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Evaluation of Chloroform Extract and Its Fractions of *Ocimum gratissimum* (Linn.) Leaves for Cardioprotective Activity

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The present study has been designed to investigate the efficacy of active extract and its fraction of *Ocimum gratissimum* (Linn.) leaves on ischaemia and reperfusion-induced myocardial injury using wister albino rats. Petroleum ether, chloroform, acetone and methanolic extracts of *Ocimum gratissimum* (Linn.) leaves were prepared and screened for their cardioprotective potential. Among all the extracts chloroform extract reduce myocardial injury. The chloroform extract further purified using column chromatographic technique that results four major fractions viz F1, F2, F3 and F4. These fractions screened for myocardial infarct size and LDH and CK-MB release in coronary effluent at dose level of 100mg/kg body weight as compared with that of standard Trimetazidine(35mg/kg body weight). The fraction F4 of chloroform extract significantly prevented myocardial infarct size and LDH and CK-MB release as compared with that of standard trimetazidine. Further, cardioprotective effect of the fraction F4 of chloroform extracts of *Ocimum gratissimum* (Linn.) was significantly ($p < 0.05$) compared to vehicle control group.

Keyword: Chromatography, Ischaemia, *Ocimum gratissimum*, Reperfusion, Trimetazidine.

1. 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].

O. gratissimum is herbaceous plant which belongs to family Labiatae. It is known by various names in different parts of the world. In India it is known by its several vernacular names, the most commonly used ones being Vriddhutulsi (Sanskrit), Ram tulsi (Hindi), Nimma tulasi (Kannada). In India, *O. gratissimum* is a plant with much potential and is useful in many diseases 5. The whole plant has been used for the treatment of sunstroke, headache, influenza and as diaphoretic, antipyretic, anti-inflammatory, chemopreventive, blood-sugar lowering, nervous system stimulation 6-8. Extracts from the leaves of *O. gratissimum* were investigated for their

phytochemical constituents. Tests for tannins, steroids, terpenoids, flavonoids and cardiac glycosides were positive in various extracts. In recent years, phytochemicals in medicinal plants have received a great deal of attention mainly on their role in preventing diseases. Therefore, In the present work, an attempt has been made to study the cardioprotective activity of chloroform extract and its fractions of *O. gratissimum* leaves.

2. Materials and Methods

2.1 Drugs and Chemicals

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

2.2 Plant material

Leaves of *Ocimum gratissimum* (Linn.) was collected from Indian Institute of Integrative Medicine, Jammu (Formerly Regional Research Laboratory), CSIR, India. The reference number is [RRL(J)OG-14].

2.3 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.

2.4 Preparation of Extracts

The fresh leaves of *O. gratissimum* (Linn.) were dried in shade and room temperature for 2 days followed by drying [40-50°C] for 3-4hrs and powdered to obtained coarse powder. 1.5kg of powder of *O. gratissimum* (Linn.) leaves was extracted with pet. ether, chloroform, acetone and methanol successively to collect four extracts of different polarity compounds. The solvent was removed by evaporation under reduced pressure to obtain a semisolid mass. The result extracts were kept in a separate desiccators followed by

weighing to give percentage yield of each extract in reference to air dried leaves of *O. gratissimum* (Linn.).

2.5 Isolation and Purification of Principle Constituent from Active Fraction

The chloroform extract showing good cardioprotective effect was subjected to column chromatography using silica gel mesh size 200-400 μ and Pet. ether: ethyl acetate 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^[9].

2.6 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 chloroform extract of *O. gratissimum* (Linn.). 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 LD₅₀. The number of animals dying during the period was noted. The LD₅₀ 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.

2.7 Isolated Rat Heart Preparation

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; 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 30min 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^[10].

2.8 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).

2.9 Assessment of Myocardial Infarct Size

Heart was removed from the Langendorff's apparatus. Both the auricles 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 infarcted portion remained unstained. Infarct size was measured by macroscopic volume method^[11].

3. 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 7days; thereafter, on the 7th day, isolated rat heart after stabilization, was subjected to 30min of global ischaemia followed by reperfusion for 120min.

Group 3: (Standard; n=5) Trimetazidine (35mg/kg) was 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 30min. of global ischaemia followed by reperfusion for 120min.

Group 4: (Chloroform extract; n=5) Chloroform extract (100mg/kg) was 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 30min of global ischaemia followed by reperfusion for 120min.

Group 5: (Fraction 4 of chloroform extract; n=5) Fraction 4 of chloroform extract (100mg/kg) was 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 30min of global ischaemia followed by reperfusion for 120min.

4. Results

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

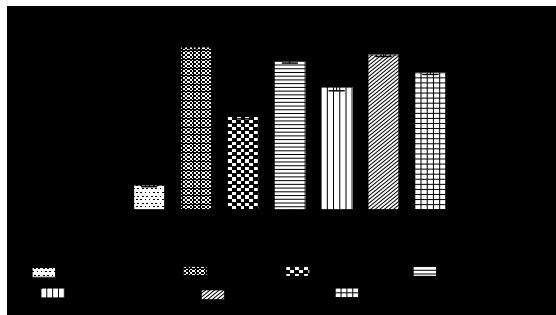


Fig 1: Effect of various extract on myocardial infarct size. Infarct size was measured by volume method. Values are expressed as mean \pm SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

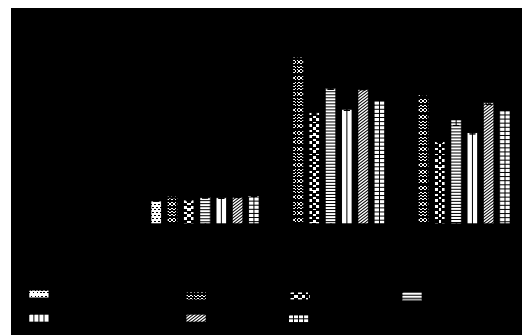


Fig 2: Effect of various extracts on LDH release. LDH was estimated in coronary effluent after stabilization (Basal), Immediately (Imm'Rep.) and 30min. after reperfusion (30' Rep.). Values are expressed as mean \pm SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

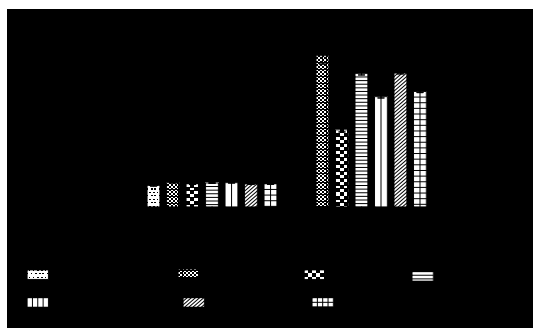


Fig 3: Effect of various extracts on CK-MB release. CK-MB was estimated in coronary effluent after stabilization (Basal) and 5min. after reperfusion (5' Rep.). Values are expressed as mean \pm SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

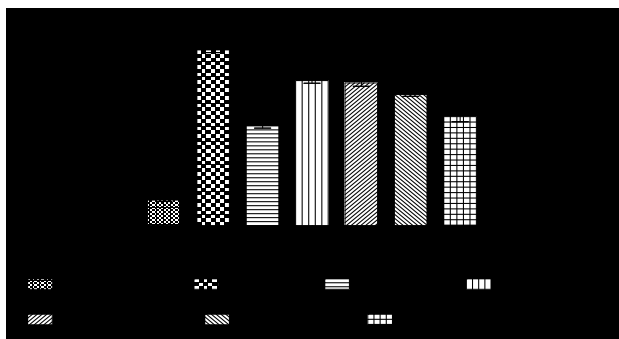


Fig 4: Effect of chloroform fractions on myocardial infarct size. Infarct size was measured by volume method. Values are expressed as mean \pm SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

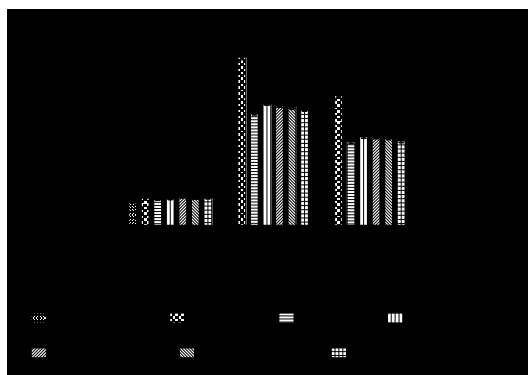


Fig 5: Effect of chloroform fractions on LDH release. LDH was estimated in coronary effluent after stabilization (Basal), Immediately (Imm'Rep.) and 30min. after reperfusion (30' Rep.). Values are expressed as mean \pm SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

4.1 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.

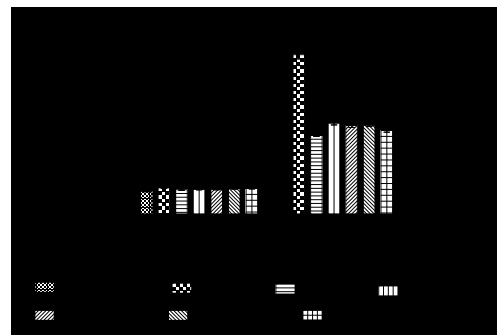


Fig 6: Effect of chloroform fractions on CK-MB release. CK-MB was estimated in coronary effluent after stabilization (Basal) and 5min. after reperfusion (5' Rep.). Values are expressed as mean \pm SEM. a= P<0.05 vs. Sham control; b= P<0.05 vs. Control; c= P<0.05 vs. Standard. ANOVA followed by Tukey's multiple comparison test.

4.2 Effect on Myocardial Infarct Size

Various extracts of *O. gratissimum* (Linn.) 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 chloroform extract of *O. gratissimum* (Linn.) leaves found to be active (Fig 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 (trimetazidine, 35mg/kg) was significantly more effective to reduce myocardial infarct size as compared to fraction 4, measured by macroscopic volume method (Fig 4).

4.3 Effect on Ischaemia and Reperfusion Induced Release of LDH

Various extracts of *O. gratissimum* (Linn.) 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 *O. gratissimum* (Linn.) leaves significantly reduced release of LDH in coronary effluent (Fig 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 (trimetazidine, 35mg/kg) markedly reduced release of LDH in coronary effluent as compared to isolated fraction F4, measured immediately and 30 min. after reperfusion (Fig 5).

4.4 Effect on Ischaemia and Reperfusion Induced Release of CK-MB

Various extracts of *O. gratissimum* (Linn.) 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 *O. gratissimum* (Linn.) leaves significantly reduced release of CK-MB in coronary effluent (Fig 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 (trimetazidine, 35mg/kg) markedly reduced release of CK-MB in coronary effluent as compared to the isolated fraction F4, collected 5 min. of reperfusion (Fig 6).

5. Discussion

In spite 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 *O. gratissimum* (Linn.) 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 chloroform extracts of *O. gratissimum* (Linn.) 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 *O. gratissimum* (Linn.). Moreover, some extensive work in this direction could also lead to explore the exact mechanism of action of these drugs.

6. Acknowledgement

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