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In vitro antioxidant activity of hemolymph from *Camponotus compressus* and its anti-hyperglycemic activity on alloxan Induced diabetic albino rats

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ABSTRACT

Objective: To evaluated the antioxidant and antihyperglycemic activity of hemolymph from ant (*Camponotus compressus*). **Materials and Methods:** The fourier transform infrared spectroscopy (FTIR) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine the peptide nature of the hemolymph. The antioxidant activity of the hemolymph was tested by DPPH radical scavenging assay and their anti-hyperglycemic activity were evaluated on animals using biochemical and histological techniques.**Result:** FTIR analysis revealed the functional group similarity between hemolymph and insulin, SDS-PAGE showed the presence of peptide which has similar molecular weight of insulin. The hemolymph was effective in inhibiting the DPPH radical, which revealed its proton-donating ability, there by acting possibly as primary antioxidants. Further, the hemolymph significantly lowered the blood glucose level of the pretreated animals compared to alloxan induced diabetic animals, suggested that the hemolymph from ant (*C. compressus*) has antihyperglycemic effect against alloxan induced diabetes in rats, which was further confirmed by histological studies. **Conclusion :** The hemolymph of *C. compressus* has potent antioxidant activity and effective in reducing the blood glucose level in experimental animals. The antioxidant and antihyperglycemic effect of hemolymph of *C. compressus* may be due to the presence of insulin like peptide / bioactive peptide as analyzed by SDS-PAGE, FTIR.

1. Introduction

Bioactive peptides present in the hemolymph of insects or animals are known for their ability to inhibit pathogenic microorganisms and reactive oxygen species (ROS)[1]. In addition, the wide spectrum of activities reported for these molecules suggested their potential benefits in treatments against bacterial, viral infections and cancer[1, 2, 3, 4].

Bioactive peptides discovered so far have been divided into several groups based on their length, secondary and tertiary structure and presence or absence of disulfide bridges[5]. The haemolymph of insects and crustacean been reported to have potent antimicrobial peptide showed diverse array of activity against several human pathogens[4,6]and plays a prominent role in host defense response including self or non self recognition, cell to cell communication, superoxide anion activity, melanisation, phagocytosis, and encapsulation[7]. The hemolymph of insects have reported to have anti-diabetic activity, may be because of presence of insulin like peptides (ILP), as it structurally related to that of insulin of mammals[6,8].

Bioactive peptides in hemolymph have been emerged as the alternative source of medicine for treating many kind of chronic disease including diabetes mellitus[6,8]. The medicinal plants are traditional used in many countries to treat diabetes mellitus, but still no effective medicine have

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been developed so far from the herbal source[9]. Further, Diabetes has recently been identified by Indian Council of Medical Research (ICMR) as one of the refractory diseases for which satisfactory treatment is not available in modern allopathic system of medicine and suitable alternative medicine are to be investigated[10]. Hence the present study was designed to evaluate the antioxidant and anti-hyperglycemic activity of aqueous extract of hemolymph from *Camponotus compressus*. The antibacterial activity of hemolymph of *C. compressus* has already been reported by our group. To our best of knowledge, the present study would be the first report on this line of study.

2. Materials and Methods

2.1. Collection and processing of *C. compressus*

The *C. compressus* were collected from the region of Kelambakam forest, Chennai, Tamilnadu, India. The collected ants were frozen in liquid nitrogen and ground into powder by using mortar and pestle. The finely ground powder were used for hemolymph extraction[6].

2.2. Extraction of Hemolymph

The ant powder was mixed with equal volume (w/v) of ice cold ethanolic Phenyl methyl sulphonyl fluoride (10 μg/ml) and was incubated in an ice-bath for 40 mins to inhibit the protease activity and centrifuged thrice at 10,000rpm (4 ℃ for 15Mins). The supernatant was extracted with an equal volume of ice cold hexane (v/v) to remove metapleural gland secretions and centrifuged at 10,000rpm for 15 min at 4℃. The aqueous phase was filtered with 0.45 μ filter. The filtrate was stored at 4℃ and was used for further analysis[6].

2.3. Protein Estimation

The protein estimation was done by Bradford dye binding assay[11]to estimate the amount of protein present in the hemolymph of *C. compressus*. The amount of protein was calculated and expressed as mg/ml.

2.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

The extract of hemolymph from *C. compressus* was subjected to Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard insulin as positive control. The SDS- PAGE was carried out according to the method of Laemmli[12], using 15% cross-linked polyacrylamide gel. After electrophoresis, gel was stained with silver staining method[13]and the band was visualized.

2.5. Fourier Transform Infrared Spectroscopy

The hemolymph and standard insulin was subjected to FTIR analysis by KBr pellet (FTIR grade) in 1:100 ratio and the spectrum was recorded in the Nicolet Impact 400 FTIR Spectrophotometer using diffuse reflectance mode[6]. All measurements were carried out in the range of 400- 4000 cm $^{-1}$ at a resolution of 4 cm $^{-1}$.

2.6. In-vitro screening of bioactive chemicals

For further confirmation of the protein nature of the extract of hemolymph from *C. compressus*, a qualitative bioactive chemical analysis was performed by the methods described by Harborne[16]and Sazada *et al*.[17].

2.7. In vitro Antioxidant Assay

2.7.1. Estimation of Radical Scavenging property using the DPPH assay

The Radical scavenging activity of hemolymph was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay according to Sharma and Bhat[16]with small modification. The decrease of the absorption at 517nm of the DPPH solution after the addition of the antioxidant was measured in a cuvette containing 2960 μl of 0.1 mM ethanolic DPPH solution mixed with 40 μL of 4 to 40 μL of hemolymph (v/v) and vortexed thoroughly. Blank containing 0.1 mM ethanolic DPPH solution without hemolymph and vortexed thoroughly, the setup was left at dark at room temperature. The absorption was monitored after 20 min. The ability to scavenge DPPH radical was calculated by the following equation.

$$\%\ \text{of DPPH radical scavenging activity (\% RSA)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

$\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical + ethanol; Abssample is the absorbance of DPPH radical + hemolymph. Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions.

2.8. In vivo anti-hyperglycemic activity

2.8.1. Experimental Animals

Male Wistar albino rats weighing 100-120 g were obtained from the animal house of Mohammed Sathak A.J. College of Pharmacy, Sholinganallur, Chennai, Tamilnadu, India. The animals were housed in standard polypropylene spacious rat cages with stainless steel top grill, under hygienic conditions. Animals were provided standard pellet food manufactured by Hindustan Lever Ltd., India and Aquagaurd ST 2000 filtered well water ad libitum. A week prior to the start of experiment, the animals were acclimated to laboratory conditions (Temp. 23±3℃, Humidity 65±5% and 12 h light and 12 h dark rhythm). All studies were conducted in accordance with Institutional guidelines of Animal Care and Welfare of animals. The present experimental protocol was approved by the local ethical committee (Ethical Committee Number is No. AJ/IAEC/2010/7).

2.8.2. Fixation of Optimum Dosage Schedule for the experiment

Animals were divided into 4 groups with 6 animals in each

group. The hemolymph of *C. compressus* was administered at doses of 0.125 mL, 0.25 mL, 0.5 mL and 1 mL/kg body weight per day for 7 days orally. The animal's survival and behavioral changes were noted, which confirms that there was no toxicity effect and the animals are healthy in all the groups. The dosage and duration of the dosage which gives significant protection of animal was fixed up as the optimum dosage for the work schedule. The fixed optimal dosage for the experiment was 0.25 mL / kg body weight.

2.8.3. Induction of Diabetes in animals

Diabetes was induced in animals using alloxan, as per the guide lines reported earlier[10]and the approval for the induction of experimental diabetes was got from the Institutional Animal Ethical Committees.

2.8.4. Experimental Design

After a week of acclimatization, the animals were distributed randomly into four experimental groups with six animals each (the groups are listed below). In order to evaluate the anti-diabetic activity of hemolymph of *C. compressus*, the animals of group 2 and group 4 was treated / co-treated with hemolymph of *C. compressus* (250 μL/kg body- 1/10 of the LD50) orally once daily for one month. The group 1 (Normal control) and group 3 animal received normal saline at a dose of 10 ml/kg body weight, orally once daily for one month.

Group 1 -Control animals were administered their normal food orally for the entire experimental period of 1 month.

Group 2 -Drug control animals were administered orally with 250 μL/kg body weight/day of the *C. compressus* hemolymph orally for the entire experimental period of 1month.

Group 3 -Animals induced for Diabetes using alloxan [single i.p. 150 mg/kg body weight in normal saline], for the entire experimental period of 1 month.

Group 4 -Animals induced for Diabetes (as Group 3 animals), and co- administered with 250 μL/kg body weight/ day of the *C. compressus* hemolymph orally, for the entire experimental period of 1 month.

The animals were sacrificed after one month under light thiophenol anesthesia. Blood was drawn from the eye orbit of the rat[10]and fresh blood was immediately collected into test tubes and serum was obtained from the blood after centrifugation at 5000 rpm for 5Min at 4℃. The level of blood glucose was tested as reported earlier[10, 17]. The excised livers and pancreas were preserved in 10% phosphate-buffer formalin for the histological examination.

2.8.5. Estimation of glucose level in experimental animals

The blood glucose level of experimental animals was estimated as reported by Schmidt[17]. Glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. In a subsequent peroxidase catalyzed reaction, the oxygen liberated is accepted by the chromogen to give a red colored quinoneamine compound. The red color so developed is measured at 505nm and is directly proportional to glucose concentration of the test sample.

2.8.6.. Histopathological examination

Histological studies of livers and pancreas of experimental rats were carried out to confirm the anti-hyperglycemic activity of hemolymph of *C. compressus*. Small portions of liver / pancreas were fixed in 10% phosphate buffer formalin and dehydrated in graded alcohol series followed by xylene treatment and the liver specimens were embedded in paraffin blocks and cut into 5 μm in thickness sections. They were stained with hematoxylin and eosin, examined under light microscope and images were recorded at 400X[18].

2.9. Statistical analysis

The experimental data were analyzed by One-way ANOVA using the AGRES statistical software[19].

3. Results

3.1 Identification of Bio Active Chemicals In The Hemolymph

The preliminary bioactive chemical screening done on the extracted hemolymph confirmed the protein nature as it showed high positive to amino acids, protein and carbohydrate, whereas alkaloids, phenols, glycosides and saponins were completely absent (Table 1).

Table 1

In-vitro screening of bioactive chemicals

Detection of chemical components	Test/ Reagents	Result
Alkaloids	Mayer's	-
Carbohydrates	Fehling's	+++
Glycoside	Borntrager's	-
Saponins	Foam's	-
Protein and Aminoacids	Millon's	+++
	Biuret	+++
Phenolic compound	Ferric Chloride	-

+++ : Present in high concentration; +: Present in trace concentration; - : Constituents not detectable using the specified assay method.

3.2 Estimation of Protein

The protein content of the crude extract of hemolymph was 2.88 mg/ml as estimated by Bradford dye binding method, revealed the protein content of the extract.

3.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the hemolymph and was compared with standard insulin. Electrophoresis in denaturing condition (SDS-PAGE) revealed the presence of 5Kda peptide which has same molecular weight of insulin. SNS PAGE method was used to visualize the band (Fig. 1).

3.4 Fourier Transform Infrared Spectroscopy

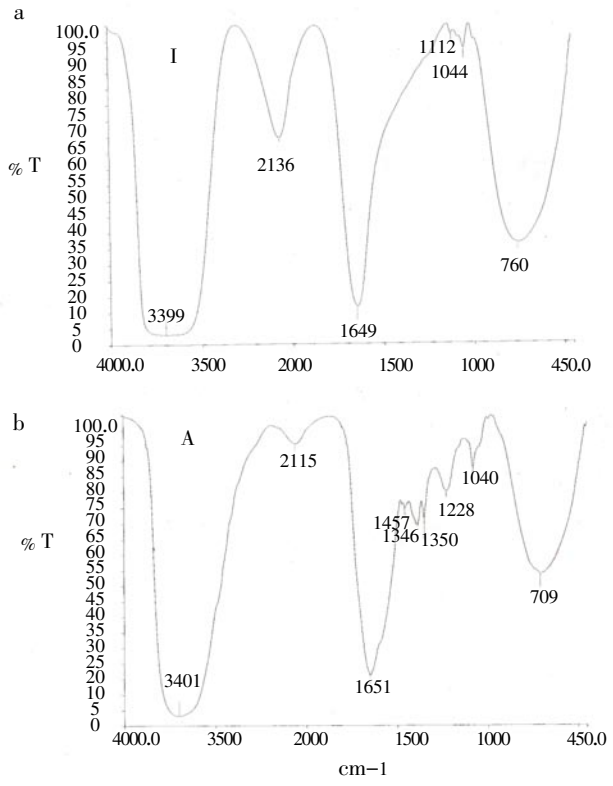
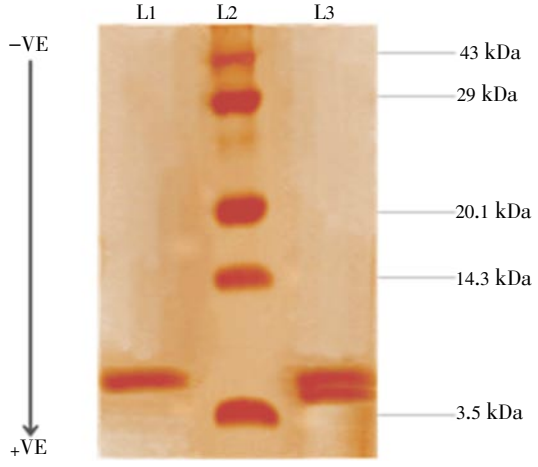


Figure 1(a). FTIR analysis of Insulin . (b). FTIR analysis of Hemolymph



Figrue 2. Electrophoresis of crude extract of hemolymph in denaturing condition (SDS-PHAGE).
L1 – Hemolymph; L2 – Standard Molecular Marker (Low molecular weight range); L3 – Insulin (Positive control)

FTIR spectral analysis of both Insulin and the *C. compressus* hemolymph showed the same peak value at the range between 3430cm^{-1}and 1630 cm^{-1}. The sample's prominent peaks at 3430cm^{-1}, 2900 cm^{-1}, 2360 cm^{-1}, 2340 cm^{-1} and 1630 cm^{-1} showed the presence of similar functional group moieties of insulin (Fig. 2a & 2b).

3.5 DPPH Assay

The *C. compressus* hemolymph was found to be a good radical scavenger with the percentage of inhibition of 52% at 24 μL concentration (Fig. 3). The DPPH radical scavenging ability of the extract of hemolymph of *C. compressus* was dose dependent manner.

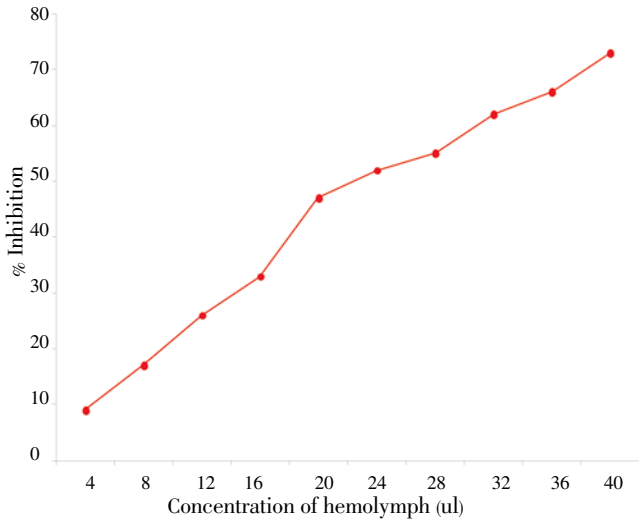


Figure 3. Free Radical scavenging property of crude extract of hemolymph from C. compressus (DPPH assay)

3.6 In vivo studies

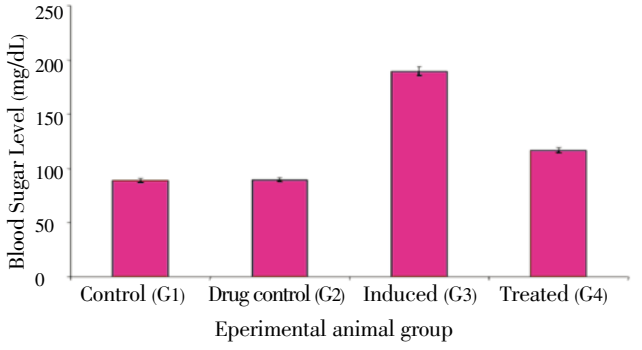


Figure 4. Reduction in blood sugar level by the hemolymph from C. compressus
Values are means of three with SD. Means bearing different alphabets are significantly different at p = 0.05 as determined by analysis of variance (One way Anova).

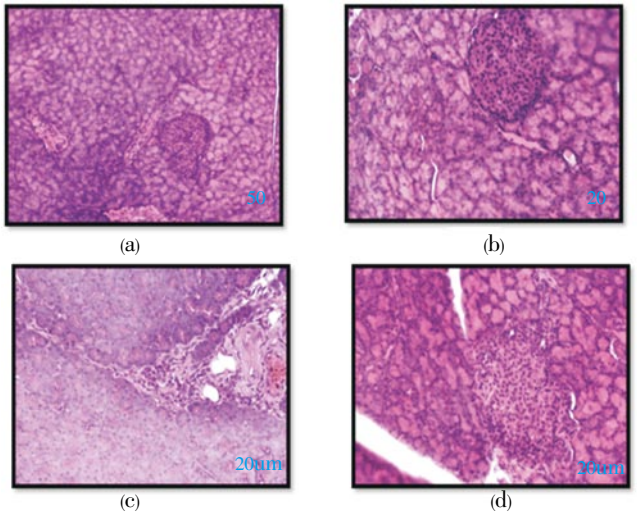


Figure 5. Histological observation of rat pancreas.
(a) Control; (b) Drug control; (c) induced and (d) Treated

There was a significant decrease in the level of blood

glucose of the experimental animals was observed in hemolymph treated diabetic animals (Group- 4) compared to alloxan treated diabetic animals (Group- 3) (Fig. 4). It is evident from the present investigation that crude extract of hemolymph of *C. compressus* is potential in lowering the blood glucose level of diabetic animals.

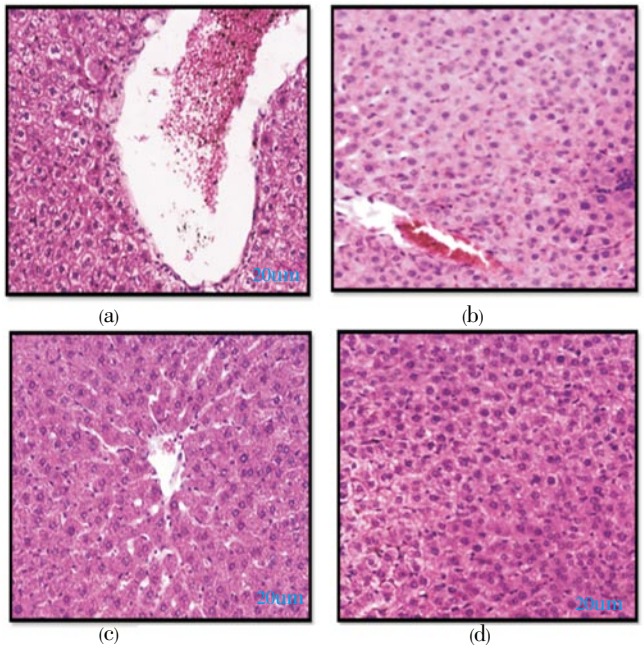


Figure 6. Histological observation of rat liver.
(a) Control; (b) Drug control; (c) Induced and (d) Treated

3.7 Histological Studies

The histological preparation of pancreas revealed that the islets of langerhans of normal control group (Group- 1), the drug control group (Group- 2) and the hemolymph treated group (Group- 4) showed normal architecture of islet cells compare to alloxan alone treated group (Group- 3). Pancreatic islets of induced diabetic group rats (Group- 3) revealed significant architectural disarray which also extended into surrounding exocrine tissue. Histologically, the islets of langerhans of normal control group were unevenly scattered in the pancreatic tissue and they were often quite abundantly distributed and were of varying sizes in the same lobule of pancreas. Well delineated islets were completely enmeshed in the surrounding acinar cells of the exocrine pancreas (Fig. 5a). The drug control group animal showed very minute destruction of islet cells. The islet cells were compactly arranged, with negligible intercellular space and no inflammatory cells were observed (Fig. 5b). Pancreatic islets of induced diabetic group rats revealed significant architectural disarray which also extended into surrounding exocrine tissue. Further, alloxan treatment brought an increase in intercellular space compared to control rats (Fig. 5c). Another significant change following alloxan treatment was the occurrence of peripheral widening between pancreatic acinar (exocrine tissue) and islet cells. Pancreatic islets of treated group rats also showed architectural disarray but to a lesser extent as compared alloxan induced diabetic rats (Fig. 5d).

The histological preparations of liver cells of the control group (Group- 1), the drug control group (Group- 2) and the hemolymph treated group (Group- 4) showed normal with intact nucleus and proper cell morphology with no abnormality detecte. In the diabetic control group (Group- 3), the liver cells showed damage in the cell nucleus with bile duct hyperplasia and kupffer cell hyperplasia The control group were normal with intact nucleus and proper cell morphology with no abnormality detected (Fig.6a). In the drug control animal, mild congestion of cells was observed (Fig.6b). In the diabetic control group, the liver cells showed damage in the cell nucleus with bile duct hyperplasia and kupffer cell hyperplasia (Fig.6c). The treated group rats showed certain morphological changes that the cellular components were returned back to normal (Fig.6d).

4. Discussion

C. compressus account for only 2% of all insect species described to date, but it is estimated that they constitute more than half of the world's insect biomass[6, 20]. At present, the number of species worldwide is approximately 9,500, but the true number may eventually reach 20,000 or more. The present study utilized the ant species for the extraction of hemolymph, as it was reported that they are the dominant life forms, particularly in the tropics[6, 20]. From table 1, the hemolymph composition clearly indicates the presence of protein and carbohydrates whereas the existance of Alkoloids, Glycoside, Saponins and Phenolic compounds are negative. In order to determine if the crude extract of hemolymph from *C. compressus* contain peptide that is structurally related to vertebrate hypoglycemic hormone insulin, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Fourier transform infrared spectroscopy (FTIR) analysis was performed and compared with standard insulin. Similar type of comparison study was reported by Karmer *et al*[8]in honeybee royal jelly, they performed an immunological assay specific for insulin like polypeptide and detected several insulin like peptides, the major immunoreactive component, which has an apparent molecular weight similar to that of bovine insulin. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals and other reactive oxygen species (ROS), which are continuously, produced in vivo, resulted in cell death and tissue damage[21]. Antioxidants are important inhibitor of lipid peroxidation, not only for protection but also as a defence mechanism of living cells against oxidative damage[22]. There are several *In vitro* methods developed in order to define the antioxidant activity of bioactive components, among which, the method based on the scavenging of DPPH are the most popular[23]. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[16, 23]. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability[16]. The present study reveals that the extract of hemolymph did show the

proton-donating ability and could serve as free radical inhibitors or scavengers, there by acting possibly as primary antioxidants. Diabetes millitus is a chronic metabolic disorder characterized by reduced availability or diminished effectiveness of insulin, characterized by hyperglycemia with or without glycosurea[24]. Alloxan induced diabetic animal model are suitable model for the studies of antidiabetic / antihyglycemic activity of molecules [10]. The hemolymph treatment effectively reduced the blood glucose level in experiemental animals compared to untreated diabetic animals which was confirmed by histopathology study. This effect may be due to the presence of insulin like peptides of hemolymph. The histological studies on both liver and pancreases of rats treated with hemolymph confirmed the anti-hyperglycemic activity of hemolymph of *C. compressus*. Further detailed studies on hemolymph from *C. compressus* using different doses and covering longer period of observation studies in animals are needed to define the antidiabetic and antioxidant activity of hemolymph from *C. compressus* and their mechanism of activity. The bioactive hemolymph form *C. compressus* may be developed as effective antidiabetic drug with further pharmacological and clinical studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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