ORIGINAL RESEARCH ARTICLE

Antihypertensive, antihyperlipidemicand antioxidantinfluence of D-carvone in L-NAME induced hypertensive rats

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ABSTRACT
This study was designed to evaluate the antihypertensive, antihyperlipidemic and antioxidant effect of D-carvone, a monoterpene against N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) induced hypertension.Hypertension was prompted in adult male albino rats of the Wistar strain, weighing 180–220 g, by oral administration of the L-NAME (40 mg/kg/ body weight/day) in drinking water for 4 weeks. Rats were treated with D-carvone (5, 10 and 20 mg/kg body weight) for four weeks. L-NAME treated rats showed significant increase in diastolic blood pressure (DBP) and significant decrease in body weight. A significant increase in the total cholesterol (TC), triglycerides (TG), levels of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH),and significant decrease in the levels of non-enzymatic antioxidants such as vitamin C, vitamin E and reduced glutathione (GSH), in plasma were observed in L-NAME induced hypertensive rats. Treatment with D-carvone (5, 10 and 20 mg/kg bw) brings back all the above parameters to near normal level, in which 20 mg/kg showed the highest effect than that of other two doses. These results suggest that D-carvone acts as an antihypertensive, antihyperlipidemic and antioxidantagent against L-NAME induced hypertension.

Key words: Hypertension, Hyperlipidemia, Antioxidant, D-carvone.

1. INTRODUCTION
Hypertension is a major risk factor for cardiovascular diseases and a large body of evidence suggests oxidative stress, an increase in the production of reactive oxygen species (ROS), as a strong underlying factor in hypertension [1,2]. Hypertension affects approximately 25% of the adult population worldwide, and its prevalence is predicted to increase by 60% by 2025 [3].In humans, hypertension and hyperlipidemia are frequent causes of CVD and major risk factors for atherosclerosis; the presence of both conditions accelerates atherosclerosis [4].Oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases[5].Nitric oxide (NO) synthesis and release by endothelial cells play an important vascular relaxation effect [6], contributing to the modulation of vascular tone. In addition, NO has been identified as important in other cellular events, such as vascular smooth muscle cell proliferation [7]. Chronic nitric oxide inhibition with L-NAME (N^{ω}-nitro-L-arginine methyl ester) can increase regional vascular resistance, raise the blood pressure,

oxidative stress, and renal damage in both *in vitro* and *in vivo* models [8].

The development of a safe and effective way to manage hypertension has challenged medical researchers for centuries. In recent times, focus on plant research has increased all over the world and a large body of evidence was collected to show immense potential of medicinal plants used in various traditional systems. A wide variety of the traditional herbal remedies are used by hypertensive patients, especially in the third world countries and may therefore represent new avenues in the search for alternative antihypertensive drugs [9]. The interest on bioactive compounds from herbal plants has increased in recent years due to their health benefits, particularly protection against a variety of ailments such as cardiovascular diseases and cancer [10].

Monoterpenes are primary compounds of plant essential oils and the effects of many medicinal herbs have been attributed to them. D-carvone (**Fig 1**) is a monoterpene, present in the essential

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oils of many medicinal and aromatic plants (Caraway, dill and spearmint) that are endowed with many biological activities including antioxidant, antimicrobial, fungicidal and insecticidal [11]and anticancer properties [12].Extensive literature survey has shown that no sufficient work has been done to study its antihypertensive effect. Therefore, the present study was designed to determine the antihypertensive, antihyperlipidemic and antioxidant effect of D-carvone in L-NAME induced hypertensive rats.
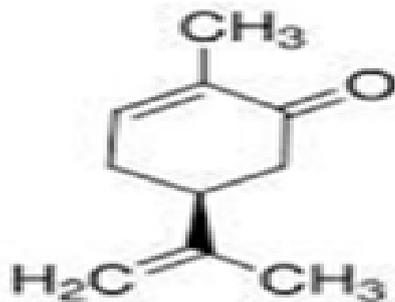

Fig 1: Structure of D-carvone

2. MATERIALS AND METHODS

2.1. Experimental Animals
Male albino (9 weeks-old) rats of Wistar strain with a body weight ranging from 180 to 220g were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and they were housed (3 rats/cage) in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h under a 12:12 h light/dark cycle at around 22 °C. Food and water were provided ad libitum to all the animals. The rats were fed on a standard pellet diet (Kamadhenu Agencies, Bangalore, India). The whole experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA, approval number: 1082) Annamalai University, Annamalainagar.

2.2. Chemicals and Drug
D-carvone and N$^{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals used in this study were of analytical grade obtained from Merck and Himedia, India.

2.3. L-NAME induced hypertensive model and experimental time line
Animals were given L-NAME in drinking water at a dosage of 40 mg/kg for 4 weeks [13]. Each of the following groups consisted of six animals. D-carvone was suspended in 1% DMSO and administered orally everyday using an intragastric tube throughout the experimental period [12].

Group I	-	Control
Group II	-	Control+D-carvone 20 mg/kg body weight (bw) of animals
Group III	-	Control animals received L-NAME-hypertensive control
Group IV	-	L-NAME+D-carvone (5 mg/kg bw)
Group V	-	L-NAME+D-carvone (10 mg/kg bw)
Group VI	-	L-NAME+D-carvone (20 mg/kg bw)

The experimental duration was 30 days. During the experimental period, body weight gain was measured every day. On 31st day, the rats were sacrificed by cervical dislocation. Blood was collected through orbital sinus in a heparinized tube and centrifuged at 1000 × g for 10 min and the plasma was separated by aspiration.

2.4. Measurement of blood pressure by non-invasive method
Before commencement of the experiment, animals were trained with instrument for measuring blood pressure. In all groups of animals, diastolic blood pressure was recorded every week during the entire period of the study by tail-cuff method (IITC, model 31, Woodland Hills, CA, USA). The animals were placed in heated chamber at an ambient temperature of 30-34°C for 15min and from each animal; 1-9 blood pressure values were recorded. The lowest three readings averaged to obtain a mean blood pressure. All the recordings and data analyses were done using a computerized data acquisition system and software (IITC Inc. /Life Science Instruments, USA).

2.5. Extraction of lipids
Total lipids were extracted from plasma according to the method of Folch *et al.*[14] using chloroform:methanol mixture (2:1, v/v). Plasma was mixed with cold chloroform–methanol (2:1, v/v) and the contents were extracted after 24 hours. The extraction was repeated four times. The combined filtrate was washed with 0.7% of potassium chloride (0.1 N) and the aqueous layer was discarded. The organic layer was made up to

a known volume with chloroform and used for the analysis of lipids.

2.6. Estimation of total cholesterol and triglycerides

The levels of total cholesterol (TC) were estimated by the method of Zlatkis et al.[15]. Lipid extract of 0.5 mL was evaporated to dryness. To this, 5.0 mL of ferric chloride - acetic acid reagent was added. The tubes were mixed well and 3.0 mL of concentrated sulphuric acid (H_2SO_4) was added. A series of standards containing cholesterol in the range 3–15μg were made up to 5.0 mL with the reagent and a blank containing 5.0 mL of the reagent was prepared. The absorbance was read after 20 minutes at 560 nm.

The content of triglycerides (TG) was estimated by the method of Fossati and Lorenzo [16]. Lipid extract of 0.5 mL was evaporated to dryness. To this, 0.1 mL of methanol was added followed by 4.0 mL of isopropanol. About 0.4 g of alumina was added to all the tubes and shaken well for 15 minutes. It was centrifuged and then accurately 2.0 mL of the supernatant was transferred to appropriately labeled tubes. The tubes were placed in a water bath at 65°C for 60 minutes for saponification after adding 0.6 mL of the saponification reagent followed by 0.1 mL of sodium metaperiodate and 0.5 mL of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for an hour. A series of standards of concentration 8-40 μg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 405 nm.

2.7. Estimation of lipid peroxidation products

The levels of thiobarbituric acid reactive substances (TBARS) in plasma were estimated by the method of Niehaus and Samuelson [17]. A total of 0.5 mL of plasma was diluted with 0.5 mL of double distilled water and mixed well, and then 2.0 mL of thiobarbituric acid (TBA) – trichloroacetic acid (TCA) - hydrochloric acid (HCL) reagent was added. The mixture was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged for 10 min and the supernatant was taken for measurement. The absorbance was read at 535nm against reagent blank.

Estimation of plasma lipid hydroperoxides (LOOH) was done by the method of Jiang et al.[18]. Fox reagent (0.9 mL) was mixed with 0.1 mL of plasma and incubated for 30 min at room temperature. The color developed was read at 560 nm.

2.8. Determination of non-enzymatic antioxidants

Vitamin C in the plasma was estimated by the method of Roe and Kuether [19]. To 0.5 mL of plasma, 1.5 mL of 6% TCA was added and allowed to stand for 5 min and centrifuged. To the supernatant, 0.3 g of acid washed Norit was added, shaken vigorously and filtered. A total of 0.5 mL of the filtrate was taken and 0.5 mL of dinitrophenylhydrazine (DNPH) was added, stoppered and placed in water bath at 37 °C for exactly 3 h, removed, placed in ice-cold water and added 2.5 mL of 85% sulphuric acid. The contents of the tubes were mixed well and allowed to stand at room temperature for 30 min. The color developed was read at 540 nm.

The level of Vitamin E in the plasma was estimated by the method of Baker et al [20]. To 0.5 mL of plasma, 1.5 mL of ethanol was added, mixed and centrifuged. The supernatant was evaporated at 80 °C and to the precipitate, 3.0 mL of petroleum ether, 0.2 mL of 2, 2pyridyl solution and 0.2 mL of ferric chloride were added. Afterwards, all the tubes were mixed well and kept in dark for 5 min and 4.0 mL of n-butanol was added. The red color developed was read at 520 nm.

Reduced glutathione (GSH) in the plasma was estimated by the method of Ellman [21]. 0.5 mL of plasma was pipetted out and precipitated with 2.0 mL of 5% TCA. A total of 2.0 mL of supernatant was taken after centrifugation and 1.0 mL of Ellman's reagent and 4.0 mL of 0.3M disodium hydrogen phosphate were added. The yellow color developed was read at 412 nm.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using statistical package for the social science (SPSS) software version 14.0. Results were expressed as mean ± S.D. for six rats in each group. A value of $P < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Blood pressure measurements

(**Fig 2**) shows the effect of D-carvone at three different doses (5, 10 and 20 mg/kg) on diastolic blood pressure in L-NAME induced hypertensive rats. The L-NAME rats showed significantly

increased diastolic blood pressure while treatment with D-carvone significantly reduced the diastolic blood pressure.

3.2. Body weight

(**Fig 3**) shows the effect of D-carvone at three different doses (5, 10 and 20 mg/kg) on body weight in L-NAME induced hypertensive rats. The L-NAME rats showed significantly decreased body weight while treatment with D-carvone significantly elevated the body weight. The 20 mg/kg dose showed better effect in reducing diastolic blood pressure and enhancing body weight than other two doses (5 and 10 mg/kg), so we have chosen 20 mg/kg dosage for further evaluation.

3.3. Plasma lipid level

(**Table 1**) shows the concentrations of plasma lipids (TC and TG) were increased in hypertensive rats as compared to the control rats. Treatment with D-carvone significantly reduced the concentrations of plasma lipids (TC and TG respectively).

3.4. Lipid peroxidation products

(**Table 2**) shows the effect of D-carvone on the levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxide (LOOH) in the plasma of L- NAME induced hypertensive rats. The L-NAME rats exhibited a significant increase in the levels of TBARS and LOOH. The administration of D-carvone reduced the levels of TBARS and LOOH significantly.

3.5. Non-enzymatic antioxidants

(**Table 3**) shows the effect of D-carvone on vitamin C, E and reduced glutathione levels in the plasma of control and L-NAME induced hypertensive rats. The levels of vitamin-C and E and reduced glutathione decreased significantly in L-NAME rats, and administration of D-carvone significantly increased these non-enzymatic antioxidants.

Table 1: Effect of D-carvone on total cholesterol and triglycerides in plasma of control and L-NAME induced hypertensive rats

Parameters		Groups			
		I	II	III	IV
Total cholesterol	Plasma (mg/dL)	$83.11 \pm 6.41^*$	$83.76 \pm 5.83^*$	$110.06 \pm 8.24^{\#}$	$95.13 \pm 5.43^{\$}$
Triglycerides	Plasma (mg/dL)	$57.17 \pm 3.87^*$	$56.11 \pm 3.97^*$	$81.16 \pm 6.33^{\#}$	$68.66 \pm 4.65^{\$}$

Group I - Control; Group II - Control + D-Carvone (20 mg/kg); Group III – L-NAME control (40mg/kg); Group IV – L-NAME + D-Carvone (20 mg/kg).Values are mean ± S.D. for six rats in each group. Values not sharing a common symbol differ significantly at P <0.05 (DMRT).

Table 2: Effect of D-carvone on TBARS and LOOH in plasma of control and L-NAME induced hypertensive rats

Parameters		Groups			
		I	II	III	IV
Thiobarbituric acid reactive substances	Plasma (mmol/dL)	$0.14 \pm 0.01^*$	$0.13 \pm 0.01^*$	$0.44 \pm 0.04^{\#}$	$0.22 \pm 0.02^{\$}$
Lipid hydroperoxides	Plasma (mmol/dL)	$8.89 \pm 0.35^*$	$8.95 \pm 0.53^*$	$22.17 \pm 1.85^{\#}$	$10.28 \pm 0.86^{\$}$

Group I - Control; Group II - Control + D-Carvone (20 mg/kg); Group III – L-NAME control (40mg/kg); Group IV – L-NAME + D-Carvone (20 mg/kg).Values are mean ± S.D. for six rats in each group. Values not sharing a common symbol differ significantly at P <0.05 (DMRT).

Table 3: Effect of D-carvone on vitamin C, vitamin E and GSH in plasma of control and L-NAME induced hypertensive rats

Parameters		Groups			
		I	II	III	IV
Vitamin-C	Plasma (mmol/dL)	$3.12 \pm 0.16^*$	$3.23 \pm 0.13^*$	$0.98 \pm 0.04^{\#}$	$2.56 \pm 0.11^{\$}$
Vitamin-E	Plasma (mmol/dL)	$2.44 \pm 0.15^*$	$2.65 \pm 0.1^*$	$0.94 \pm 0.08^{\#}$	$2.28 \pm 0.13^{\$}$
GSH	Plasma (mmol/dL)	$35.11 \pm 1.7^*$	$36.5 \pm 1.9^*$	$19.4 \pm 1.18^{\#}$	$31.28 \pm 1.80^{\$}$

Group I - Control; Group II - Control + D-Carvone (20 mg/kg); Group III – L-NAME control (40mg/kg); Group IV – L-NAME + D-Carvone (20 mg/kg).Values are mean ± S.D. for six rats in each group. Values not sharing a common symbol differ significantly at P <0.05 (DMRT).
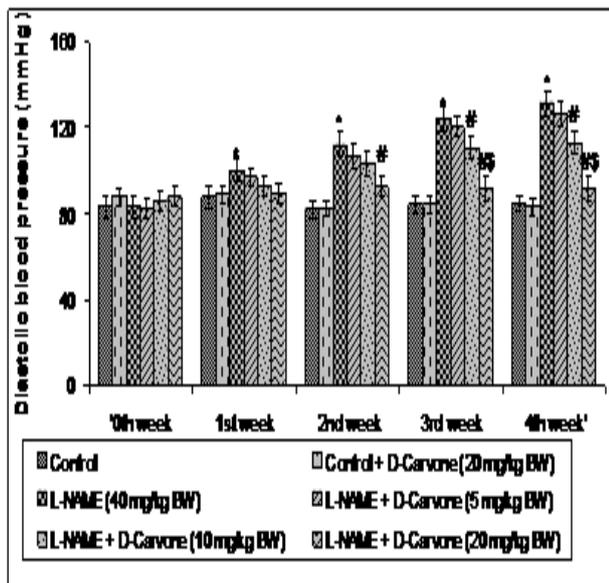
Fig 2: Effect of D-carvone on diastolic blood pressure in control and L-NAME induced hypertensive rats.

Values are mean ± S.D. for six rats in each group. * differs significantly at P <0.05 compared with control. # differssignificantly at P <0.05 compared with L-NAME rats. $ differs significantly at P <0.05 compared with 10 mg/kg D-carvonetreated rats (DMRT).
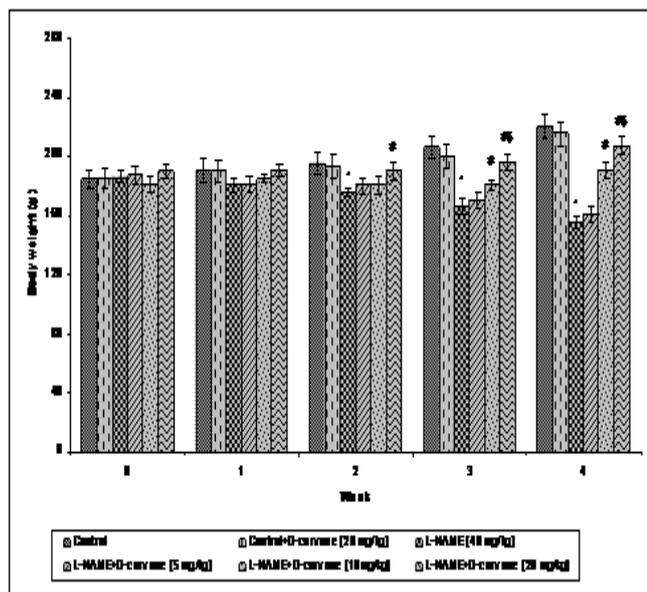

Fig 3: Effect of D-carvone on body weight in control and L-NAME induced hypertensive rats.

Values are mean ± S.D. for six rats in each group. * differs significantly at P <0.05 compared with control. # differs significantly at P <0.05 compared with L -NAME rats. $ differs significantly at P <0.05 compared with 10 mg/kg D-carvone treated rats (DMRT).

4. DISCUSSION

The present study was performed to evaluate the antihypertensive, antihyperlipidemic and antioxidant effect of D-carvone, a monoterpene against N�-nitro-L-arginine methyl ester hydrochloride (L-NAME) induced hypertension.

During the experimental period of 4 weeks, no adverse effects were observed in the D-carvone alone supplemented rats suggesting that D-carvone is well tolerated.

The results show for the first time that D-carvone betters L-NAME induced hypertension by reducing blood pressure, oxidative stress and dyslipidemia. Chronic inhibition of NO produces volume-dependent elevation of blood pressure; and its physiological and pathological characteristics resemble essential hypertension [22]. Besides, it is well established that acute inhibition of NO biosynthesis by in vivo administration of L-NAME, an L-arginine analog, leads to arterial hypertension and vasoconstriction [22]. In this study, diastolic blood pressure of L-NAME rats was increased significantly with respect to weeks, whereas D-carvone treatment at 20 mg/kg effectively prevents elevated diastolic blood pressure than other two doses. L- NAME rats showed significantly decreased body weight. After treatment with D-carvone, the weight loss improved which might be as a result of its ability to reduce the loss or degradation of structural proteins [23].It clearly indicate its ability to protect blood pressure and body weight from free radicals due to its antioxidant potential [11].

Abnormalities in plasma lipids and lipoprotein metabolism play a central role in the pathogenesis of hypertension. The presence of high BP and hyperlipidemia is so common in hypertension that many have argued that the high BP itself may play a role in altering lipid metabolism, resulting in abnormalities [24].A number of publications support the concept that a low-dose supplementation of terpene is sufficient to produce significantly lower plasma cholesterol concentrations [25]. The effect of terpenes on reducing plasma cholesterol levels has been recognized for many years [26].High levels of circulating cholesterol and its accumulation in tissues are well associated with cardiovascular damage [27]. In our study, we observed increased levels of TC in plasma of hypertensive rats. D-carvone supplementation decreased the levels of TC in hypertensive rats. Accumulation of TGs is one of the risk factors of CVD. The mechanism of observed increase in TGs after hypertension may be due to elevated flux of fatty acids and impaired removal of VLDL from the plasma. Treatment with

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D-carvone decreased the levels of TGs in hypertensive rats.

Lipid peroxidation is an important pathogenic event in hypertension, and accumulation of lipid hydroperoxides reflects the various stages of diseases and its complications [28]. Previous studies in our laboratory show that, the levels of lipid peroxidation products were elevated in L-NAME-induced hypertensive rats [29]. Our results showed that the lipid peroxidation end products, measured as TBARS and LOOH were increased in plasma of L-NAME induced hypertensive rats. Thus, the antihypertensive effect of D-carvone may be due to decreased oxidative stress. From these evidences, we come to a decision that the protective action of D-carvone would be at least partially due to its antioxidant potential.

The second line of defense consists of non-enzymatic antioxidants namely; vitaminC, vitamin E, and reduced glutathione which scavenge the residual free radicals escaping from decomposition by the antioxidant enzymes [30].The non-enzymatic antioxidants scavenge the residual free radicals escaping from decomposition enzymes [31]. The major antioxidant of the aqueous phase is vitamin C, which acts as the first line of defense during oxidative stress. Vitamin E appears to be the most effective lipid soluble antioxidant in the biological system. Glutathione plays a marked role in detoxification reaction because it is a direct radical scavenger [32].The lowered concentrations of vitamin C, vitamin E, and GSH observed in L-NAME induced hypertensive rats. Treatment with D-carvone significantly elevated the level of this non-enzymatic antioxidant suggests that this compound might be potentially useful in counteracting free radical mediated oxidative stress caused by lipid peroxidation.

In conclusion, our results demonstrated that D-carvone at a dose of 20 mg/kg exhibited a greater antihypertensive, antioxidant and antihyperlipidemic effect than the other two doses(5 mg and 10 mg/kg) as evidenced by a considerable decrease in the blood pressure, oxidative stress, lipid levels and improve antioxidant status in L-NAME induced hypertensive rats.Our study also showed that the monoterpene compound, D-carvone was better effect in hypertensive rats. Further studies are needed to find out the exact mechanism of action of D-carvone.

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