Pharmacognosy Magazine
ISSN: 0973-1296

PHCOG MAG.
An official Publication of Phcog.Net

PHCOG MAG.: Research article

In vivo and *In vitro* evaluation of free radical scavenging potential of methanol extract of *Desmodium gangeticum* root: A possible explanation through GC Mass analysis

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ABSTRACT

Desmodium gangeticum root is considered extensively in the indigenous system of medicine as an anti inflammatory, anti microbial agent in many Asian countries. Oxidative stress plays a major role in the biochemical and pathological changes associated with myocardial ischemic-reperfusion injury. The current investigation focuses on the in vitro and in vivo free radical scavenging property of the aqueous extract of *Desmodium gangeticum* (DG) root on experimentally induced ischemic reperfused rat heart. The free radical scavenging potential was studied in vitro by using different antioxidant models such as DPPH, super oxide scavenging activity, hydroxide scavenging activity and nitric oxide scavenging activity. Aqueous extract of DG, 50 or 100 mg/kg body weight was administrated once daily for 30 days orally in Wister rats to evaluate the in vivo free radical scavenging potential. Thereafter, hearts were isolated and subjected to ischemic reperfusion injury with the help of Langendroff apparatus. The experimental group consists of normal, ischemic control, reperfusion control and drug. Lipid peroxide products (thiobarbituric acid reactive substances) were significantly increased during ischemia and ischemic-reperfusion. The enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in the myocardial tissue homogenate showed a significant decrease during ischemia and ischemic-reperfusion. Pre treatment of rats with DG (50 or 100mg / kg b.wt.) orally for 30 days daily caused a significant effect in the activity of antioxidant enzymes. The in vitro antioxidant study was found to inhibit lipid per-oxidation (LP) and scavenge hydroxyl and superoxide radicals. Concentration needed for 50% inhibition of DPPH, superoxide scavenging activity, hydroxide scavenging activity and nitric oxide scavenging activity were found to be 38.5, 21, 50.5, 50µg/ml respectively. Administration of DG to normal rats did not have any significant effect on any of the parameter studied. The results of our study showed that DG possesses activity to scavenge the free radical generated during ischemia and ischemic-reperfusion.

KEYWORDS: *Desmodium gangeticum*, Myocardial ischemia, Myocardial ischemic reperfusion injury, Antioxidants

INTRODUCTION

Free radicals induced peroxidation has gained much importance because of their involvement in several pathological conditions such as cancer, cardiovascular disease, cataracts, immune system decline, etc., (1). The imbalance between the antioxidant defence system and reactive oxygen species (ROS) may increase the oxidative burden and lead to the damage of macromolecules.

Recent success in experiments on antioxidant therapy in myocardial ischemic reperfusion injury suggested the significant role of free radical in the mediation of the pathology of revascularization injury (2). Natural antioxidants including superoxide dismutase, catalase and glutathione peroxidase are depleted accompanied by accumulation of ROS. Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have restricted use in foods as they are suspected to be carcinogenic. Therefore, the importance of search for natural antioxidants has greatly increased in the recent years (3). Natural products can play an important role in two aspects: enhance the activity of original natural antioxidants and neutralize ROS by non-enzymatic mechanisms (4). Many natural products like magnolol (5) and honokiol

(6) showed their effect on inhibition of ventricular tachycardia, ventricular fibrillation and / or extra systole during the period of ischemic reperfusion injury. Miller et al (7) has reported that many plants often contain substantial amounts of antioxidants including vitamins C and E, carotenoids, flavonoids and tannins etc. and thus can be utilized to scavenge the excess free radicals from human body.

Desmodium gangeticum (DG) (Leguminosae) is an herb or shrub common on the lower hills and plains throughout India; on the Himalayas it ascends to 5,000 feet (8). DG is widely used as a medicinal herb in the treatment of ischemic heart diseases (9). Moreover, the plant is reported to have antileishmanial and immunomodulatory activities (10). Dharmani and his co-worker experimentally showed the anti-ulcerogenic property of ethanolic extract of DG (11). There are reports suggesting nineteen compounds of various classes, such as flavonoid glycosides, pterocarpanoids, lipids, glycolipids, and alkaloids, were isolated and identified from the *Desmodium gangeticum* whole plant (12). With this observation, the present study was designed to investigate the anti oxidant activity of DG root extract in different in-vitro antioxidant models. However, apart from the direct scavenging activity of the compounds, other factors such as transition metal chelating and uptake into membrane may play an important role in the antioxidant potential of the extract. Hence, the study also focused on the in-vivo free radical scavenging potential of DG root extract by inducing oxidative stress in ischemic reperfusion rat heart model. GC Mass analysis was also included in the study to predict the reasoning for the activity of the DG root extract.

MATERIALS AND METHODS

Plant material

The plant *Desmodium gangeticum* (Leguminousea), after collection from the botanical garden maintained in the botany department of Assumption College, Changanacherry was washed and cleaned with water. The plant material was taxonomically identified by Prof. James Joseph, Head of the Department, Department of Botany, Saint Berchman's College, Mahatma Gandhi University, Kerala. The voucher specimen A/C no. 3908 was retained in our laboratory for future reference.

The plant extract was prepared as follows: One kilogram (1 kg) of fresh secondary roots of DG was sliced and air dried at room temperature. The sliced, air dried roots of the plant were milled into fine powder in a warring commercial blender. The powdered plant material was soaked in 2L methanol for 72 hrs and the extract was filtered and distilled on a water bath. The last traces of the solvent were removed under vacuum drier and the solid brown mass obtained was stored at –4°C until further use. The yield of the extract was 6.1% w/w of powdered methanolic extract. The extract was lyophilized. For in-vitro antioxidant assay, the methanolic extract was dissolved in 0.9% saline. For in-vivo antioxidant assay, DG was suspended in water and administered orally.

Dosage fixation

A pilot study was conducted to determine the effect of DG at four different doses (25, 50, 100 and 150 mg/kg b.wt.) for a period of 30 days in ischemic reperfused rats. Since 50 and 100 mg/kg b.wt. showed significant ($P < 0.05$) effect, we used these doses for further studies.

Chemicals

All chemicals used were of analytical grade.

Animals

Adult male albino rats of the Wistar strain, weighing approximately 250-280 g were obtained from King Institute of Preventive Medicine, Chennai, India. They were acclimatized to animal - house conditions and were fed commercial pelleted rat chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water (ethically approved by Ministry of Social Justices and Empowerment, Government of India).

Heart Preparation

Wister male rats weighing 250-280 g were anesthetized with 40 mg/kg sodium thiopentenone. After an intravenous injection of 300U heparin, the heart was rapidly excised via a mid-sternal thoracotomy and arrested in the ice cold Krebs-Henseleit buffer (KH) containing (mM/L) NaCl: 118, KCl: 4.7, $MgSO_4$: 1.2, KH_2PO_4: 1.2, $CaCl_2$: 1.8, $NaHCO_3$: 25 and $C_6H_{12}O_6$: 11. The heart was attached to a Langendroff apparatus via an aorta for retrograde perfusion with KH buffer maintained at 37°C and pH = 7.4 and saturated with a gas mixture of 95% O_2 and 5% CO_2. The coronary perfusion pressure was maintained at 80 mm Hg.

Experimental Protocol

The rats were divided into four groups (n = 6 in each group): group 1, control; group 2, ischemic control; group 3, reperfusion; and group 4, drug.

Normal control

In normal control group, hearts were perfused for 90 minutes with KH buffer and used for the biochemical analysis.

Ischemic control

Ischemic control groups were further sub divided into

five groups (n=6 in each group) as 5 minutes global ischemia (2.1), 15 minutes global ischemia (2.2), 30 minutes global ischemia (2.3), 45 minutes global ischemia (2.4) and 60 minutes global ischemia (2.5) respectively. In all these subgroups, hearts were perfused with KH buffer for 20 minutes before insulting global ischemia by stopping the KH buffer inflow to the heart.

Reperfusion

In reperfusion group, the 30 minutes ischemic hearts (n=6 in each sub groups) were subjected to 15 minutes reperfusion (3.1), 30 minutes reperfusion (3.2) and 45 minutes reperfusion (3.3) respectively.

Drug

The animals in the drug group were subdivided into 8 groups.

Group 4.1: Rats (n=6) in this group were pretreated orally with DG at a dose of 50mg /kg b. wt. for thirty days. Hearts were perfused for 90 minutes with KH buffer and used for the biochemical analysis.

Group 4.2: Rats (n=6) in this group were pretreated orally with DG at a dose of 100mg /kg b. wt. for thirty days. Hearts were perfused for 90 minutes with KH buffer and used for the biochemical analysis.

Group 4.3: Rats (n=6) in this group were pretreated orally with DG at a dose of 50mg /kg b. wt. for thirty days. Hearts were subjected to 30 minutes of global ischemia after equilibration and followed by 30 minutes of reperfusion.

Group 4.4: Rats (n=6) in this group were pretreated orally with DG at a dose of 50mg /kg b. wt. for thirty days. Hearts were subjected to 30 minutes of global ischemia after equilibration and followed by 45 minutes of reperfusion.

Group 4.5: Rats (n=6) in this group were pretreated orally with DG at a dose of 100mg /kg b. wt. for thirty days. Hearts were subjected to 30 minutes of global ischemia after equilibration and followed by 30 minutes of reperfusion.

Group 4.6: Rats (n=6) in this group were pretreated orally with DG at a dose of 100mg /kg b. wt. for thirty days. Hearts were subjected to 30 minutes of global ischemia after equilibration and followed by 45 minutes of reperfusion.

Group 4.7: Rats (n=6) were perfused with KH buffer for 20 minutes and were infused with standard drug, verapamil (0.2 mg/kg b.wt.) for 10 minutes. They were subjected to 30 minutes global ischemia followed by 30 minutes reperfusion.

Group 4.8: Rats (n=6) were perfused with KH buffer for 20 minutes and were infused with standard drug, verapamil (0.2 mg/kg b.wt.) for 10 minutes. They were subjected to 30 minutes global ischemia followed by 45 minutes reperfusion.

Acute toxicity studies

Wister albino rats (150-250 g) maintained under standard laboratory condition was used. A total of five animals were used which received a single dose (2000 mg/kg, b.wt.) of DG. Animals were kept on overnight fasting prior to drug administration. After the administration of DG, the food was with-held for 3-4 hrs. Animals were observed individually at least once during the first thirty minutes after dosing, periodically during the first 24 hrs (with special attention during the first 4hrs) and daily there after for a period of 14 days. Daily cage side observation included changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory (heart and blood pressure), autonomic salvation, lacrimation, perspiration, pilorection urinary incontinence and defecation changes.

Biochemical assays

Thiobarbituric acid reactive substances (TBARS) (13) was measured as a marker of lipid peroxidation, and endogenous antioxidants, such as superoxide dismutase (SOD): Cu-Zn SOD and Mn SOD (14:15), catalase (16) and glutathione peroxidase (GPx) (17) were estimated in a UV-1601 Shimadzu spectrophotometer. Protein concentration was measured with Folin phenol reagent, following the procedure described by Lowry (18).

In vitro anti oxidant activity

Determination of super oxide radical scavenging activity

Superoxide scavenging was determined by the nitroblue tetrazolium (NBT) reduction method of Mc Cord and Fridovich (19). The reaction mixture contained EDTA (6 µM) containing NaCN (3 µg), riboflavin (2µM), NBT (50 µM), various concentration of extracts (5-50 µg/ml) and phosphate buffer (67 µM, pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent visible light for 15 min and the optical density was measured at 530 nm before and after the illumination. The percentage inhibition of super oxide generation was evaluated by comparing the absorbance vales of the control and experimental tubes.

Determination of hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell (20). Stock solutions of EDTA (1 mM), $FeCl_3$

(10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10mM) were prepared in distilled de-ionized water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of the extract (10 – 100 µg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37 °C for 1 hr. A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation.

Lipid peroxide scavenging activity

Reaction mixture (0.5ml) containing rat liver homogenate (0.1ml, 25%w/v) in Tris –HCl buffer (40mM, pH 7.0), KCl (30 mM), ferrous iron (0.16mM) and ascorbic acid (0.06 mM) was incubated for 1hr at 37ºC in the presence and absence of extract (20-180µg/ml). The lipid peroxidation was measured by TBARS formation (13). For this incubation mixture, 0.4ml was treated with sodium dodecyl sulphate (8.1%,0.2 ml), TBA (0.8%,1.5 ml) and acetic acid (20%,1.5 ml, pH 3.5).The total volume was then made upto 4ml by adding distilled water and kept in a water bath at 100ºC for 1hr. After cooling, 1ml of distilled water and 5ml of a mixture of n- butanol and pyridine (15:1 v/v) was added and shaken vigorously. After centrifugation, the absorbance of the organic layer was measured at 532 nm. The percentage inhibition of LP was determined by comparing results of the test compounds with those of controls and not treated with the extracts.

DPPH radical scavenging activity

The free radical scavenging activity of the DG extract and butylated hydroxyl toluene (BHT) was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH (21). 0.1mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in water at different concentrations (10-100 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extract that inhibits the formation of DPPH radicals by 50%.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (22), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (22). Sodium nitroprusside (5mM) in phosphate-buffered saline (PBS) was mixed with 3.0ml of different concentrations (10 – 320 µg/ml) of the drugs dissolved in the suitable solvent systems and incubated at 25^0 C for 150 min. The samples from the above were reacted with Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of potassium nitrite, treated in the same way with Griess reagent.

GC Mass Analysis

All analysis was conducted with a Perkin Elmer Clarus 500 GC equipped with mass spectrometry. The chromatographic conditions were as follows: Column : Elite -1 (100% dimethyl poly siloxane). Helium was used as the carrier gas with a flow rate of 1ml/minute. The 1µL DG methanolic root extract was injected into the GC –MS in split less mode at 250°C. The column oven temperature was held at 110°C for 2 minutes, then programmed at 75°C/min to 200°C for 1 min, 5°C / min to 280°C and held for 9 minutes. Helium carrier gas was maintained at a flow rate of 1.0ml/min

Statistics

All data are reported as mean ± SD. Results were statistically analyzed by a one-way analysis of variance (ANOVA) by SPSS software 12.00. $P< 0.05$ was considered to be significant. Linear regression analysis was used to calculate IC_{50} values whenever needed.

RESULTS

GC/MS analysis resulted in the identification of 64 compounds. Major compounds comprises of 4-[2-(dimethylamino)ethyl] phenol -(Cactine), glycerine, sucrose, asarone, trans –Z- □□ bisabolene epoxide, 2,5-bis (1,1-dimethyl ethyl) phenol, trans-2-methyl-4-n-pentylthiane, S,S-dioxide, decahydro-1,1 dimethylnaphthalene, 4,5 dihydro-2-(phenyl methyl) 1-H-imidazole, (-)-nortrachelogenin, 2-methyl-9,10-anthracene dione and Piperine. It represents around 33%. Minor compounds such as conhydrin, oxirane, 2,5-dihydro-1-H-pyrrole, thymol, Eugenol, apiol, eicosane, 3- methyl-2-(2-oxo propyl) furan and1-methoxy-10-H-

phenothiazine were identified.

Several concentrations ranging from 2-500 µg/ml of methanolic extract of DG were tested for their antioxidant activity in different in-vitro models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The maximum inhibitory concentration (IC_{50}) in all models viz DPPH, superoxide scavenging activity, hydroxide scavenging activity and nitric oxide scavenging activity were found to be 38.5, 21, 50.5, 50 µg/ml respectively.

The in-vivo antioxidant effect of the extract was determined by administering the rats with DG orally for about 30 days and then sacrificed the animal for the induction of reperfusion induces ischemic injury. The observations in the present study suggested a potent in-vivo antioxidant capacity for DG as compared to standard drug namely verapamil against revascularization injury.

Table 1 : Chemical composition of aqueous extract of Desmodium gangeticum root by GC Mass Analysis.

No.	R.T	Name	Molecular formula
1	3.00	Butane, 1,1-diethoxy-	$C_8H_{18}O_2$
2	4.07	Glycerin	$C_3H_8O_3$
3	4.44	Phenol	C_6H_6O
4	6.08	Propane, 1,1-diethoxy-	$C_7H_{16}O_2$
5	6.16	Thymine	$C_5H_6N_2O_2$
6	6.24	Cyclobutanone, 2,3,3,4-tetramethyl-	$C_8H_{14}O$
7	6.78	1-H-Pyrrole, 2,5-dihydro-	C_4H_7N
8	7.71	4-H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$
9	8.13	Octanoic Acid	$C_8H_{16}O_2$
10	8.32	Oxirane, 2-methyl-2-pentyl-	$C_8H_{16}O$
11	8.61	2,3,4-Trimethyl-2-pentanol	$C_8H_{18}O$
12	9.09	-Hepten-3-one, 4-methyl-	$C_8H_{14}O$
13	9.55	Conhydrin	$C_8H_{17}NO$
14	9.95	1-Propanone, 1-phenyl-3-[2-(phenylmethoxy)phenyl]-	$C_{22}H_{20}O_2$
15	10.21	2H-Pyrrol-2-one, 1,5-dihydro-1-methyl-	C_5H_7NO
16	10.47	Nonanoic acid	$C_9H_{18}O_2$
17	10.64	2-Azetidinone, 3,4,4-trimethyl-	$C_6H_{11}NO$
18	11.11	Thymol	$C_{10}H_{14}O$
19	12.09	Benzenepropanoic acid	$C_9H_{10}O_2$
20	12.75	Eugenol	$C_{10}H_{12}O_2$
21	13.08	iso-Butyl tiglate	$C_9H_{16}O_2$
22	13.99	Sucrose	$C_{12}H_{22}O_{11}$
23	14.99	1-Methylene-2b-hydroxymethyl-3,3-dimethyl-4b-(3-methylbut-2-enyl)-cyclohexane	$C_{15}H_{26}O$
24	15.41	Phenol, 4-[2-(dimethylamino) ethyl]- (Anhalin, Peyocactine. Cacitine)	$C_{10}H_{15}NO$
25	15.61	Etilefrin-propionyl	$C_{19}H_{27}NO_5$
26	17.50	Benzenepropanol, 4-hydroxy-α-methyl-, (R)- (Betuligenol4.Rhododendrol)	$C_{10}H_{14}O_2$
27	17.63	Undecanoic acid	$C_{11}H_{22}O_2$
28	18.66	Asarone	$C_{12}H_{16}O_3$
29	18.72	Caryophyllene oxide	$C_{15}H_{24}O$
30	19.06	Apiol	$C_{12}H_{14}O_4$
31	19.90	3-O-Methyl-d-glucose	$C_7H_{14}O_6$
32	20.19	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-	$C_{15}H_{26}O$

		(2α,4α,8β)]- β-Eudesmol	
33	20.55	trans-Z-à-Bisabolene epoxide	$C_{15}H_{24}O$
34	20.87	Curlone	$C_{15}H_{22}O$
35	21.39	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$
36	21.89	Phenol, 2,5-bis (1,1-dimethylethyl)-	$C_{14}H_{22}O$
37	22.05	Tetradecanoic acid	$C_{14}H_{28}O_2$
38	22.86	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	$C_{11}H_{22}O_2S$
39	23.04	Eicosane	$C_{20}H_{42}$
40	23.49	2-Methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane	$C_{15}H_{26}O$
41	23.80	3-Methyl-2-(2-oxopropyl)furan	$C_8H_{10}O_2$
42	24.01	Dibutyl phthalate	$C_{16}H_{22}O_4$
43	24.35	9-Heptadecanone	$C_{17}H_{34}O$
44	24.51	Cyclohexane, tetradecyl-	$C_{20}H_{40}$
45	25.33	Naphthalene, decahydro-1,1-dimethyl-	$C_{12}H_{22}$
46	25.44	10H-Phenothiazine, 1-methoxy-	$C_{13}H_{11}NOS$
47	25.75	Methyl 3,5-di-t-butylsalicylate	$C_{16}H_{24}O_3$
48	26.17	n-Hexadecanoic acid(Palmitic acid)	$C_{16}H_{32}O_2$
49	26.84	5-Eicosene, (E)-	$C_{20}H_{40}$
50	28.56	9,12-Octadecadienoic acid, methyl ester, (E,E)- (Linoleic acid ester)	$C_{19}H_{34}O_2$
51	28.68	9-Octadecenoic acid (Z)-, methyl ester (Oleic acid ester)	$C_{19}H_{36}O_2$
52	29.10	9,10-Anthracenedione, 2-methyl-	$C_{15}H_{10}O_2$
53	29.39	Valeric acid, undec-2-enyl ester	$C_{16}H_{30}O_2$
54	30.79	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$
55	31.97	Naphthalene, 2-(1,1-dimethylethyl) decahydro-4a-methyl-	$C_{15}H_{28}$
56	32.17	1H-Imidazole, 4,5-dihydro-2-(phenylmethyl)-	$C_{10}H_{12}N_2$
57	32.93	2-(3,4-Methylenedioxyphenyl) cyclohexanone	$C_{13}H_{14}O_3$
58	33.09	Dihexadecyl phosphate	$C_{32}H_{67}O_4P$
59	34.95	2-Hydroxy-4-methoxy-7-methyl-7, 8,9,10,11,12,13,14-octahydro-6-oxabenzocyclododecen-5-one	$C_{17}H_{24}O_4$
60	35.29	Benzene, 1-methoxy-4-(1-propenyl)- (Anise camphor)	$C_{10}H_{12}O$
61	36.08	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$
62	39.23	(-)-Nortrachelogenin	$C_{20}H_{22}O_7$
63	41.84	Squalene	$C_{30}H_{50}$
64	43.55	Piperine	$C_{17}H_{19}NO_3$

Table 2: The activities of Catalase, Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and the concentration of TBARS in the tissue homogenate of isolated rat heart during myocardial ischemia and ischemic reperfusion

Group	TBARS (mM/100g wet tissue)	Catalase (µM of H_2O_2 consumed/min/mg protein)	SOD ($U^{\#}$/mg protein)		GPx (µg of GSH consumed/min/mg protein)
			Mn SOD	Cu-Zn SOD	
1	0.60 ± 0.04	7.61 ± 0.54	8.2 ± 0.61	52.2 ± 4.2	1.85 ± 0.18
2.1	0.49 ± 0.03*	4.11 ± 0.33*	8.5 ± 0.63	53.6 ± 4.1	1.05 ± 0.10*
2.2	0.70 ± 0.05*	3.89 ± 0.31*	5.5 ± 0.51*	33.5 ± 3.5*	1.21 ± 0.20*
2.3	0.54 ± 0.04	7.11 ± 0.50	5.6 ± 0.49*	35.4 ± 3.6*	1.05 ± 0.15*
2.4	0.62 ± 0.05	6.35 ± 0.54*	3.7 ± 0.41*	23.3 ± 3.1*	0.85 ± 0.10*
2.5	0.73 ± 0.06*	5.89 ± 0.47*	3.2 ± 0.42*	20.5 ± 2.9*	0.52 ± 0.09*
3.1	0.78 ± 0.06*	4.08 ± 0.34*	5.2 ± 0.52*	32.4 ± 3.2*	1.22 ± 0.14*
3.2	0.74 ± 0.05*	5.17 ± 0.47*	6.0 ± 0.55*	36.1 ± 3.3*	1.11 ± 0.11*
3.3	0.70 ± 0.05*	5.20 ± 0.41*	5.7 ± 0.58*	35.2 ± 3.4*	1.21 ± 0.11*

$^{\#}$SOD unit: One unit is defined as the enzyme concentration required for inhibiting 50% of the OD produced by the chromogen at 560 nm in 1 min.; Values are mean ± SD for 6 rats in each group. Significantly differing values (from normal control group) are expressed as (*) p< 0.05

Table 3 : Effect of aqueous root extract of DG on TBARS, Catalase, Superoxide dismutase (SOD), Glutathione peroxidase (GPx) in the tissue homogenate of isolated rat heart

Group	TBARS (mM/100g wet tissue)	Catalase (µM of H_2O_2 consumed/min/mg protein)	SOD ($U^{\#}$/mg protein)		GPx (µg of GSH consumed/min/mg protein)
			Mn SOD	CuZn SOD	
1	0.60 ± 0.03	7.61 ± 0.54	8.2 ± 0.72	52.2 ± 4.2	1.85 ± 0.18
4.1	0.59 ± 0.04	7.85 ± 0.54	8.1 ± 0.73	51.6 ± 4.1	1.85 ± 0.17
4.2	0.58 ± 0.04	7.57 ± 0.53	8.1 ± 0.71	51.0 ± 4.1	1.80 ± 0.18
4.3	0.47 ± 0.03*	6.17 ± 0.55*	7.2 ± 0.65*	47.3 ± 4.0	1.57 ± 0.17*
4.4	0.44 ± 0.03*	5.86 ± 0.47*	7.4 ± 0.62	44.5 ± 3.8	1.51 ± 0.16*
4.5	0.58 ± 0.04	5.51 ± 0.45*	6.9 ± 0.62*	41.1 ± 3.6*	1.43 ± 0.16*
4.6	0.55 ± 0.05	5.23 ± 0.50*	6.8 ± 0.68*	35.6 ± 3.3*	1.39 ± 0.16*
4.7	0.59 ± 0.05	5.11 ± 0.48*	6.5 ± 0.62*	39.7 ± 3.1*	1.48 ± 0.15*
4.8	0.64 ± 0.06	5.02 ± 0.43*	6.1 ± 0.71*	34.1 ± 3.2*	1.40 ± 0.16*

$^{\#}$SOD unit: One unit is defined as the enzyme concentration required for inhibiting 50% of the OD produced by the chromogen at 560 nm in 1 min. ; Values are mean ± SD for 6 rats in each group. Significantly differing values (from normal control group) are expressed as (*) p< 0.05

Table 4 : Effect of methanolic extract of DG on ferrous sulphate induced lipid peroxidation in rat liver homogenate

Extract concentration	TBARS (nmol/mg protein) [a]	Inhibition (%)[a]
Control	2.41 ± 0.15	
1000	0.18 ± 0.01	92.53 ± 1.38
800	0.51 ± 0.07	78.83 ± 2.45
600	0.85 ± 0.17	64.73 ± 2.0
400	1.18 ± 0.11	51.03 ± 3.2
200	1.28 ± 0.27	46.88 ± 4.2
Tocopherol (10µmol/L)	0.06 ± 0.01	97.51 ± 4.3

"a " Mean ± SEM, 6 independent analysis

Table 5 : Free radical scavenging activity of DG extract

Extract concentration	Inhibition (%)			
	DPPH	Nitric oxide	Super-oxide	Hydroxyl radical
1000	96.11± 4.21	93.42± 3.54	97.58± 3.25	90.38± 4.62
500	93.36± 5.75	89.75± 5.61	94.46± 5.24	89.54± 5.67
250	89.53± 4.62	81.21± 3.35	86.42± 5.23	85.37± 5.73
125	85.23± 3.55	70.87± 4.72	73.99± 4.34	76.43± 3.61
62	57.86± 4.73	56.33± 5.46	69.38± 3.74	62.76± 4.83
32	44.35± 5.32	47.58± 4.61	56.57± 3.85	41.54± 3.63
16	15.63± 5.64	28.74± 3.27	45.65± 4.81	32.55± 2.73
10	3.69± 1.51	5.88± 1.63	33.63± 2.39	8.13± 1.88
7	1.02± 0.31	3.11± 0.61	17.04± 2.26	4.21± 1.55
5	0.35± 0.01	1.38± 0.21	5.35± 1.15	1.06± 0.23
Ascorbic acid (100□g)	95.02± 4.01	86.47± 4.21	88.65± 5.87	95.44±4.81
BHT (20□g)	92.63± 3.01	91.7±3.11	21± 1.16	50.5± 3.41
Curcumin IC_{50}	38.5±1.81	50± 3.24		

Values are mean ± S.E.M of 3 replicates. NT: Not tested.

DISCUSSION

The chemical composition of DG root extract was analyzed by employing GC-MS, leading to a comparison of the relative retention times and the mass spectra of the extract components with those of authentic samples and mass spectra from the data library. As shown in Table 1, GC/MS analysis resulted in the identification of 64 compounds.

DG extract caused a dose dependent protection against lipid peroxidation with 92.5% protection at 1mg/ml concentration. Initiation of lipid peroxidation by ferrous sulphate takes place through ferryl perferryl complex (23).

The effect of DG root extracts on inhibition of hydroxyl radical production was assessed by the iron (II)-dependent deoxyribose damage assay. Ferrous salts can react with hydrogen peroxide thus forming hydroxyl radical via Fenton's reaction (24). The iron required for this reaction is obtained either from the pool of iron or the heme containing proteins. The hydroxyl radical thus produced may attack the sugar of DNA bases, which leads to sugar fragmentation; base loss and DNA strand breakage. Addition of transition metal ions such as iron at low concentrations to deoxyribose causes degradation of the sugar into malondialdehyde and other related compounds which form a chromogen with thiobarbituric acid (TBA). Table 5 presents the effects of the examined DG root extracts on hydroxyl scavenging actvity. DG was capable of reducing hydroxyl radical production at all concentrations (IC_{50} = 50.5 µg/ml). Ascorbic acid, used as a standard was highly effective in inhibiting the oxidative DNA damage, showing an IC_{50} = 15 µg/ml.

The DPPH radical is considered to be a model of lipophilic radical. A chain reaction in lipophilic radicals was initiated by lipid autoxidation. The radical scavenging activity of DG was determined from the reduction in absorbance at 517 nm due to scavenging of stable DPPH free radical. The dose dependent inhibition of DPPH radical indicated that DG causes reduction of DPPH radical in a stoichometric manner. A 125 µg/ml of DG and BHT (20µg) exhibited 85.23 and 92.63% inhibition, respectively and the IC_{50} values were found to be 38.5µg and 15 µg/ml for DG and BHT respectively.

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological processes. Some of the physiological effects may be mediated through the intermediate formation of S-nitroso-cysteine or S-nitroso-glutathione (25). In the present study, the extract competes with oxygen to react with nitric oxide and thus inhibits the generation of the anions. Curcumin was used as a reference compound. The concentration of DG needed for 50% inhibition was found to be 50 µg/ml whereas for curcumin, it was found to be 32.5 µg/ml. A novel bisabolene epoxide present in the DG extract is known to inhibit nitric oxide synthesis in lipopolyaccharide activated macrophages (26).

It was apparent that superoxide was not itself very reactive, at least compared to the rates of many free-radical reactions, which often occur immediately on collision of the reactive species (diffusion- controlled). Superoxide might thus be viewed as like a pro-drug, not itself directly damaging but leading to the production of a species much more reactive. The emergence of nitric oxide on the physiological scene has added a new twist to the pivotal role superoxide now seems to occupy in diverse biological processes. In the present study, super oxide radicals reduce NBT to a blue colored formazan that is measured at 560nm. The effect of DG in this regard is shown in Table 5. The probable mechanism of scavenging the super-oxide anion may be due to the inhibitory effect of DG towards generation of super-oxides in the in-vitro reaction mixture.

The free radical scavenging effects of the examined extracts in different antioxidant model could be mainly due to the presence of compounds like eugenol, 3-pyrroline derivatives (27), maltol (28), piperine (29) (Table 1) and other secondary biomolecules present in the extracts. Asarone, one of the constituent of DG extract (around 2%) is known to have hypolipidemic and antithrombotic activity in-vivo (30).

Myocardial damage induced by ischemia-reperfusion is due, at least in part, to the generation of ROS. Table 2 shows the concentration of TBARS in the myocardium of ischemic and reperfused rat heart. Inadequate perfusion of a tissue/organ leads to oxygen (O_2) and adenosine triphosphate (ATP) depletion, and the accumulation of toxic metabolites. Another effect of hypoperfusion is the conversion of xanthine dehydrogenase to xanthine oxidase, which upon reperfusion, catalyzes the conversion of hypoxanthine to xanthine with the concomitant production of ROS. ROS also increase in concentration upon reperfusion of the ischemic myocardium. However in the present study, the oxidative stress induced by ischemic reperfusion was found to decrease upon DG pretreatment. Moreover, the protective nature of the extract can be explained by their constituents such as eugenol, thymol, asarone and cactine. Report suggested that eugenol can induces dose-dependent hypotension and bradycardia(31). The calcium-sensitizer effect of thymol suggest that it can induce negative ionotropic effect to myocardium. The alkaloid cactine is reported to have a digitalis-like activity on the heart. It frequently gives prompt relief in functional heart disease. It is also used in the treatment of palpitations and angina pectoris. Experimental evidences suggested that one of the causative factors for ischemic reperfusion injury is calcium overload in the myocytes (32). Our GC Mass spectrum of DG showed the presence of caryophyllene oxide and it is reported to have relaxant effect on smooth muscle and can also block calcium channels of the heart (33).

In conclusion, this study shows the presence of phenolic compounds and biologically active alkaloids in methanolic extract of DG can render antioxidant property. Different in-vitro antioxidant models also suggested the free radical scavenging potential possessed by methanolic extract of DG. Moreover the in-vivo ischemic reperfusion rat model re-emphasizes the antioxidant capacity exhibited by methanolic extract of DG roots.

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