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Original Research Article

Evaluation of Cardioprotective potential of Methanolic Extracts and its Fractions of *Allium humile* Leaves

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Abstract: The present study has been designed to investigate the efficacy of *Allium humile* leaves active extract and its fraction on ischaemia and reperfusion-induced myocardial injury. Petroleum ether, chloroform, acetone and methanolic extracts of *Allium humile* leaves were prepared and screened for their cardio protective potential. Among all the extracts methanolic extract attenuated myocardial injury. The methanolic extract was further purified using column chromatographic technique that results four major fractions viz F1, F2, F3 and F4. These fractions were again screened for myocardial infarct size, LDH and CK-MB release in coronary effluent at dose level of 100mg/kg body weight and compared with standard drug Ramipril (1mg/kg body weight). The fraction F4 of methanolic extract significantly prevented myocardial infarct size, LDH and CK-MB release.

Keywords: Ischaemia, Reperfusion, *Allium humile*, Ramipril, Chromatoghraphy.

Introduction

The term "cardioprotection" specifically describe interventions that preserve or enhance the viability of myocardium during ischaemia and reperfusion and thus limit the extent of acute myocardial infarction. During ischaemia and reperfusion two forms of cell death in the pathology of myocardial infarction are reported i.e. necrosis and apoptosis (programmed cell death, cell suicide)[1,2]. Although reperfusion is prerequisite for tissue salvage, reperfusion of the ischaemic myocardium results in irreversible tissue injury and cell necrosis, leading to decreased cardiac performance[3,4]. *A. humile (A.nivale; A.gowanianum),* family Alliaceae, is a species of onion found in the Himalayas, at altitudes of 3000-4000m. Flowers are white, star-shaped, in a rather lax umbel 2.5-4cm across, borne on a leafy stem. Narrow-elliptic petals, about 1 cm long, spread outwards, and are much longer than the stamens. Leaves are many, flat, 2-5mm broad, blunt, usually shorter at flowering than the stem. The stem itself is 7-25cm tall. Bulbs are clustered, cylindrical, covered with fibrous leaf-bases. Edible plant part used includes flowers, leaves, root and bulb. Leaves and inflorescences are also used as seasoning agents. Other reports describe its anti-bacterial[5], blood purification, anti-inflammatory, antioxidant[6], anti- asthamatic and anti-diabetic activities[7, 8].

Materials and Methods

Drugs and chemicals:

Ramipril is taken as a gift sample from USV Baddi, Himanchal Pradesh, India. All the reagents used in this study were of analytical grade and were always freshly prepared before use.

Plant material:

Leaves of *Allium humile* was collected from Chamoli District Uttranchal, India. The plant material was identified from Botanical Survey of India, Northern Regional Centre, Dehradun, India with the reference number BSI/NRC 9 (Tech.)/2010-03/839/12796.

Animals:

Adult Wister rats of either sex, weighing 250 to 300g were used in the study. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (Reg.no.273/CPCSEA). Animals were obtained from IVRI Bareilly, India and were maintained under standard laboratory conditions in the departmental animal house of SBSPGI, Dehradun, Uttarakhand, India.

Preparation of extracts:

The fresh leaves of *A. humile* were dried in shade and room temperature for 2days followed by drying [40-50^{o}C] for 3-4hrs and powdered to obtained coarse powder. 980g of powder of *A. humile* leaves were extracted with pet ether, chloroform, acetone

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and methanol to get four extracts. The solvent was removed by evaporation under reduced pressure to obtain semisolid masses. The resultant extracts were kept in a desiccator followed by weighing to give percentage yield of each extract.

Isolation and purification of principle constituent from active fraction:

The methanolic extract showing good cardioprotective effect was subjected to column chromatography using silica gel mesh size 200-400µ as stationary phase and eluting with chloroform: methanol as mobile phase in different ratio lead in to the isolation of four fractions, F1, F2, F3 and F4. The cardioprotective activity was evaluated for all four fractions in which fraction F4 of chloroform extract was found significantly effective than other fractions.

Acute toxicity study:

Albino mice of 10 animals per group and weighing 20-25g were administered graded dose (100-2000 mg/kg body weight, orally) of the methanolic extract of *A.humile*. After administration of extract mice were observed for toxic effects after 48hr of treatment. The toxicological effects were observed in terms of mortality expressed as LD_{50}. The number of animals dying during the period was noted. The LD_{50} of the extract was determined by Litchfield and Wilcoxon, 1949 method. No mortality was observed therefore the extract is safe to use even at the doses of 2000mg/kg of body weight orally.

Isolated rat heart preparation[9]:

Rats were heparinised (500 IU/L, i.p.) and sacrificed after 20min by cervical dislocation. The heart was rapidly excised and immediately mounted on Langendorff's apparatus. The temperature was maintained at 37°C by circulating hot water. The preparation was perfused with krebs Henseleit (K-H) buffer (NaCl 118 Mm; KCl 4.7 Mm; $CaCl_2$ 2.5 Mm; $MgSO_4.7H_2O$ 1.2 mM; KH_2PO_4 1.2mM; $C_6H_{12}O_6$ 11 mM), pH 7.4 and bubbled with 95% O_2 and 5% CO_2. The coronary flow rate was maintained 6-9 ml/min and perfusion pressure was kept constant at 70 mmHg. Global ischemia was produced for 30min by completely closing the inflow of physiological solution and followed by 120min of reperfusion. The coronary effluent was collected before ischaemia, immediately, 5min, 30min and 120min after reperfusion for estimation of LDH and CK-MB.

Assessment of myocardial injury:

The myocardial infarct size was measured using the triphenyltetrazolium chloride (TTC) staining method. The level of LDH and CK-MB (Siemens Medical Solution Diagnostic Ltd., Baroda, India) in coronary effluents was estimated using commercially available kits. Values of LDH and CK-MB were expressed in international units per litre (IU/L).

Assessment of myocardial infarct size[10]:

Heart was removed from the Langendorff's apparatus. Both the auricals and the root of aorta were excised, and ventricles were kept overnight at temperature of -4°C. Frozen ventricles were sliced into uniform sections of 1-2mm thickness. The slices were incubated in 1% w/v TTC solution in 0.2M Tris-chloride buffer, pH 7.8 for 20min at 37°C. The normal myocardium was stained brick red while the infracted portion remained unstained. Infarct size was measured by macroscopic volume method.

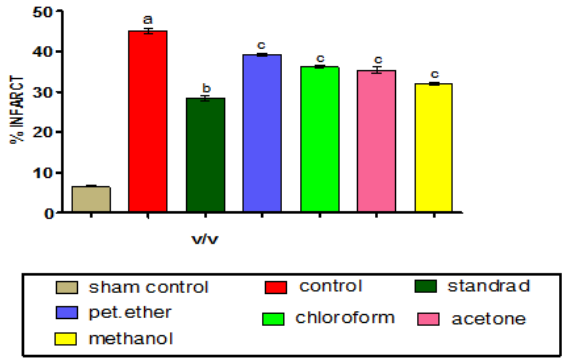
Experimental protocol:

In all groups, isolated rat heart was perfused with K-H solution and allowed to stabilize for 10 min. After stabilization isolated rat heart was perfused continuously with K-H buffer for 160 min. without subjecting it to global ischaemia in sham control group. In vehicle control group rats were administered 1% Tween 80 orally for 7 days and various extracts (100mg/kg) and standard (1 mg/kg) were dissolved in 1% Tween 80 and administered orally once daily to rats for 7days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min. of global ischaemia followed by reperfusion for 120 min. Further, various fractions of methanol extract (100mg/kg) were dissolved in 1% Tween 80 and administered orally once daily to rats for 7days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min of global ischaemia followed by reperfusion for 120min.

Results

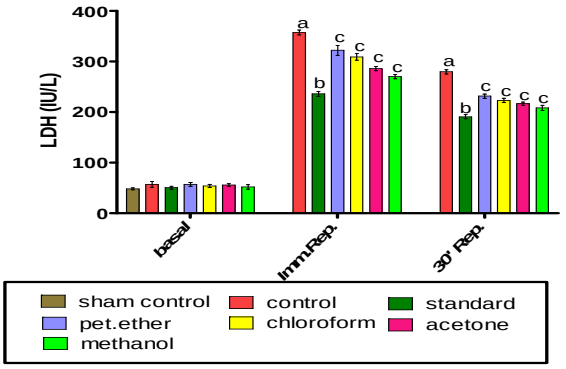
The study was carried out at three dose levels (50mg/kg, 100mg/kg, and 150mg/kg) and the results are depicted for the optimal dose (100mg/kg).

Figure.1: Effect of Ischaemia and Reperfusion on Myocardial Infarct Size. Infarct size was measured by volume method.



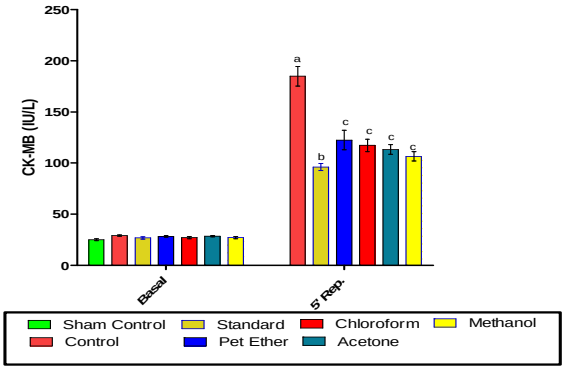
Values are expressed as mean±SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

Figure.2: Effect of Ischaemia and Reperfusion on LDH release. LDH was estimated in coronary effluent after stabilization (Basal), Immediately (Imm'Rep.) and 30min. after reperfusion (30' Rep.).



Values are expressed as mean±SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

Figure.3: Effect of Ischaemia and Reperfusion on CK-MB release. CK-MB was estimated in coronary effluent after stabilization (Basal) and 5min. after reperfusion (5' Rep.).



Values are expressed as mean±SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

Statistical Analysis:

All values for enzymatic data (LDH and CK-MB) and infarct size were expressed as mean ±SEM. Statistical analysis was performed using Graph Pad Prism Software. The values were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Value of P<0.05 was considered to be statistically significant.

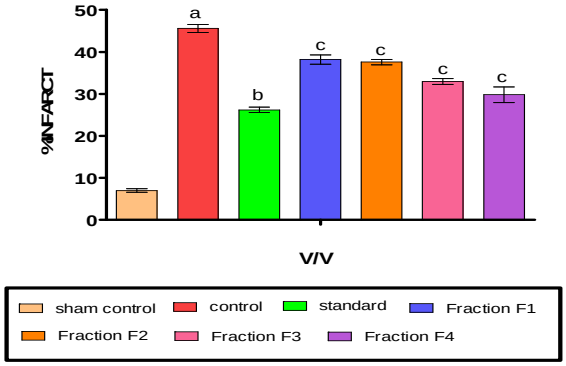
Effect on Myocardial Infarct Size:

Various extracts of *A. humile* leaves viz. petroleum ether, chloroform, acetone and methanol were evaluated on ischaemia and reperfusion induced increase in myocardial infarct size, respectively. Among all the extracts methanol extract of *A. humile* leaves found to be active (Figure 1). Further purification of active extract was carried out using column chromatography which resulted isolation of four fraction viz. F1, F2, F3 and F4. Which were again evaluated for above said effect and among all the fractions fraction F4 significantly attenuated ischaemia and reperfusion induced increase in myocardial infarct size. However, treatment with standard (ramipril,1mg/kg) was significantly more effective to reduce myocardial infarct size as compared to fraction 4, measured by macroscopic volume method (Figure 4).

Effect on Ischaemia and Reperfusion Induced release of LDH

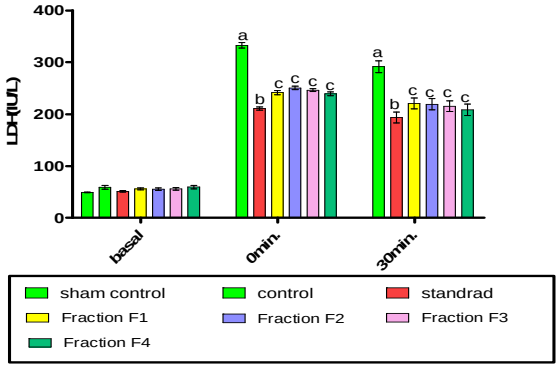
Various extracts of *A. humile* leaves viz. Petroleum ether, chloroform, acetone and methanol were evaluated on ischaemia and reperfusion induced increase in release of LDH in coronary effluent measured immediately and 30 min. after reperfusion, respectively. Similarly, among all the extracts chloroform extract of *A. humile* leaves significantly reduced release of LDH in coronary effluent (Figure 2). Further the fraction F4 of chloroform extract among all other fractions significantly attenuated release of LDH in coronary effluent measured immediately and 30 min. after reperfusion. Moreover, treatment with standard (ramipril,1mg/kg) markedly reduced release of LDH in coronary effluent as compared to isolated fraction F4, measured immediately and 30 min. after reperfusion (Figure 5).

Figure.4: Effect of Ischaemia and Reperfusion on Myocardial Infarct Size. Infarct size was measured by volume method.



Values are expressed as mean±SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

Figure 5: Effect of Ischaemia and Reperfusion on LDH release. LDH was estimated in coronary effluent after stabilization (Basal), immediately (Imm'Rep.) and 30min. after reperfusion (30' Rep.).

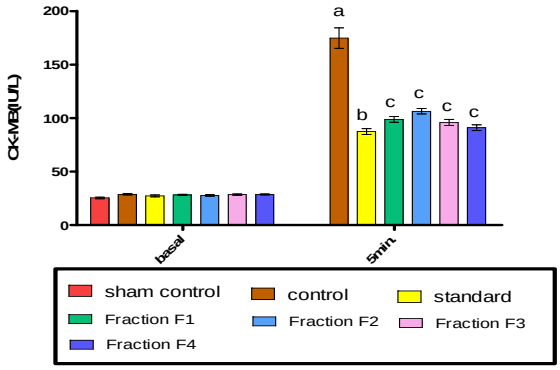


Values are expressed as mean±SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple compariso test.

Effect on Ischaemia and Reperfusion Induced release of CK-MB

Various extracts of *A. humile* leaves viz. Petroleum ether, chloroform, acetone and methanol were evaluated on ischaemia and reperfusion induced increase in release of CK-MB measured in coronary effluent collected after 5min. of reperfusion, respectively. Similarly, among all the extracts chloroform extract of *A. humile* leaves significantly reduced release of CK-MB in coronary effluent (Figure 3). Further the isolated fraction F4 of chloroform extract among all other fractions significantly attenuated ischaemia and reperfusion induced increase in release of CK-MB in coronary effluent collected after 5 min. of reperfusion. Moreover, treatment with standard (ramipril, 1mg/kg) markedly reduced release of CK-MB in coronary effluent as compared to the isolated fraction F4, collected 5 min. of reperfusion (Figure 6).

Figure.6: Effect of Ischaemia and Reperfusion on CK-MB release. CK-MB was estimated in coronary effluent after stabilization (Basal) and 5min. after reperfusion (5' Rep.).



Values are expressed as mean±SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

Discussion

Inspite of the disadvantage of high mortality, high heart rate and high rate of drug metabolism, albino rats are used in the present study because they are small in size having low cost and readily available. Moreover histological sectioning and quantification is easy in rat hearts due to small size. Isolated perfused rat heart preparation has been employed in the present study because it permits the use of pharmacological interventions without any interference due to change in systemic circulation. Various extracts of *A.humile* leaves viz. petroleum ether, acetone, chloroform and methanol at a dose level of 100mg/kg were evaluated for ischaemia and reperfusion induced myocardial injury. Further purification of active extract was carried out using column chromatography which resulted isolation of four fraction viz. F1, F2, F3 and F4. These fractions were again evaluated for above said effect and among all the fractions F4 significantly attenuated ischaemia and reperfusion induced increase in myocardial infarct size. Fraction F4 significantly decreased the infarct size, release of LDH and CK-MB in coronary effluent during perfusion period compared to control group. The present findings suggests that methanolic extracts of *A. humile* leaves and its fraction F4 significantly attenuated ischaemia and reperfusion induced increase in myocardial infarct size. Further, investigation on this could lead to identification of novel

Cardioprotective agent(s) from *A. humile*. Moreover, some extensive work in this direction could also lead to explore the exact mechanism of action of these drugs.

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