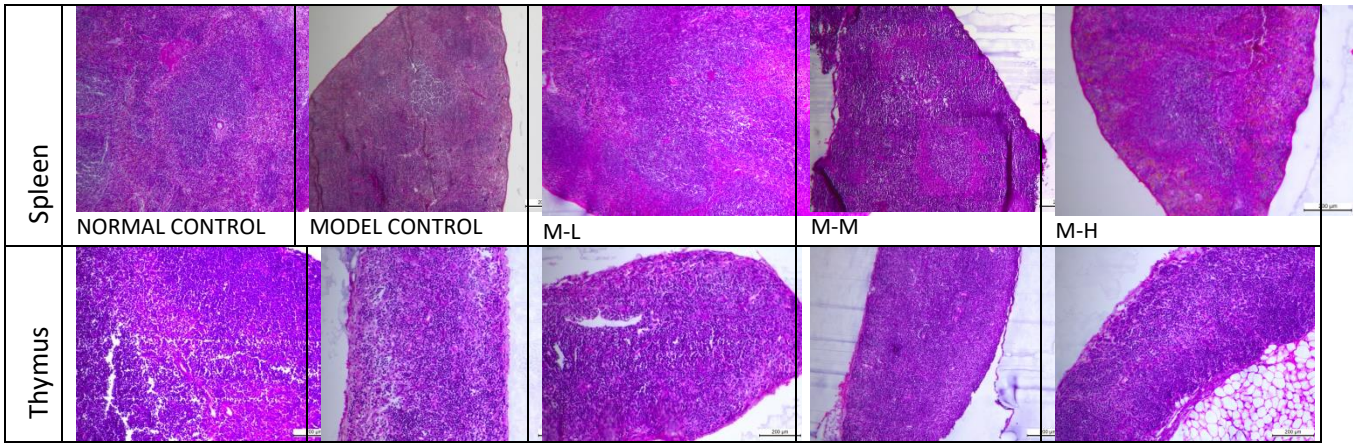

Fig 1: Effects of Aqueous extract of *Mimela* sp. on histomorphology of spleen and thymus of mice

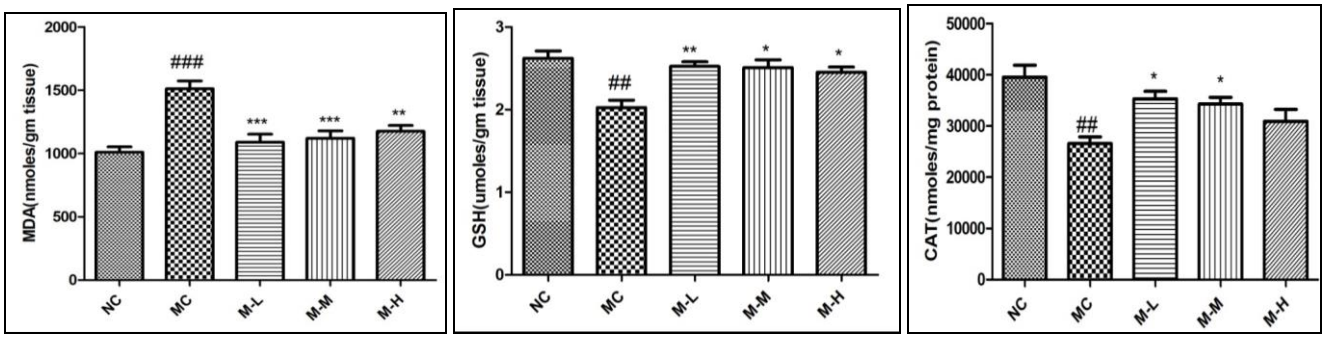

Fig 2: Effects of Aqueous extract of Insect M (*Mimela* sp.) on oxidative stress parameters, MDA= Melonaldehyde, GSH=Reduced Glutathione, CAT=Catalase, NC=Normal comtrol, MC= Model control, M-L= low dose, M-M= medium dose, M-H=high dose. Values are expressed in Mean +_ SEM (n=5). Further the level of significance was calculated using Tukey's multiple comparison test. $P^{\#}<0.05$, Significant vs control and $P^{*}<0.05$, Significant vs Model group.

Effects of *Mimela* sp. aqueous extract on Blood parameters: The cyclophosphamide injected group showed significant decrease in WBC, RBC and HGB compared to control group. On the other hand the combined cyclophosphamide and insect extract treated group showed increase in RBCs, HGBs and significant increase in WBCs ($P<0.05$); Table 2.

Delayed type hypersensitivity reactions (DTH): The cell mediated immune response of the insect extract was assessed by delayed type hypersensitivity reactions on the foot pad of mice. As shown in Table.3, the extract treated group showed dose related increase in DTH reactivity in the sequence M-L> M-M >M-H though not significant.

Hemagglutinating antibody (HA) Titer: The HA titer was used to assess the humoral immune response. As shown in the Table.3, the cyclophosphamide treated group showed decrease in HA titer compared to control group after incubation with SRBCs. The extract treated group showed increase in HA titer; M-L= M-M>M-H compared to model group as evident from hemagglutination reaction after 2hrs incubation with SRBCs.

Table 2: Effect of Aqueous Insect Extract on Blood parameters

Groups	Treatment	WBC x 10^6/ul	RBC x 10^6/ul	HGB(g/dl)
I	**Control- Normal saline(NS)**	**3.66 +0.94**	**8.63 + 0.89**	**14.50 +1.81**
II	Model- NS + CYP	2.08[a] +0.68	6.04[a] +0.63	10.68[a] +0.67
III	M-L (500mg/kg + CYP)	3.07[d] +0.12	7.26 +1.06	12.20[c] +1.15
IV	M-M (1000mg/kg + CYP)	3.14[c] +0.63	7.53 +1.09	12.38[c] +0.96
V	M-H (2000mg/kg + CYP)	3.13[c] +0.23	7.38 +1.05	11.87[d] +0.78

Values are presented as Mean +_ SD of 5 animals.[a] $P<0.05$ Significant difference against Normal control; [c]$P<0.05$ represent Significant difference against Model control and [d]$P<0.05$ Significant difference of M-H verses NC group.

Table 3: Effect of Insect aqueous Extract on humoral and delayed type hypersensitivity response

Groups	Treatments	Hemagglutination antibody titer	Mean of left footpad thickness(mm)
I	Control-Normal saline(NS)	1:2048	0.28 +0.037
II	Model- NS + CYP	1:128[a]	0.38[a] +0.034
III	M-L (500mg/kg + CYP)	1:1024[c]	0.42[a] +0.066
IV	M-M (1000mg/kg + CYP)	1:1024[c]	0.45[d] +0.050
V	M-H (2000mg/kg + CYP)	1:512[d]	0.48[d] +0.058

Values are expressed as Mean and Mean +_ SEM for HA Titer and DTH respectively.[a]$P<0.05$ Significant difference verses NC; [d]$P<0.05$ Significant difference verses MC Groups.

Effects on Pro-inflammatory cytokines: The effect of insect extract on proinflamatory cytokine (IL-6 and TNF-α) levels in model group were significantly decreased compared to control and immunocompressed but extract treated groups. Amongst the extract treated groups, the TNF- α of lower dosed (M-L) group were

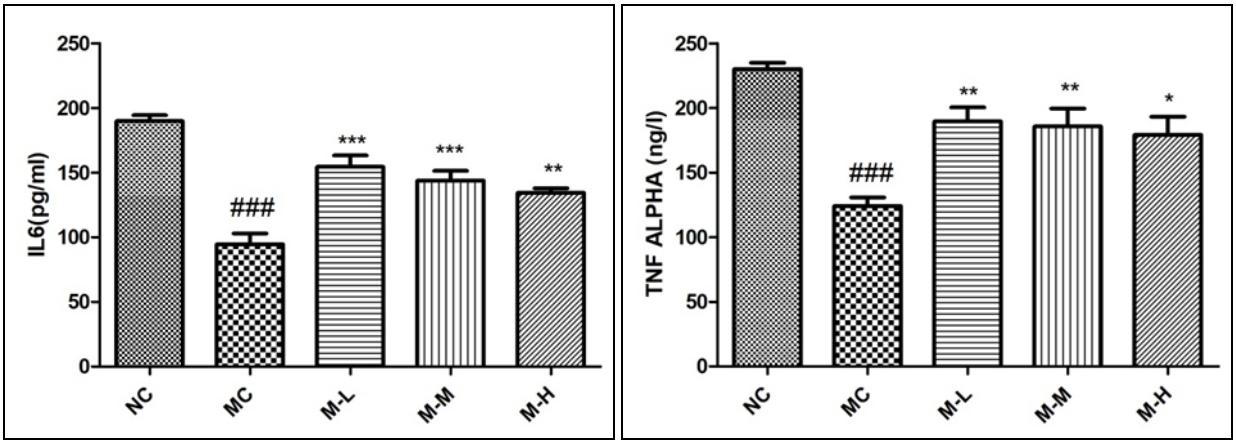

Fig 2: Effect of Aqueous extract of *Mimela* sp. on Proinflamatory cytokine levels, IL-6=Interleukin 6, TNF=Tumor Necrosis Factor alpha. Values are expressed as Mean +_SEM (n=5). Additional post hoc test done using Tukey's multiple comparison test; $P^{\#}$<0.05 vs. NC and P^{*}<0.05 vs MC.

Significantly higher than that of model group followed by medium dosed (M-M) and higher dosed group (M-H). The IL-6 Level for the sample treated groups shows down regulation in a dose dependent manner compared to control group i.e; M-L>M-M>M-H.

Discussion

The upsurge of interest in pharmacognosy considering adverse effects of conventional drugs has led to lot of researches in recent years establishing Insect and Plants as promising therapeutic agents. Insect and its various chemical extracts had shown to have antiviral, immunomodulatory and free radical scavenging activity (Ai *et al*. 2014) [21]. All these properties of insect could be expected due to various bioactive chemical it possess and sequester from their food plants. Besides, the insects are also nutritionally rich as reported by several studies (Chakravorty *et al.*, 2014; Chung 2010; Oyegoke *et al.*, 2006; Anand *et al.*, 2008; Ramos-Elorduy *et al.*, 2002) [32, 23, 24, 25] and nutrition serve as an important factor affecting immune system thereby modulating its action against infection (Scrimshaw 1997) [26]. The *Mimela* sp. taken for the present study are rich in proteins, essential amino acids, fats, vitamins and minerals consumed as a relish by the people of Arunachal Pradesh. The antioxidant studies on this insect has shown it to have good antioxidant potential as evident by significant decrease in MDA content whereas rise in GSH content and Catalase activity compared to cyclophosphamide treated immunosuppressed model group. The reduction in spleen and thymus weight of model group was shown to get improved in extract treated mice groups thus, improving the immune status that was impaired by Cyp and enhancing the immunogenic capacity of the mice. The thymus and spleen are the primary and secondary lymphoid organs where T lymphocytes develop, differentiates, mature and settle eliciting immune responses, immune cell differentiation and proliferation leading to increase in their weights (Kraus MD, 2003) [27]. The decline in their weight signifies reduced immune cell number and response. Any abnormal clinical distortion or lesions in the tissues of spleen and thymus as may be detected by histological observation also causes decline in immune functions. The histomorphological observation in the present studies revealed abnormal morphology of cyp group which was shown being improved in extract treated groups bringing it closer to the normal control group.

Leukocytes, an important component of immune system makes antibodies and kills tumor cells thus keeping immune responses in control and its counting is considered a valid method to evaluate immune functions (Lammermann T, 2014) [28]. The present studies shows decline in its count in cyp treated group which was inhibited in extract treated groups that showed increase in WBC count in mice immunocompromised by cyp.

The hemagglutination assay is used to quantify the relative concentration of antibodies in a serum sample by observing the agglutination or clumping of red blood cells. The highest dilution of the sample where the clumping is seen due to lattice formation is considered as hemagglutination titer (HA titer) and is used to evaluate the humoral immune responses. The present study revealed increase in HA titer in immunosuppressed mice treated with insect extract.

Delayed type hypersensitivity assessed by change in foot volume of experimental animal was used to study cell mediated immune response (CMI). The CMI is critical to defend against pathogens, transplant rejection, tumor immunity etc. In the present study there was enhancement of footpad volume in extract treated mice compared to cypimmunocompromised one.

Cytokines are broad categories of small protein that include chemokines, interleukins, interferons and tumor nacrosis factors. Tumor necrosis factor alpha (TNF-α) is a pro inflammatory cytokine generated by activated macrophages, T lymphocytes and natural killer cells to bacterial infection or other immunogens and are involved in death of the cells (Kouakou K, 2013) [29]. Interleukin 6 (IL-6) act both as pro-inflammatory cytokine and anti-inflammatory myokines secreted mainly by monocytes in response to infections and tissue injuries (Toshio T, 2014) [30]. The level and persistence of TNF-α and IL-6 cytokines play an important role in determining the behavior of a given factor in immunomodulation (Wahab *et al*. 2014). Our study showed significant increase in cytokine production (both TNF-α and IL-6) in immuno compressed mice treated with insect extract.

Conclusion

This study shows that the aqueous extract of *Mimela* sp. increases antioxidant activity, enhances the immune functions both humoral and cell mediated immunity, reduces histopathological changes, increases the number of leukocytes and elevates cytokines levels TNF-α and IL-6 in imunoc ompromised mice. Thus it can be said that the *Mimela* sp. has both antioxidant and immunomodulatory potentials that could set up new avenues in research of insect food as medicines. However, further studies are required on the immunomodulatory mechanism of this insect and the specific compounds involved.

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Ethical approval and consent to participate

The animal experiments were approved by the institutional animal care and ethical committee, Rajiv Gandhi University, Arunachal Pradesh, India.

Competing interests

The authors declare that they have no competing interests.

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