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Cardioprotective effect of various extract of *Rhododendron arborium Sm* flower on Albino rats

Versha Parcha, Neelam Yadav, Amita Sati, Yogita Dobhal and Navneet Sethi

Abstract
In the current study, *Rhododendron arborium Sm* flowers were explored for its effect on ischemic heart. Total ethanolic and aqueous extract were prepared and subjected to qualitative and phytochemical studies and were screened for cardioprotective potential i.e., infarction size, release of LDH and CK-MB. Increase in infarction size, decrease in release of LDH and CK-MB are parameters for good cardioprotective action. Total ethanolic extract is found to be rich in carbohydrates, glycoside, flavanoids and saponins while total aqueous extract is composed of carbohydrates, glycosides, amino acids, tannins & saponins. Total ethanolic extract significantly attenuated ischemia reperfusion induced increase in myocardial infarct size, decrease in release of LDH and CK-MB coronary effluent as compared to aqueous extract at the dose level of 70mg/mL IP. Standard drug used for the study was Enalapril 2.5mg/mL. Thus, from above results it was observed that total ethanolic extract of flower of *Rhododendron arborium Sm* has good cardioprotective effect as compared to aqueous extract which is sold in the market as squash. Further ethanolic extract was fractionated with petroleum ether, dichloromethane, n-butanol in search of active principle and screened. For above said parameters, among all the extracts n-butanol showed the maximum cardioprotective potential and can thus result in isolation of active principle from the same.

Keywords: *Rhododendron arborium*, Cardioprotective, Ischemia, Reperfusion, Enalapril

Introduction
Among all the major health problems found in the population of developed and developing countries, cardiovascular diseases (CVDs) remains the principle cause of death accounting for roughly 20% [1-3]. CVDs includes ischemic heart disease (ie., angina & Myocardial infarction (MI)) which is actually an interruption of blood supply to the heart which causes heart cells to die due to occlusion of a coronary artery usually due to thrombus formation over an atherosclerotic plaque that has got dissected, ulcerated or hemorrhage[4-6]. The major cause of CVDs or the damage of cellular biomolecules is the accumulation of reactive free radicals in the body [7-10].

Reactive free radicals are generated in our body due to endogenous (metabolic pathways) and exogenous (exposure to radiations, pollutions, etc.,) sources. Exogenous sources elevate the free radical formation in the body leading to their accumulation [4-6]. To prevent their overloading, our body exerts many enzymatic and non-enzymatic defence systems. Under any pathophysiological conditions, if the delicate equilibrium between free radical and antioxidant capability gets altered, it may lead to oxidative stress and increased tissue injury which is the major cause of pathogenesis of cardiovascular disease [11-12]. Thus, antioxidants play a key role to scavenge free radicals and are associated with reduced risk of cancer and cardiovascular diseases [13].

Considering the aforesaid, recently there has been an increasing interest in the protective function of dietary antioxidants for extending life span. A broad variety of remedial plant components are thus being used having diverse medicinal properties with low side effects [14]. *Rhododendron arboreum Sm.* being investigated for the presence of bioactive constituents in different parts of the plant [15-17]. Three biologically active components ie, quercetin ($C_{15}H_{10}O_7$), rutin ($C_{27}H_{30}O_{16}$) and coumaric acid ($C_9H_8O_3$) have been reported in flowers of *R. arboreum* using high-performance thin layer chromatography (HPTLC) [18]. Exploration of more bioactive constituents gave a futuristic pathway to use the Rhododendron family for the medicinal purpose [19]. Investigations were done on *Rhododendron dauricum* L. flavonoids which exerted vasodilation and myocardial preservations [20]. However, very little scientific information is available regarding the cardioprotective effect of *R. arboreum* [21]. So, in present study attempts were made to explore cadioprotective potential of *R. arboreum* flowers extracts (ethanolic and aqueous extracts sold in the market in the form of squash).

Materials and Methods

Drugs and chemicals

Enalapril maleate was used as standard drug for study and was given IP at the dose level of 2.5 mg/mL. All the reagents used in this study were of analytical grade and were always freshly prepared before use.

Plant material

The Rhododendron flowers were procured from hilly region of Dhaulaghiri near Dhanaulti, Uttrakhand and authenticated by Botanical Survey of India, Uttrakhand, Dehradun, India with the reference number BSI/NRC/ Tech. (Indent.) /2011-12/ 415/ Acc. No. 113625. Petals were separated from the flowers of *Rhododendron arboreum Sm.* and were dried under shade for about one week and then on tray drier at 40˚C for 6 hours. The dried material was crushed and then subjected to extraction.

Preparation of various extracts

a. Preparation of total aqueous extract

The dried crushed flower petals of *Rhododendron arboreum Sm.* (20 gm) was taken in a flask. To it added 400 ml of distilled water and the mixture was boiled on water bath for 2.5 hrs. The mixture was filtered using muslin cloth and the marc was pressed to obtain the pulp. The pulp was concentrated to obtain 9.2 gm dried material. The dried material was used to perform the phytochemical screening and to evaluate the cardio protective activity.

b. Preparation of total ethanolic extract

The cold extraction was performed using ethanol. The 300 g of crushed powder of *Rhododendron arboreum Sm.* flower petals were placed with 2 liters of ethanol in a conical flask. The obtained homogeneous blend was stored at room temperature for two days, filtered and the obtained residue was restored in ethanol under similar conditions for two more days. Obtained filtrate was evaporated using distillation assembly to yield 56.25 gm dry material.

c. Fractionation of total ethanolic extract

The dried material (8 gm) was taken in a flask and added 150ml of distilled water and petroleum ether (150 ml). The mixture was agitated and separated by the pet-ether layer and water residue using separating funnel. To the water residue layer, added 150ml of dichloromethane (DCM) and agitated in separating funnel to separate DCM layer and water residue layer. The water residue layer obtained is mixed with n-butanol (150 ml) and shaking was done to obtain the water residue and n-butanol layer. On drying the pet-ether and DCM layer the yield was negotiable whereas the n-butanol layer was dried to give 24% of yield. The dried material obtained after evaporating the solvents was used to perform the phytochemical screening and to evaluate the cardio protective activity.

Pharmacological studies (Cardioprotective activity)

The present study "Cardio protective effect of various *Rhododendron arboreum sm.* flower extracts" (total aqueous extract, ethanolic extract & its fraction (n-butanol)) was carried out on albino rats of waster strain of either sex. Animals were kept in animal house. They were fed with water and *add libitum*. Animal were divided in to different groups having 5 animals each. The experimental protocol used in the present study was approved by Institutional Animal Ethics Committee.

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. The temperature was maintained at 37ºC 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 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 Lactate Dehydrogenase (LDH) and Creatine Kinase (CK-MB) [22].

Assessment of myocardial injury

The myocardial infarct size was measured using the triphenyl tetrazolium 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 apparatus. Both the auricles and the root of aorta were excised, and ventricles were kept overnight at temperature of -4 ºC. Frozen ventricles were sliced into uniform sections of 2-3 mm thickness. The slices were incubated in 1% w/v tetraphenyl tetrazolium chloride (TTC) solution in 0.2 M Tris-chloride buffer, pH 7.8 for 30 min at 37ºC. Dehydrogenase enzyme and cofactor NADH present in the viable myocardium react with tetrazolium salts to form a form zone 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 [23, 24].

Experimental protocol

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

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 vehicle 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)

Enalapril (2.5 mg/kg) was 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 ischemia followed by reperfusion for 120 min.

Group 4: (Ethanolic extract 70mg/ml; n=5)

Ethanol extract (70mg/ml) was 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 ischemia followed by reperfusion for 120 min.

Group 5: (Aqueous extract 70mg/ml; n=5)
Aqueous extract (70mg/ml) was 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 ischemia followed by reperfusion for 120 min.

Group 6: (n-butanol fraction 50mg/ml; n=5)
n-butanol fraction (50mg/ml) was 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 ischemia followed by reperfusion for 120 min.

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.

Result & Discussion

Qualitative Chemical analysis of various Extracts of *Rhododendron Arboreum Sm.* flowers

Table 1: Qualitative Chemical analysis of various extracts of *Rhododendron Arboreum Sm.* flowers.

Test performed	Aqueous extract	Total ethanolic extract	n-butanol fraction
Test for Flavanoids			
Shinoda's test	-	-	+
Lead Acetate Test	-	+	+
Sodium Hydroxide test	-	+	+
Test for Carbohydrates			
Fehling test	+	+	+
Molish test	+	+	+
Benedict test	+	+	+
Test for Glycosides			
Leagal's test	+	+	+
Liberman- Burchard's test	+	+	+
Keller-Kilani's test	+	+	+
Test for saponin			
Form test	+	+	-
Test for tannin			
$FeCl_3$ test	+	-	-
Test for Amino acids			
Ninhydrin test	+	-	-
Biuret test	+	-	-
Millions test	+	-	-

Effect of *R. arboreum* flower extract on myocardial infarct size
Total aqueous extract, ethanolic extract and its n-butanol fraction were evaluated for ischemia reperfusion induced myocardial infarct size. Total ethanolic extract of flower of *Rhododendron arboreum Sm.* significantly attenuated ischemic reperfusion induced increase in myocardial infarct size, as compared to aqueous extract. However treatment with standard Enalapril (2.5 mg/mL) was significantly less effective as measured by volume method. Further its n-butanol fraction was studied for the assessment of myocardial infarct size. Studies revealed that n-butanol fraction of ethanolic extract significantly attenuated ischemia reperfusion (I/R), induced increase in myocardial infract size as compared with standard enalapril (2.5 mg/kg) (Fig. 1).

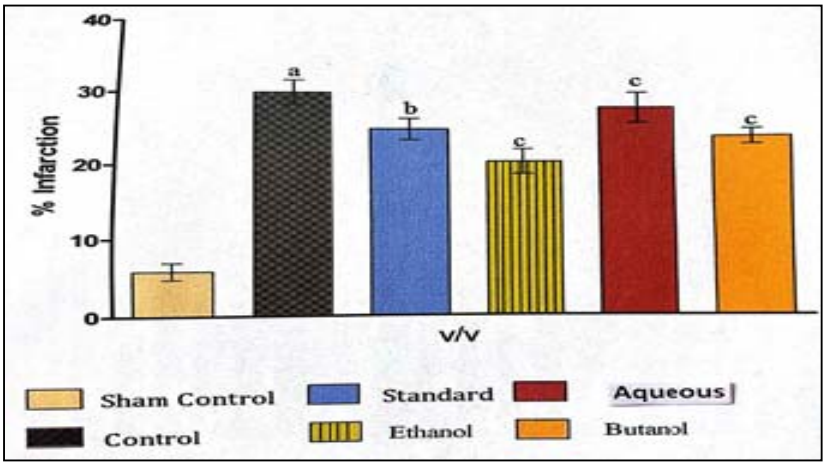

Fig 1: % Infarction in all test group measured by volume method.

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 Turkey's multiple comparison test.

Effect of *R. arboreum* flower extract on the release of LDH

Total aqueous extract, ethanolic extract and its n-butanol fraction were evaluated on ischemia and reperfusion induced decrease in release of LDH in coronary effluent measured immediately and 30 min. after reperfusion, respectively. The total ethanolic extract of *Rhododendron arboreum* flower significantly attenuated ischemia reperfusion induced decrease in release of LDH in coronary effluent measured immediately (0 min.) and 30 min. after reperfusion as compared to aqueous extract. Further treatment of standard Enalapril markedly reduced release of LDH in coronary effluent as compared to total ethanolic extract measured immediately (0 min.) and 30 min. after reperfusion. Since ethanolic extract showed good result, it was further fractionated and n-butanol was again studied for the same effect. It was found that n-butanol significantly attenuated ischemia reperfusion (I/R) induced decrease in LDH in coronary effluent when measured immediately (0 min.) and 30 min. after reperfusion. (Fig. 2).

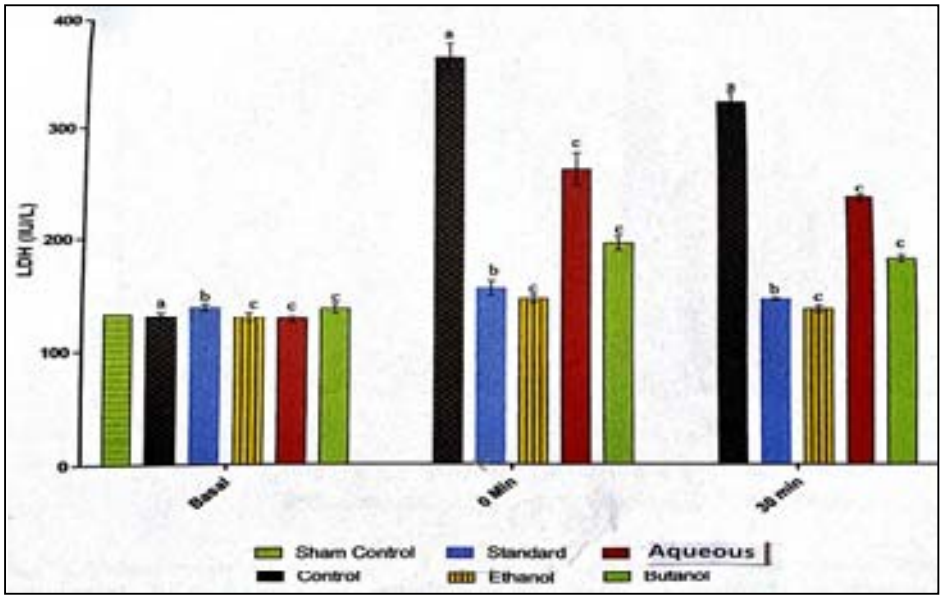

Fig 2: Comparison of LDH activity in all group.

LDH was estimated in coronary effluent after stabilization (Basal), Immediately (Immure.) and 30 min. 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 Turkey's multiple comparison test.

Effect of R. arboreum flower extract on CK-MB release

Total aqueous extract, ethanolic extract and its n-butanol fraction were evaluated for decrease in release at CK-MB in coronary effluent measured after 5 min. of reperfusion. The total ethanolic extract of *Rhododendron arboreum* flower significantly attenuated ischemia reperfusion induced decrease in release of LDH in coronary effluent measured after 5 min. of reperfusion as compared to aqueous extract. Further treatment with standard enalapril markedly reduce release of LDH in coronary effluent measured after 5 min. of reperfusion. Further treatment with standard Enalapril markedly reduce release of CK-MB in coronary effluent as compared to total ethanolic extract measured after 5 min. of reperfusion. N-butanol fraction of total ethanolic extract was again studied for the same effect. It was found that n-butanol significantly attenuated ischemia reperfusion (I/R) induced decrease in CK-MB measured after 5 min of reperfusion. (Fig. 3).

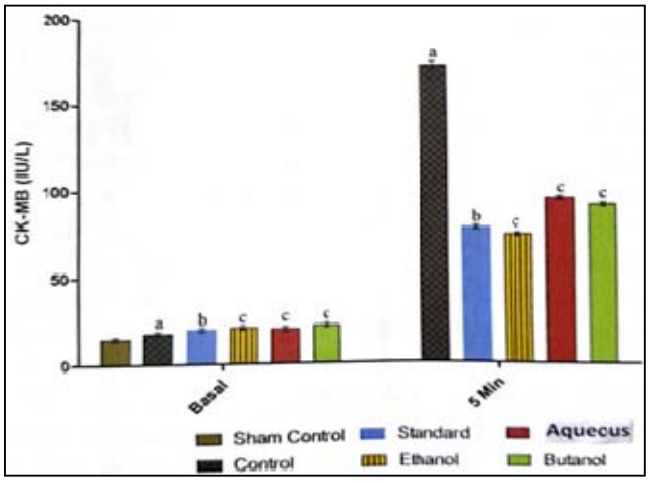

Fig 3: Comparison of CK-MB activity in all group

CK-MB was estimated in coronary effluent after stabilization (Basal) and 5 min. 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 Turkey's multiple comparison test.

Oxygen-derived free radicals are known to play a vital role in the genesis of various cardiovascular disorders. In the present study we have taken *Rhododendron Arboreum* for exploring its effect on ischemic heart. Total aqueous extract, ethanolic extract and its n-butanol fraction were prepared and subjected to qualitative and phytochemical screening. The results of qualitative and phytochemical studies showed that the aqueous extract is composed of carbohydrates, glycosides, amino acids, tannins and saponins while total ethanolic extract & its n-butanol fraction is rich in carbohydrates, glycosides, flavonoids and saponins. (Table 1).

Further total aqueous extract, ethanolic extract and its n-butanol fraction were evaluated for cardio protective activity on ischemia reperfusion, release of LDH in coronary effluent measured after 5 min. of reperfusion. Total ethanolic extract & its n-butanol fraction significantly attenuated ischemia reperfusion induced increase in myocardial infarct size (Fig. 1), decrease in release of LDH (Fig. 2) and CK-MB coronary effluent (Fig. 3) as compared to aqueous extract at the dose level of 70mg/mL and 50mg/mL in case of n-butanol. Standard drug used for the study was Enalapril 2.5 mg/mL.

Conclusion

From the above study it could be concluded that ethanolic extract of *Rhododendron arborium Sm* flowers and further its n-butanol fraction has good cardioprotective effect as compared to aqueous extract which is sold in market as squash. Further attempts can be made to isolate active principle from the active n-butanol fraction of *Rhododendron arboreium* flowers which can be an important entity in itself or a lead on which further work towards drug discovery could be carried out.

Conflict of Interest

The authors declare that there is no conflict of interest.

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