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Antioxidant and Free radical Scavenging Effect of D-carvone in Hypertensive Rats. *In Vivo* and *In Vitro* study

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ABSTRACT

This study was planned to assess the antioxidant and free radical scavenging effect of D-carvone against L-NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) induced hypertension. Hypertension was encouraged in adult male albino rats of the Wistar strain, considering 180–230 g, by oral administration of the L-NAME (40 mg/kg/ body weight/day) in drinking water for 4 weeks. Rats were cured with D-carvone (5, 10 and 20 mg/kg body weight) for four weeks. A significant reduction in the levels of non-enzymatic antioxidants such as vitamin C, vitamin E and reduced glutathione (GSH), in plasma were perceived in L- NAME induced hypertensive rats. Moreover, *in vitro* free radical scavenging activity of $ABTS^{+}$ and $DPPH^{\bullet}$ radical scavenging possible of D-carvone was also quantified. Treatment with D-carvone (5, 10 and 20 mg/kg bw) carries back all the above parameters to near usual level, in which 20 mg/kg displayed the highest effect than that of other two doses. Further, D-carvone displays concentration dependent antioxidant potential. These results suggest that D-carvone acts as an antioxidant and free radical scavenging agent against L-NAME induced hypertension.

Keywords: D-carvone; Free radicals; Antioxidant; Hypertension

1. INTRODUCTION

Hypertension is a main risk factor for cardiovascular diseases and a huge body of proof proposes oxidative stress, and rise in the production of reactive oxygen species (ROS), as a strong fundamental factor in hypertension (Ceriello, 2008; Cutler et al., 2008). Hypertension disturbs approximately 25% of the adult population worldwide, and its occurrence is predicted to increase by 60% by 2025 (Kearney et al., 2005). Oxidative stress plays a vital role in the pathogenesis and development of cardiovascular diseases (Higashi et al., 2009). 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 (Harrison, 1997).

Oxidation and production of free radicals are an integral part of normal cell metabolism. A free radical is defined as any atom or molecule possessing unpaired electrons. Free radicals such as O_2^{-} (superoxide anion), ^{-}OH (hydroxyl radical) and $^{1}O_2$ (singlet oxygen) are formed as a part of the normal metabolic process. Free radicals can cause oxidative damage to lipids,

proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans (Olayinka et al., 2010). Thus, free radicals result in the consumption of antioxidant defenses which may lead to disruption of cellular functions and oxidative damage to membranes and enhance susceptibility to lipid peroxidation (Valko et al., 2006).

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 (Mukherjee, 2001).

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 (Gorinstein et al., 2005). D-carvone (Fig. 1) is a monoterpene, present in the essential oils of many medicinal and aromatic plants (Caraway, dill and spearmint) that are endowed with many biological activities including antioxidant, antimicrobial, fungicidal and insecticidal (Johri, 2011) and anticancer properties (Vinothkumar et al., 2013). Therefore, the present study was designed to determine the antioxidant and free radical scavenging effect of D-carvone in L-NAME induced hypertensive rats.

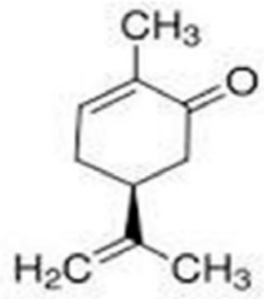

Fig. 1. Structure of D-carvone.

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 230g 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^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-dipyridyl 2,4-dinitro phenyl hydrazine (DNPH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and butylated hydroxytoulene (BHT) 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 (Saravanakumar and Raja, 2011). 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 (Vinothkumar et al., 2013).

Group I - Control
Group II - Control+D-carvone 20 mg/kg body weight
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. 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. Determination of non-enzymatic antioxidants

Vitamin C in the plasma was estimated by the method of Roe and Kuether (1943). 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. (1980). 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, 2 ′ dipyridyl 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 (1959). 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.

2.5. *In Vitro* free radical scavenging assay

The ability to scavenge the stable free radical (O_2^-), total antioxidant potential was determined by the $ABTS^+$ assay, as described by Miller et al. (1996) and $DPPH^\bullet$ was determined by the method Mensor et al. (2001). Concentration dependent free radical scavenging potential of D-carvone was performed (20, 40, 60, 80, 100 µM concentration). The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. All the tests were performed for three times and the graph was plotted with the average of three observations.

2.6. Statistical analysis

Data were scrutinized 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

3.1. Non-enzymatic antioxidants

Table 1 displays 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 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).

3.2. *In Vitro* antioxidant activity of D-carvone

Figure 2 shows the free radical scavenging efficiency of D-carvone. In this study, the antioxidant potential of D-carvone was examined *in vitro* by $DPPH^{\bullet}$ and $ABTS^{+}$ scavenging effect in different concentrations (20, 40, 60, 80 and 100 µM). D-carvone scavenged the $DPPH^{\bullet}$ and $ABTS^{+}$ radicals in a concentration dependent manner.

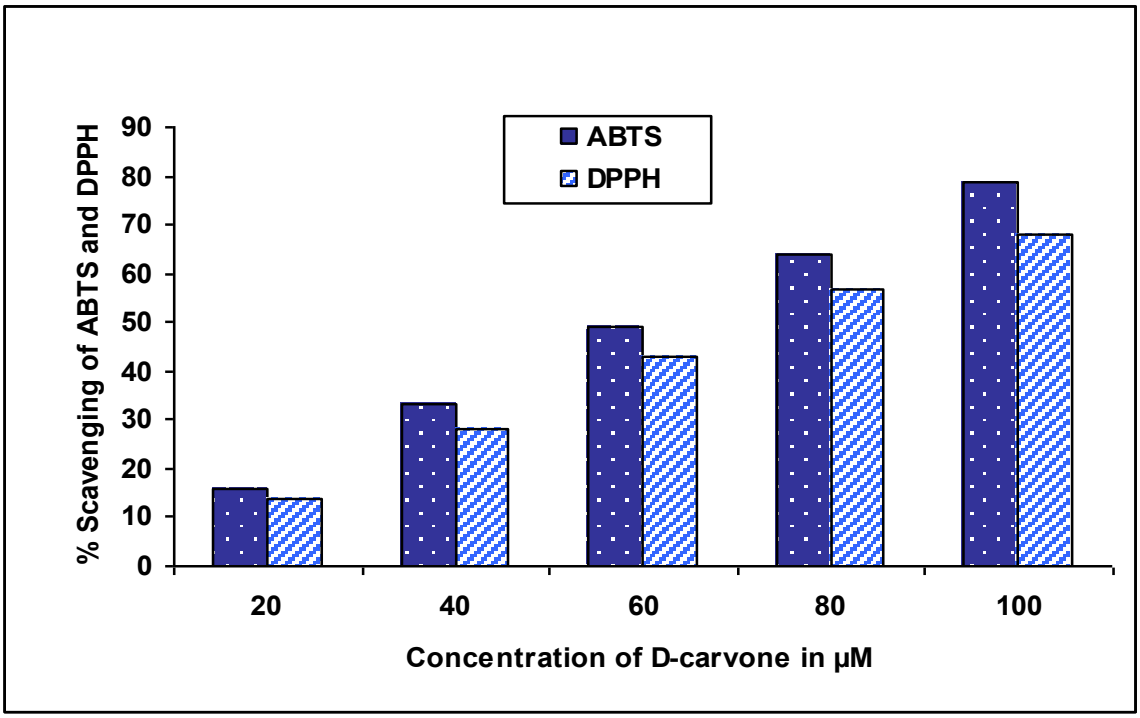

Fig. 2. Antioxidant assay (*In Vitro).* Percentage scavenging activity of D-carvone on $DPPH^{\bullet}$ and $ABTS^{+}$ (Average of triplicate experiments).

4. DISCUSSION

The current study was implemented to assess the antioxidant and free radical scavenging effect of D-carvone against 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 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 (Mahdi, 2002). The non-enzymatic antioxidants scavenge the residual free radicals escaping from decomposition enzymes (Roy et al., 1994). 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 (Kitts et al., 1998). 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.

The preliminary *in vitro* study of antioxidant effect of D-carvone on $DPPH^{\bullet}$ and $ABTS^{+}$ indicates that D-carvone showed concentration-dependent antioxidant potential. Oxidative stress, originally described as an altered balance between the production of free radicals and antioxidant defenses, is an important phenomenon in different physiological and pathological processes (Chang and Wu, 2006). From this evidence, the scavenging effect of D-carvone during oxidative explode might play a probable protective role in preventing the oxidative stress. To know the mechanism of action, the $DPPH^{\bullet}$ radical scavenging and total antioxidant ($ABTS^{+}$) activity of D-carvone was investigated *in vitro*. Thus, as an antioxidant, D-carvone has the potential to scavenge free radicals and increases the functions of non-enzymatic antioxidant network.

In conclusion, our results demonstrated that D-carvone was found to be a free radical scavenging ability in different *in vitro* assays and D-carvone at a dose of 20 mg/kg exhibited a greater antioxidant effect than the other two doses (5 mg and 10 mg/kg) as evidenced by a 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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