International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 7(6): November-December 2017

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Pharmaceutical Sciences **Research Article……!!!**

Received: 05-08-2017; Revised: 31-10-2017; Accepted: 01-11-2017

POTENTIAL OF *RHEUM EMODI* WALL.EX MEISSN AS ANTIHYPERLIPIDEMIC AND ANTIOXIDANT AGAINST HIGH-FAT DIET INDUCED OXIDATIVE STRESS

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Keywords:

Rheum emodi, antioxidant, *in vitro* evaluation, *in vivo* evaluation, hyperlipidemia, oxidative stress

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ABSTRACT

Background: Hyperlipidemia and underlying high cholesterol has been reported among the top five leading cause of global mortality. Many drugs and extracts from plant origin and specifically from Indian plants have been reported and used for hyperlipidemia alleviation.

Objective: The present study was aimed to evaluate antioxidant and anti-hyperlipidemic activity of hydroalcoholic and aqueous extract of *Rheum emodi* Wall. exMeissn against high-fat diet induced oxidative stress and hyperlipidemia in Wistar rats.

Materials and Methods: Hyperlipidemia and oxidative stress was induced in wistar rats with high-fat diet *ad libitum.* The hyperlipidemic rats were then treated with gavage feeding with aqueous and hydroalcoholic extract of *R. emodi*at different doses (100, 250 and 500 mg/kg b.wt./day). The effect was compared with standard drug Lovastatin (7.2mg/Kg).

Results: The high-fat diet showed marked increase in the lipid profile levels in rats as measured by enzyme analytical methods. Both the extracts of the plants showed marked lowering of LDL and VLDL while the aqueous extract showed a substantial increase in HDL (Good Cholestrol). In addition, promising radical scavenging effects were observed through *in vitro* superoxide, hydroxyl, and hydrogen peroxide assays.

Conclusion: The present study established rhizome/root extract of*R. emodi*as a promising anti-oxidant and anti-hyperlepedimic agent. However, further investigations are needed to understand the mechanistic basis of this effect of the extract and its chemical constituents thereof.

INTRODUCTION

Under physiological and pathological aerobic conditions, reactive oxygen species (ROS), Reactive Nitrogen Species (RNS) and Reactive Chlorine Species (RCS) are generated by the organism[1]. The most important of them being the ROS, which comprise hydroxyl, superoxide, peroxyl ($RO_2^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$). Less common include the nitrogen free radicals as Nitric Oxide and Nitrogen Dioxide ($^{\bullet}NO_2$). These free radicals are then usually converted into nonradical reactive species, such as, hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). Thus, both ROS and RNS include a radical as well as non-radical species, which, under normal conditions are maintained at a safe base level by the inherent anti-oxidant mechanisms of the organism. However, when this homeostasis is breached by pathological conditions, there is a consequent decrease in the efficiency of this anti-oxidant system and the complete dwindling of protective system leading to oxidative stress and eventual biological target damage.

Plasma lipoproteins, which are involved in hyperlipidemia, are protected against oxidative stress by antioxidant defense system found innately in the organism. Several enzymes make up this antioxidant defense system, including but not limited to glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase, in addition to hydrophilic antioxidants such as reduced glutathione, ascorbate and urate. Plasma Lipoproteins also facilitate the carriage of lipophilic antioxidants like tocopherols and caroteniods. All of these mechanism work together to alleviate free radical stress and also block free radical chain reactions in the process[2].

Phospholipids oxidative stress in response to several underlying factors released through different types of cells and organisms, these include, endothelial cells, smooth muscle cells, and macrophages. In particular, monocytes and T Lymphocytes are tethered, activated and attached to endothelial cells by induction and expression of adhesion molecules released due to damage to endothelial cells in response to Ox-LDL. These adhesion molecules encompass P-selectin and chemotactic factors like monocyte chemoattractant protein-1 and macrophage colony stimulating factor[3]. Macrophages in the atherosclerotic lesions have also been indicated to express myeloperoxidases, resulting in the formation of unique pattern of protein oxidation products. The myeloperoxidases released have also been associated with LDL-oxidation[4].

Anti-oxidant potential of natural products from plants in particular have been thoroughly researched and proven to be effective in metabolic syndromes, including altered lipid profile, hypertension, insulin resistance, diabetes and obesity[5-7]. *Rheum emodi*has been researched thoroughly and the active compounds and extracts have shown promising results as antidiabetic, antimicrobial, antifungal, cytotoxic, hepatoprotective, immune enhancing, antimicrobial, nephroprotective and above all, found to have substantial antioxidant properties [8-16]. Methonalic extract of *Rheum emodi*has been found to not only possess anti-oxidant but also anti cancer properties[17]. Several compounds found in the root/rhizome of *Rhememodi*, especially marsupsin and maesopsinhave shown antioxidant properties[18]. There is however

a knowledge gap in the form of nonexistence of any research or data that correlates anti-oxidant properties with antihyperlipedemic potential of *Rheum emodi*and this research was intended to address that very gap.

2. MATERIALS AND METHODS

2.1. Plant Materials. Dried rhizome of the plant *Rhuememodi*was collected from Pahalgam area of Jammu and Kashmir during the month of May 2012. The curator authenticated the plant material and a voucher specimen (KASH-bot/Ku/PH-1201) was deposited in the Centre for Plant Taxonomy (KASH), University of Kashmir.

2.2. Chemicals and Reagents. DPPH, catchin and enzyme kits were obtained from Sigma-Aldrich ChemieGmbh, Industriestrasse 25, Postfach, CH-9471, Buch, Switzerland. Cholestrol was purchased from Merck KGaA, Darmstadt, Germany. All the other analytical grade reagents used in the experiment were obtained from HiMedia, A-516, SwastikDisha Business Park, LBS Marg, Mumbai 400086, India.

2.3.Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee, (no. KU/Pharm/CPCSEA/2009-01),University of Kashmir.All the wistar rats (200 ± 50g) used in the experiment were obtained from Indian Institute of Integrative Medicine, Jammu, India. Rats were grouped in a number of 6 rats per group and placed in rat cage with water and food, *ad libitum.*The environment was also regulated at 25 ± 1^0C with 12/12 h (light/dark) cycle.

A period of one week was decided for rats to be acclimatized to the laboratory environment, this was followed by random selection of rats into 6 groups (each group with 6 animals). The extract was suspended in normal saline at a dose level of 200mg/Kg body weight. Gavage was used to feed the rats with each dose at a final volume of extract at 1ml. Group I received vehicle only at 5ml/kg/day (NC); Group II received high fat diet (86.8% basic diet, 8% butter, 5%cholesterol, and 0.2% sodium cholate) only (HC). Group III received high-fat diet along with lovastatin

(10mg/kg/day) (HC/LOVA); Group IV received high-fat diet and 1ml hydroalcoholic extract of *Rheum emodi*(HC/HARE);and Group V received high-fat diet and 1 ml aqueous extract of *Rheum emodi*(HCAQRE).

The experiment was conducted continuously for a period of 5 weeks with rats having free access to food and water. Food consumption and body weight was constantly measured on a daily basis. At the end of the experiment, all animals were put on fasting for 14h before anesthesia and sacrifice. Opthalmic venous plexus was used to draw blood sample, which was then centrifuged (5000g, 15 min, and 4^0 C). The serum samples obtained were collected and stored at -20^0 C.

2.4. Extract Preparation

The extract was prepared by washing, drying and powdering in heavy-duty Willy-mill (Bells India Ltd.) the Rhizome if *R.emodi*and then soaking the powder overnight in 70% ethanol.

After filtration, re-extraction of the powder was carried out through the same method. All the filtrates thus obtained were collected and combined and solvent removed in a rotary vacuum evaporator at 40^0 C to

obtain the hydroalcoholic extract. For aqueous extract, the same procedure was followed with water used in place of ethanol.

2.5. Acute Oral Toxicity Testing. Albino rats were used to carry out *in vivo* oral acute toxicity test. Dried extracts were converted into solutions using normal saline. The study was carried out keeping in view the guidelines set by Organization of Economic Corporation and Development (OECD) with computer-aided statistical analysis program (AOT425StatPgm, version 1.0). Up and down procedurewas conducted, using the dose increment of 175mg/kg p.o.,550mg/kg p.o., and 2000mg/kg p.o. of the aqueous andhydroalcoholic extracts (acute oral toxicity (AOT) (OECDTest guideline 425) statistical program (AOT425StatPgm, 2001).

2.6. Phytochemical Screening of the Crude Extracts. Proper techniques for phytochemical screening were followed to determine various phytoconstituents from the hydroalcoholic and aqueous extracts of *R.emodi[19, 20]*.

*2.7. Total Phenolic Content (TPC).*Total Phenolic Content of HARE and AQRE was determined by Folin-Ciocalteu reagent, according to the method ofOrthofer,Singleton, andLamuela-Raventos (1999). The result was expressed as mg gallic acid equivalents/g extract[20].

2.8 In Vitro Anti-oxidant studies

2.8.1 DPPH Radical Scavenging Activity. Minor modifications were made to the method given by Blois for conducting the assay on the basis of scavenging activity of stable DPPH free radical[21, 22]. To 1mL of thesample (10, 20, 30, 40, and 50 μg/mL) was added 3mL ofa 0.1mmol/L methanol solution of DPPH.The absorbanceof all the samples was determined at 517nm (UVD 2960,Labomed, Inc.) after an incubation period of 30minutes.Thepercentage inhibitory activity was determined according to the following formula:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\text{Ae}}{\text{Ao}}\right)\right] x\ 100, \qquad (1)$$

Where A_o is the absorbance without sample and A_e is the absorbance with the sample.

2.8.2 Reducing Power. Oyaizu's method was used to conduct the assay for reducing power[23].According to this method, the reducing power of the sample can be determined by measuring the absorbance of Pearl's Prussian blue complex associated with reduction of Fe^{3+} to Fe^{2+}. The principle of this method is reduction of Ferricyanide (Fe^{3+}) in stoichiometric excess in relation to the antioxidants. Different concentrations of the extract were taken (10, 20, 30, 40, and 50 μg/mL) and was added to 2.5 mL of 1% potassium ferricyanide [$Ke_3Fe(CN)_6$] in 2.5 mL of 0.2 M phosphate buffer (pH 6.6). The entire mixture was incubated for 20 min at 50^0 C and 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. The upper layer (2.5 mL) was addedto (2.5 mL) distilled water and FeCl3 (0.5 mL, 0.1%), and theabsorbance was measured at 700 nm.For standard, BHT was used and the percentage reduction in relation to that was calculated using the following formula:

$$\% \text{ reduction} = \left[1 - \left(1 - \frac{\text{Ac}}{\text{As}}\right)\right] \text{x } 100 \qquad (2)$$

2.8.3 Hydroxyl Radical Scavenging. Method used by Halliwell et al. was used to conduct the hydroxyl radical scavenging assay[24]. It is basedon the measurement of thiobarbituric acid reactive species(predominantly malondialdehyde producing pink color onreaction with thiobarbituric acid) generated from the degradationof deoxyribose on exposure to hydroxyl radical.Hydroxyl radical is generated from Fe^{3+}-ascorbate-H_2O_2system (Fenton reaction).

The reaction mixture contains 25mM deoxyribose,10mM ferric chloride, 100mM ascorbic acid, 2.8mM H2O2in 10mM KH2PO4 (pH 7.4), and various concentrations ofplant extracts (10–50 μg/mL).The reactionmixture was incubatedat 37∘C for 1 h. 1%thiobarbituric and 3%trichloroaceticacid (1mL each) were added, and the mixture was heatedat 100∘C for 20min. The graded intensity of the color was measured spectrophotometrically at 532 nm. Theresults wereexpressed as percentage inhibition of deoxyribose oxidationusing the formula below:

$$\% \text{ inhibition} = \left(\text{Ac} - \frac{\text{A}}{\text{Ac}}\right) x \text{ } 100, \qquad (3)$$

Where A_c represents absorbance in presence of control and A is the absorbance in the presence of extract.

2.8.4. Superoxide Radical ScavengingActivity. The capacity of substrates to inhibit the formation of formazan by scavenging the superoxide radicals generated in riboflavin-light-NBT system[25]. The reaction mixture was prepared by sequentially adding 50 mM phosphate buffer (pH 7.6), 20 μg riboflavin,12mM EDTA, and NBT 0.1mg/3 mL.Reaction started by illuminating the reaction mixture with different concentrations of sample extract/standard for 90 seconds. This was immediately followed by measurement of absorbance at 590nm. For positive control, Rutin was used and the percentage of superoxide anion scavenged was calculated using the following equation:

$$\% \text{ inhibition} = \left(1 - \frac{\text{As}}{\text{Ac}}\right) x \text{ } 100 \qquad (4)$$

Where A_s and A_c are absorbance in presence of extract and control, respectively.

2.8.5. H_2O_2 Scavenging Activity. Following the procedure laid down by Ruch et al. hydrogen peroxide scavenging assay was carried out[26]. This method is based on the principle that upon oxidation of H_2O_2 there is a decrease in absorbance. For this purpose, a solution of 43mM H2O2 was prepared in 0.1M-phosphatebuffer (pH 7.4). 1mLextract (each at different concentrations)in 3.4mL phosphate buffer was added to 0.6mL of H2O2solution (43mM), and absorbance of the reaction mixturewas recorded at 230 nm. For blank solution sodium phosphate buffer without H_2O_2 was used.

The percentage of H2O2 scavenging by the extracts andstandard was calculated using the following equation:

$$\% \text{ inhibition} = \left(1 - \frac{\text{As}}{\text{Ac}}\right) x \text{ } 100 \qquad (5)$$

Where A_s and A_c are absorbance in presence of extract and control, respectively.

2.9. In Vivo Studies

2.9.1. Lipid Profile. After collecting the blood samples, they were subjected to centrifugation at 3500 rpm for 15 min to obtain serum. The levels ofserum total cholesterol (TC), triglycerides (TG), and highdensity lipoprotein cholesterol (HDL-c) were determinedusing commercially available kits (purchased from Sigma-Aldrich Chemie GmbH, Industriestrasse 25, Postfach, CH-9471, Buchs, Switzerland) according to the manufacturer's instructions.

Low-density lipoprotein cholesterol (LDL-c) was calculated using Friedwald equation:

$$[\text{LDLc}] = [\text{Total Cholestrol}] - [HDLc] - \frac{[\text{TG}]}{5} \quad (5)$$

The atherosclerosis index (AI) as calculated by the equation:

$$\text{AI} = \text{Tc} - \frac{\text{HDLc}}{\text{HDLc}} \quad (6)$$

*2.9.2. Statistical Analysis.*The data has been analysed using descriptive statistics, t-test, and one-way ANOVA followed by post hoc analysis (Dunnett's test), using SPSS-20. Statistical significance was considered at $P< 0.05$.

3. RESULTS

3.1. Acute Oral Toxicity Study. Both, aqueous as well as hydroacoholic extract were found to be safe even up to 2000mg/Kg body weight and did not cause any mortality.

3.2. Phytochemical Investigation. Qualitative tests on the rhizomes of *R. emodi*revealed the presence of carbohydrates, reducing sugars, tannins and phenolics, Anthrquinone glycosides, flavonoids, resins, oxalic, tartaric and ascorbic acid.

3.3. Total Phenolic Content. Total Phenolic Content of the hydroalcoholic extract was found to be 414.67± 4.65 while that of aqueous extract was reported as 365.00± 4.58. The phenolic contents in thesamples were expressed asmg of gallic acid equivalent (GAE)for every g of sample (mg GAE/g).

3.4. DPPH Radical Scavenging. Both the aqueous as well as hydroalcolic extract of *R. emodi*showed a concentration dependent scavenging of DPPH radicals as shown in Figure 1. The scavenging effect of both hydroalcoholic and aqueous extract was found to be 90% at a concentration of 100 μg/mL. This effect was highly comparable to the standard catechin, at the same concentration.

Insert Figure 1 here

*3.5. Reducing Power.*The reducing power of the plant extracts and standard antioxidants (BHT and Catechin) was estimated on Fe^{3+} and found to be concentration dependent (Figure 2). The concentration dependent increase in reducing power was highly comparable to standard BHT positive control.

Insert Figure 2 here

3.6 Hydroxyl Radical Scavenging. A concentration dependent hydroxyl radical scavenging activity was noted as shown in Figure 3. The hydroalcoholic and aqueous extract of plant showed 58.3% and 49.2%

effect at a concentration of 20 µg/mL and 40 µg/mL, respectively. This effect was comparable to the standard Rutin, which is a well-known antioxidant.

Insert Figure 3 here

3.7. Superoxide Radical Scavenging. Both the hyroalcoholic and aqueous extract of the plant showed superoxide-scavenging activity. A concentration dependent radical scavenging activity was found for both the extracts and is presented in Figure 4. The highest activity was shown by the hydroalcolic extract followed by the aqueous extract and the standardrutin.

Insert Figure 4 here

*3.8. Effect on H_2O_2 Scavenging.*As is evident from Figure 5, the hydrogen peroxide scavenging activity of hydroalcoholic extract was closely comparable to the standard α-tocopherol followed by aqueous extract that showed only moderate activity.

Insert Figure 5 here

3.9 In vivo Lipid Parameters. A high level of serum triglycerides, total and LDL cholesterol was induced by a high-fat diet in Male Wistar rats. The hydroalcoholic and aqueous extract of *R. emodi*as well as lovastatin significantly restored the elevated lipid levels to a considerable extent as shown in Table 1 ($P < 0.05$). The group administered with hydroalcoholic extract showed a marked enhancement of good cholesterol (HDL).

Insert Table 1 Here

3.10. AtherogenicInndex. The highest value for atherogenic index (AI) was shown by lovastatin control followed by hydroalcoholic extract and aqueous extract.

4. DISCUSSION

The aqueous and hydroalcoholic extract of *R.emodi*showed anti-oxidant activity, both *in vitro* as well as *in vivo* without any acute or long-term toxic effect. The anti-oxidant effect was found to be concentration dependent, however with different levels of intrinsic efficacy. Based on the amount of total phenolic content present, it would be safe to assume that the DPPH, Hydroxyl radical, hydrogen peroxide and Ferric reduction potential of the extracts might most probably be due to polyphenolics present.

Superoxide shows selective reactivity due to the presence of an oxygen atom at the center. Although, the superoxide is a weak oxidant per se, its limited reactivity can lead to generation of more dangerous species, including the dreaded singlet oxygen and hydroxyl radicals, which eventually cause the peroxidation of lipids[27]. Superoxide anions are thus precursors to active

Free radicals that have potential for reacting with biologicalmacromolecules and thereby inducing tissue damage [28]. In addition, superoxide has also been associated with direct initiation of lipid peroxidation and it has been established that the antioxidant properties of flavonoids in plants show activity by indirectly or directly scavenging superoxide[29]. Superoxide opens up a Pandora's box is the cascade of free radical formation by producing other kinds of free radicals and oxidizing agents[30]. Superoxide anions derived fromdissolved oxygen by theriboflavin-light-NBT system will reduce NBT in this

system.In this method, superoxide anion reduces the yellow dye(NBT2+) to produce the formazan, which is measured spectrophotometricallyat 590 nm.Antioxidants are able to inhibitthe blue NBT formation[31]. The decrease of absorbanceat 560 nm with antioxidants indicates the consumption ofsuperoxide anion in the reaction mixture. Figure 4 showsthe inhibition of superoxide radical generation by differentconcentrations of extracts and standards.

Lipid peroxidation has also been known to initiate by DNA cross-links and strand breaks that occur due to hydroxyl radicals, and the extracts were shown to scavenge these hydroxyl radicals[32]. The ability of the extractsto scavenge ˙OH radicals can be related to the prevention of lipid peroxidation.It can be inferred that the extracts might preventreactive radical species from damaging biomolecules such aslipoproteins, polyunsaturated fatty acids, DNA, amino acids,proteins, and sugars in biological systems [32].The aim of this study was to relate this scavenging potential of the extracts and therefor catechin, □-tocopherol, and BHT were used as standards.

In addition of the ability of the extracts to substantially lower the LDL and triglyceride levels, the marginal increase in HDL by hydrolcoholic extract presents the plant as a promising plant to control hyperlipidemia and a propitious starting point for further research.

CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest regarding the publication of this paper.

ACKNOWLEDGMENT

The financial support from CCRUM (AYUSH), Ministry of Health and Family Welfare, India, is highly acknowledged.

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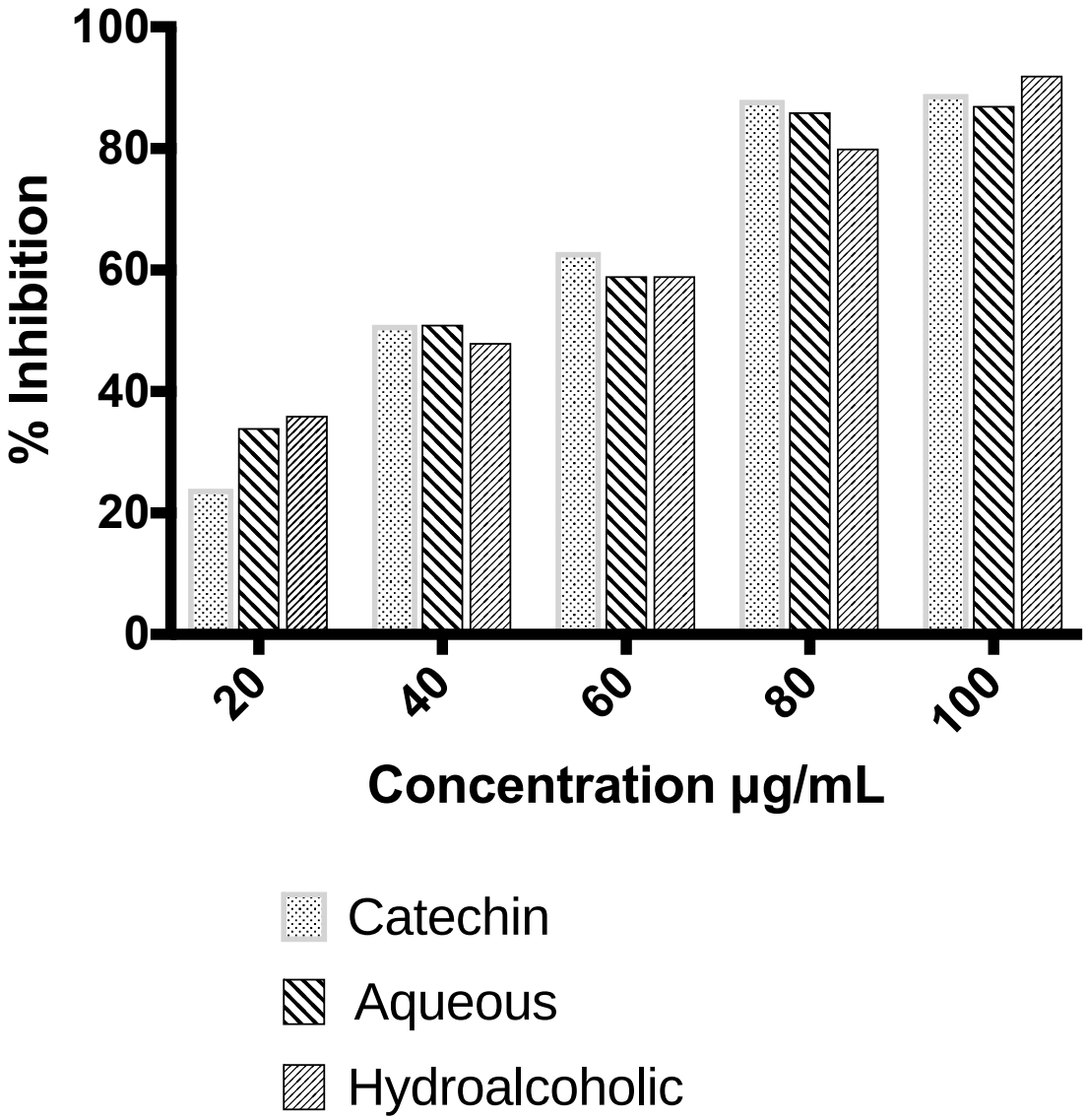

Figure 1. DPPH radical scavenging activity of hydroalcoholic and aqueous extract of *R. emodi.*

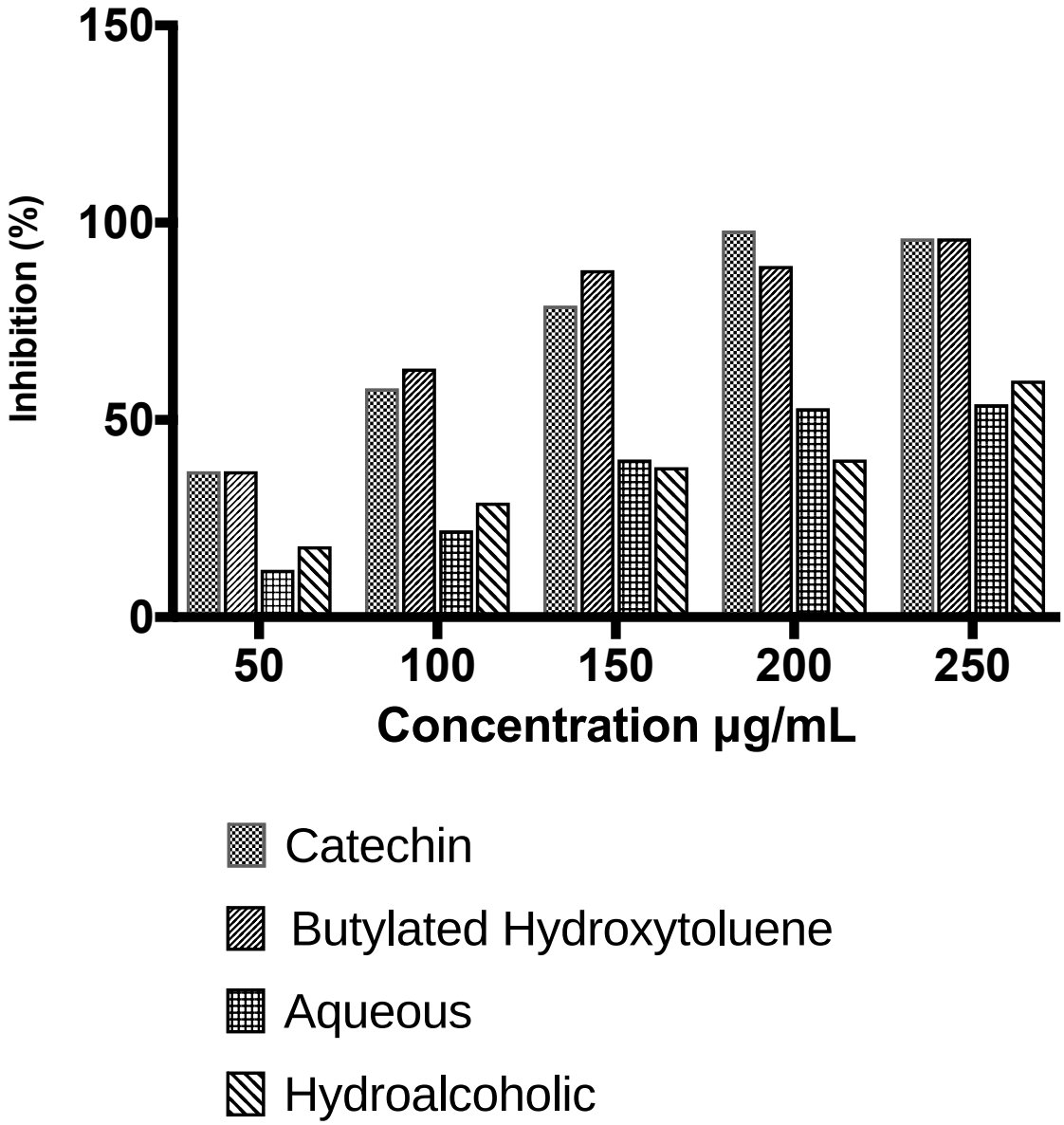

Figure 2. Reducing power of aqueous and hydroalcoholic extract of *R. emodi*

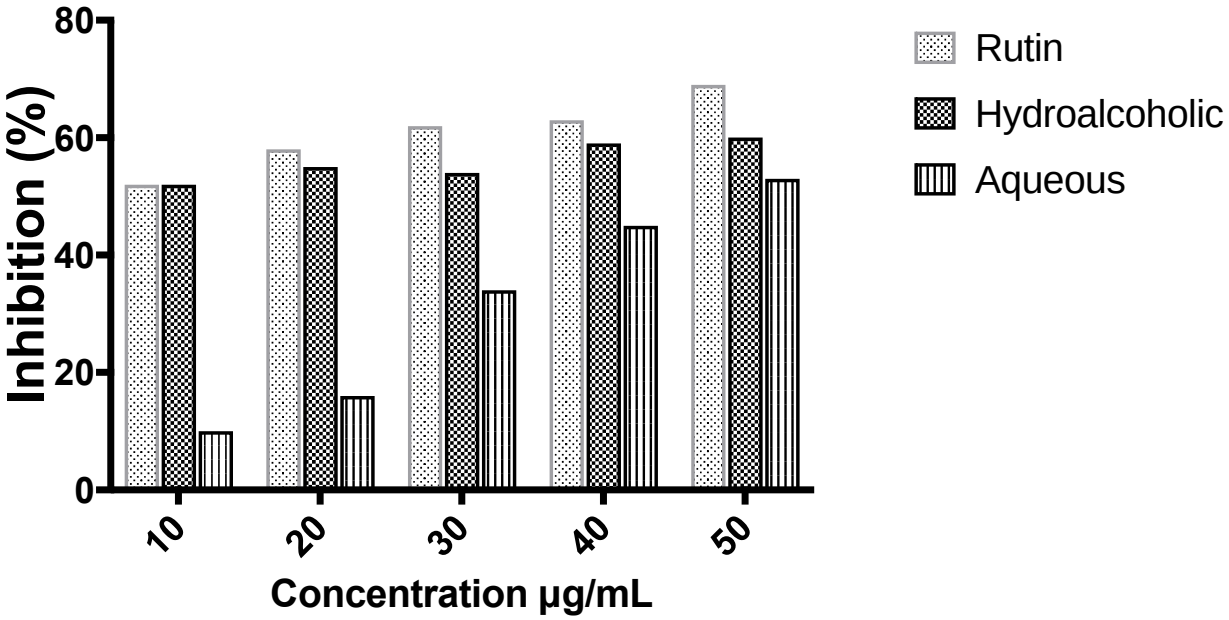

Figure 3. Hyroxyl Scavenging activity of hydroalcoholic and aqueous extract of *R. emodi.*

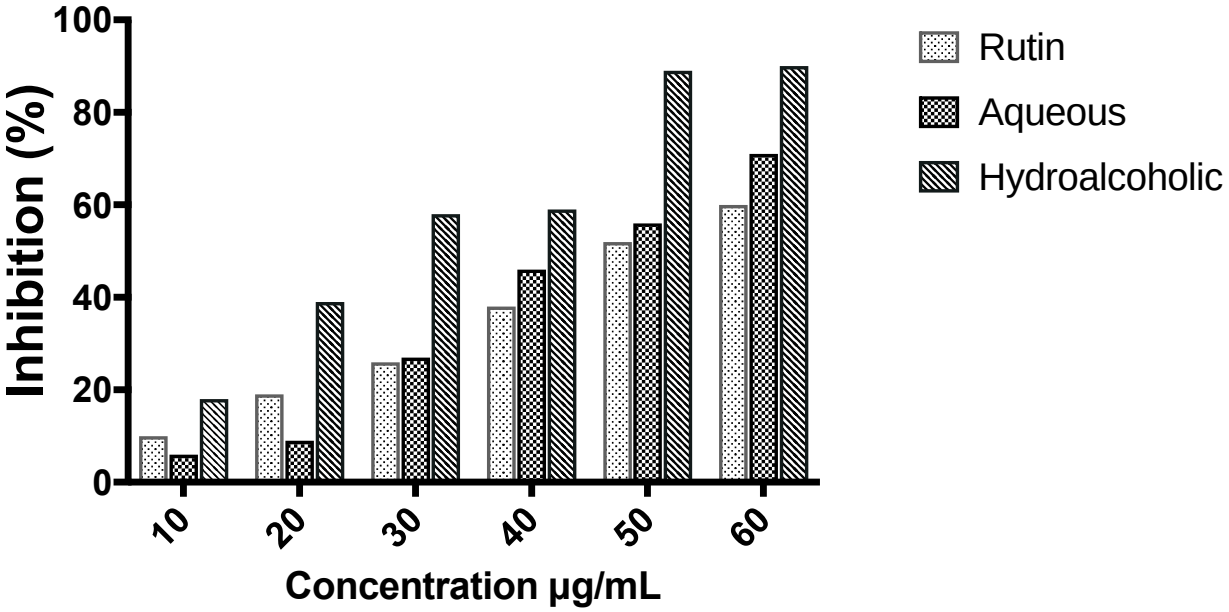

Figure 4. Superoxide scavenging activity of hydroalcoholic and aqueous extract of *R. emodi.*

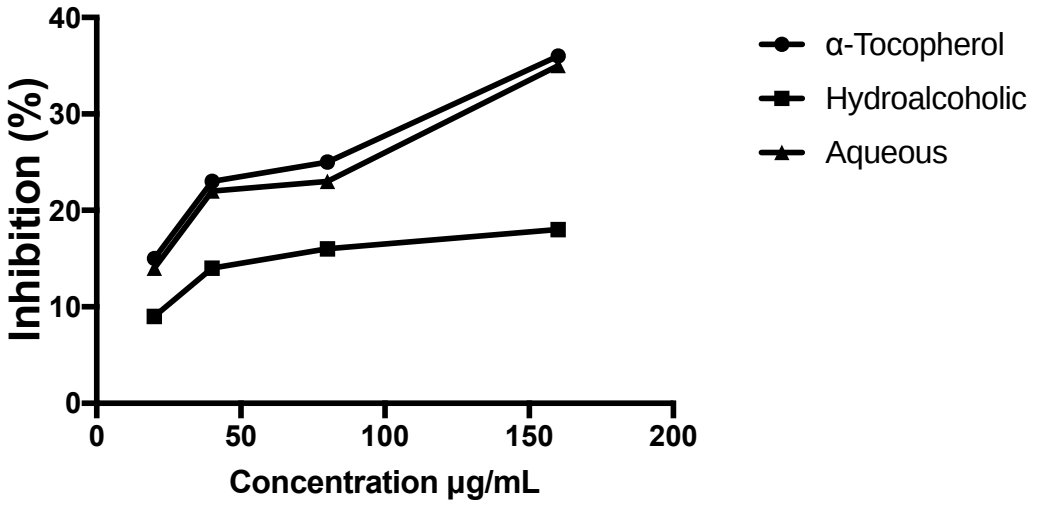

Figure 5. Hydrogen Peroxide scavenging activity of Hydroalcoholic and Aqueous extracts of *R. emodi.*

Table 1. Effect of Hydroalcoholic and Aqueous extract rhizome/root extract of *R. emodi*on lipid Profile and atherogenic index in make Wistar Rats.

Parameters	NC	HC	HC/LOVA	HC/HARE	HC/AQRE
TG	39.18 ± 1.08	73.39 ± 4.24	49.80 ± 2.12	52.92 ± 3.01	50.29 ± 2.38
TC	58.14 ± 2.51	69.65 ± 2.35	53.73 ± 1.27	33.14 ± 4.80	59.28 ± 3.70
HDL	27.55 ± 2.14	18.41 ± 0.74	27.73 ± 1.66	40.30 ± 1.02	38.03 ± 1.13
LDL	22.75	36.56	16.04	17.74	11.19
AI	57.14	68.65	52.73	32.14	58.28

Values are expressed as mean ± SEM. At $p < 0.05$, results were found to be significantly different from control group. NC (Normal control), HC (Hyperlipidemic Control Group), HC/LOVA (group fed with high fat diet+Lovastatin), HC/HARE (group fed with high fat diet + hydroalcoholic extract of *R. emodi*), HC/AQRE (group few with high fat diet + aqueous extract of *R. emodi*). TG= Triglycerides, TC= Total Cholestrol, HDL= High density Lipoprotein Cholestrol, LDL = low density lipoprotein cholesterol and AI= Atherogenic Index.