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Effect of Cardioactive Principle of Methanolic Extract of *Allium Humile* Leaves on Global Ischaemic Rat Heart

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ABSTRACT

Objective: In present study efficacy of active principle (AH-1) of Methanolic extract of *Allium humile* leaves has been evaluated to limit myocardial injury in ischaemic rat heart.

Material and Methods: Various extracts of *A. humile* leaves were prepared using solvents of increasing polarity. Methanolic extract among all other extracts further purified using chromatographic technique results four major fractions A1, A2, A3, and A4. These fractions screened for

Cardioprotective potentials. Among these fractions A4 was found to be active fraction. AH-1 (active principle) was isolated from the fraction A4 using column chromatography technique and evaluated for Cardioprotective effect.

Results: The Methanolic extract of *A. humile* leaves and further fraction A4 of Methanolic extract significantly prevented myocardial infarct size as compared with that of standard Ramipril in the study. From fraction A4 of Methanolic extract, the isolated active principle AH-1 showing cardio protective effect at dose level of 50 mg/kg body weight.

Conclusion: The study suggests that AH-1 is active Cardioprotective principle of Methanolic extract of *Allium humile* leaves and was found significant ($p < 0.05$).

INTRODUCTION

As per Ayurveda, Indian medicinal plants are rich sources of substances that have several therapeutic properties including cardio protection. About 75-85 % of the world's population, plant derived products is still play an essential role in primary health care, mainly in the developing countries. This is primarily because of the general belief that use of herbal preparation are without any adverse effect, cheap, easily and locally available [1,2]. The use of herbal preparation is increasing in the treatment of cardiovascular disease because of various possible mechanism involved in the cardio protection. Therefore, herbal extracts that are traditionally used, evaluated against in limiting the deleterious effects of ischemia and reperfusion- induced myocardial injury. Furthermore, the results are statistically analyzed and validated for prophylactic approaches and as an adjunct to standard treatment of ischemia and reperfusion-induced myocardial injury [3,4].

Allium genus has attracted particular attention of modern medicine because of its widespread health use around the world, and the cherished belief that it helps in maintaining good health warding off illnesses and providing more vigor. To date, many favorable experimental and clinical effects of allium species have been reported [5-7]. *Allium humile* (*Alliaceae*), found at high altitudes, 3000-4000 m of the Himalayas [8]. Edible plant part includes flowers, leaves, root and bulb. Leaves and inflorescences are used as seasoning agents and other reports describing antimicrobial [9], blood purification, anti- inflammatory, antioxidant [10], anti- asthmatic and anti-diabetic activities [11,12]. *A. humile* accounted for 70-75% of the total land area in medicinal plant cultivation. Among the cultivated species, *Allium humile* yielded the highest economic returns than other species but less explored for medicinal uses [13]. Therefore, in the present study *Allium humile* has been taken to isolate the active constituents that may be probably responsible for Cardioprotective effect.

MATERIALS AND METHODS

Drugs and chemicals

Ramipril is long acting angiotensin converting enzyme inhibitor. It is extensively distributed in tissues and may thus inhibit renin angiotensin system to a greater extent and possessed cardioprotective qualities. The plasma $t_{1/2}$ of its active metabolite is 8-18 hrs, duration of action is >24 hrs and bioavailability is 60% as compared to other drugs of the same category.

Ramipril is taken as a gift sample from USV Baddi, Himanchal Pradesh, and Himanchal, 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, Uttaranchal, India. The plant material was identified from Botanical Survey of India, Northern Regional Centre, and Dehradun India with the reference number BSI/NRC 9 (Tech.)/2010-03/839/12796.

Preparation of extracts

The fresh leaves of *A. humile* were dried in shade at room temperature for 2 days followed by drying [40-50°C] for 3-4 hrs and powdered to obtained coarse powder. 980 g of powder of *A. humile* leaves were extracted with petroleum ether, chloroform, acetone and methanol successively to collect four extracts of different polarity compounds. The solvents were removed by evaporation under reduced pressure to obtain a semisolid mass. The result extracts were kept in a separate desiccators followed by weighing to calculate the percentage yield of each extract in reference to air dried leaves of *A. humile*.

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 200-400 μ and chloroform: methanol as mobile phase in different ratio lead in to the isolation of four fractions, A1, A2, A3 and A4. The cardioprotective activity was evaluated for all four fractions in which fraction A4 of methanol extract was found significantly effective. Further purification of fraction A4 as above said process resulted in isolation of AH-1, AH-2 and AH-3. All these compounds further evaluated for cardioprotective activity. Among all these the compound AH1 was found significantly effective than other compounds^[14].

Acute toxicity study

Albino mice of 10 animals per group and weighing 20-25 g were administered graded dose (100-2000 mg/kg body weight, orally) of the methanol extract of *A. humile*. After administration of extract mice were observed for toxic effects after 48 hr of treatment. The toxicological effects were observed in terms of mortality expressed as LD₅₀. The number of animals dying during the period was noted. The LD₅₀ of the extract was determined by Litchfield and Wilcoxon^[15]. No mortality was observed therefore the extract is safe to use even at the doses of 2000 mg/kg of body weight orally.

Isolated rat heart preparation

Rats were heparinised (500 IU/L, i.p.) and sacrificed after 20 min by cervical dislocation. The heart was rapidly excised and immediately mounted on Langendorff's apparatus [16]. 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; CaCl₂ 2.5 mM; MgSO₄·7H₂O 1.2 mM; KH₂PO₄ 1.2mM; C₆H₁₂O₆ 11 mM), pH 7.4 and bubbled with 95% O₂ and 5% CO₂. The coronary flow rate was maintained 6-9 ml/min and perfusion pressure was kept constant at 70 mmHg. Global ischemia was produced for 30 min by completely closing the inflow of physiological solution and followed by 120 min of reperfusion. The coronary effluent was collected before ischaemia, immediately, 5 min, 30 min and 120 min after reperfusion for estimation of LDH and CK-MB [17].

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

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 0.2 M Tris-chloride buffer, pH 7.8 for 20 min at 37 °C. Dehydrogenase enzyme and cofactor NADH present in the viable myocardium react with tetrazolium salts to form a formazone pigment which is intensely coloured. The enzyme and the cofactor are lost from the infarcted cardiac cells. Therefore, infarcted portion remains unstained while the viable myocardium was stained brick red with TTC. Infarct size was measured by macroscopic volume method [18,19].

Experimental Protocol

In all groups, isolated rat heart was perfused with K-H solution and allowed to stabilize for 10min.

Group 1: (Sham control; n=5) after stabilization isolated rat heart was perfused continuously with K-H buffer for 160min. without subjecting it to global ischaemia.

Group 2: (Vehicle control; n=5) Rats were administered 1% Tween 80 orally for 7 days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min of global ischaemia followed by reperfusion for 120 min.

Group 3: (Standard; n=5) Ramipril (1 mg/kg) was dissolved in 1% Tween 80 and administered orally once daily to rats for 7 days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min. of global ischaemia followed by reperfusion for 120 min.

Group 4: (Methanol extract; n=5) Methanol extract (100 mg/kg) was dissolved in 1% Tween 80 and administered orally once daily to rats for 7 days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min of global ischaemia followed by reperfusion for 120 min.

Group 5: (Fraction 4 of Methanol extract; n=5) Fraction A4 of methanol extract (100mg/kg) was dissolved in 1% Tween 80 and administered orally once daily to rats for 7 days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min of global ischaemia followed by reperfusion for 120 min.

Group 6: (Active principle AH-1 of methanol extract; n=5) Active principle AH-1 of methanol extract (50 mg/kg) was dissolved in 1% Tween 80 and administered orally once daily to rats for 7 days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30 min of global ischaemia followed by reperfusion for 120 min.

Statistical analysis

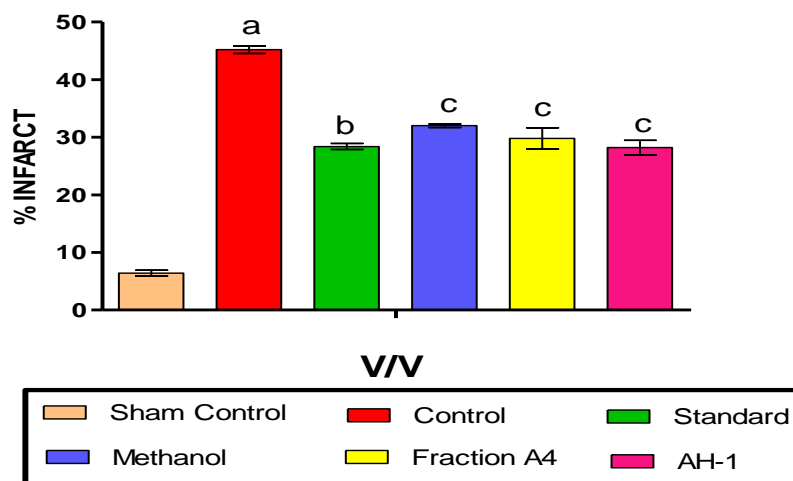
All values for enzymatic data (LDH and CK-MB) and infarct size were expressed as mean \pm SEM. Statistical analysis was performed using Graph Pad Prism Software. The values were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Value of P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

RESULTS AND DISCUSSION

Effect of AH-1 Cardioactive Principle from Methanolic Extract of *A. humile* Leaves 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. Further purification of active extract was carried out using column chromatography which resulted isolation of four fraction viz. A1, A2, A3 and A4. Which were again evaluated for above said effect and among all the fractions fraction A4 significantly attenuated ischaemia and reperfusion induced increase in myocardial infarct size? Further purification of fraction A4 resulted in isolation of AH-1, AH-2 and AH-3. AH-1 further evaluated for Cardioprotective activity and it was more significant than other compounds measured by macroscopic volume method (**Figure 1**).

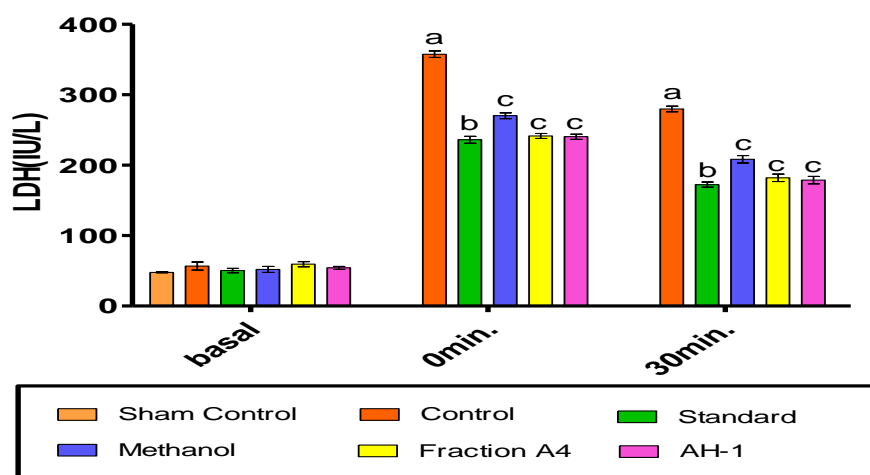
Figure 1: Effect of AH-1 Cardioactive Principle from Methanolic Extract of *A. humile* Leaves on Myocardial Infarct Size. Infarct size was measured by volume method. Values are expressed as mean \pm SEM. a= P <0.05 vs. Sham control; b= P <0.05 vs. Control; c= P <0.05 vs. Standard. ANOVA followed by Turkey's multiple comparison tests.



Effect of AH-1 Cardioactive Principle from Methanolic Extract of *A. humile* Leaves on 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 methanol extract of *A. humile* leaves and the isolated fraction A4 from methanol extract significantly reduced release of LDH in coronary effluent. Further the active principle AH-1 isolated from fraction A4 of methanol extract significantly attenuated release of LDH in coronary effluent measured immediately and 30 min. after reperfusion. Moreover, treatment with standard (ramipril, 1 mg/kg) markedly reduced release of LDH in coronary effluent as compared to active compound AH-1, measured immediately and 30 min. after reperfusion (Figure 2).

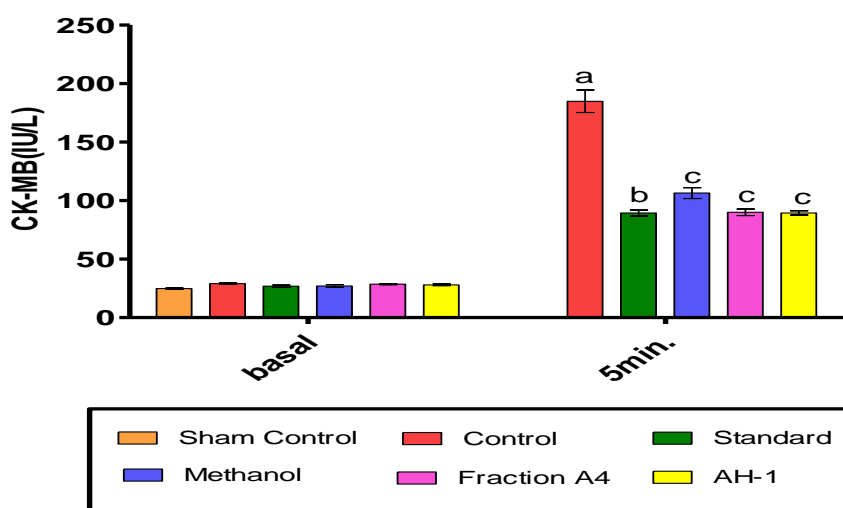
Figure 2: Effect of AH-1 Cardioactive Principle from Methanolic Extract of *A. humile* Leaves on LDH release. LDH was estimated in coronary effluent after stabilization (Basal), immediately (Imm'Rep.) and 30 min. after reperfusion (30' Rep.). Values are expressed as mean \pm SEM. a= P <0.05 vs. Sham control; b= P <0.05 vs. Control; c= P <0.05 vs. Standard. ANOVA followed by Turkey's multiple comparison tests.



Effect of AH-1 Cardioactive Principle from Methanolic Extract of *A. humile* Leaves on CK-MB release

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 5 min. of reperfusion, respectively. Similarly, among all the extracts methanol extract of *A. humile* leaves and the isolated fraction A4 from methanol extract significantly reduced release of CK-MB in coronary effluent. Further the active principle AH-1 isolated from fraction A4 of methanol extract 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, 1 mg/kg) markedly reduced release of CK-MB in coronary effluent as compared to the active compound AH-1, collected 5 min. of reperfusion (Figure 3).

Figure 3: Effect of AH-1 Cardioactive Principle from Methanolic Extract of *A. humile* Leaves on CK-MB release. CK-MB was estimated in coronary effluent after stabilization (Basal) and 5 min. after reperfusion (5' Rep.). Values are expressed as mean \pm SEM. a= P <0.05 vs. Sham control; b= P <0.05 vs. Control; c= P <0.05 vs. Standard. ANOVA followed by Turkey's multiple comparison tests.



CONCLUSION

Acute myocardial infarction (AMI) with subsequent left ventricular dysfunction and heart failure continues to be a major cause of morbidity and mortality in the Western world. Rapid advances in the treatment of AMI, mainly through timely reperfusion, have substantially improved outcomes in patients presenting with acute coronary syndrome and particularly ST-segment elevation myocardial infarction. A vast amount of research has been published on various pharmacological and interventional techniques to prevent myocardial cell death during the time of ischemia and subsequent reperfusion [20]. Traditional drugs with additional pre-clinical and clinical research are needed to further investigate newer Cardioprotective strategies to continue the current trend of improving outcomes following AMI.

Epidemiological study shows an inverse correlation between *Allium* Genus consumption and reduced risk of cardiovascular disease progression. Consumption of *A. humile* suggested significant Cardioprotective effect which includes animal studies. But certain issues regarding the proper use of traditional herbs, i.e. use of different preparations available, dose, duration and interaction with generic drugs should be optimized. In the present study 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 fractions viz. A1, A2, A3 and A4. These fractions were again evaluated at dose level of 100 mg/kg for above said effect and among all the fractions A4 significantly attenuated ischaemia and reperfusion induced increase in myocardial infarct size and release of LDH and CK-MB in coronary effluent. Further purification of fraction A4 resulted in isolation of three compounds viz. AH-1, AH-2 and AH-3. All the compounds were screened for above said activity at dose level 50 mg/kg. Compound AH-1 significantly decreased the infarct size, release of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) in coronary effluent during reperfusion compared to control group. The present findings suggests that compound AH-1 from fraction A4 of methanol extract of *A. humile* leaves significantly effective to ameliorate myocardial ischemic injury as compared to ischaemia and reperfusion induced control group. Moreover, some extensive work in this direction could also lead to characterize the nature of the isolated compounds and to explore the possible mechanism against ischaemia and reperfusion induced myocardial injury.

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