

## RESEARCH ARTICLE

### INHIBITION OF NITRIC OXIDE PRODUCTION IN LPS-STIMULATED RAW 264.7 CELLS BY *DOLICHANDRONE ATROVIRENS* BARK

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#### ABSTRACT

In traditional systems of medicine, the bark of *Dolichandroneatrovirens* has been used to treat various disorders. The main aim of this study was to determine the anti-inflammatory effect of *D. atrovirens* bark through the inhibition of nitric oxide (NO) production in lipopolysaccharide-stimulated RAW 264.7 cells. For this purpose, preliminary phytochemical composition and *in vitro* antioxidant activities of various solvent extracts of *D. atrovirens* bark were evaluated to select the most effective extract. The methanol extract of *D. atrovirens* registered the highest amount of total phenolics (476.2 mg GAE/g) and flavonoid (129.0 mg RE/g) contents with a strong antioxidant activity as measured in DPPH (IC<sub>50</sub> of 19.52 µg/mL) and ABTS (IC<sub>50</sub> of 10.82 µg/mL) scavenging activities. Hence, the methanol extract was selected for cell line study. Further, the methanol extract of *D. atrovirens* effectively inhibited the production of NO in RAW 264.7 cells induced by LPS (13.1 µM at the concentration of 80 µg/mL). It could be concluded that the presence of higher level of total phenolic components in the methanol extract of *D. atrovirens* bark might be responsible for reducing the NO level in cells.

**Keywords:** Antioxidant, *D. atrovirens*, Nitric oxide, lipopolysaccharide, RAW 264.7 cells.

#### 1. INTRODUCTION

In the last few decades, numerous studies have been documented for the utilization of natural antioxidants as potential disease preventing agents to reduce the risk of cardiovascular diseases, neurodegenerative diseases, inflammations, diabetes and cancers (1,2). The protective effects of the plants are mostly related to the antioxidant components such as phenolics, carotenoids, phytates, isothiocyanates, phytosterols, phytoesterogens and organosulfur (3). Hence, the search continues for the novel and effective antioxidants from the plant source to reduce the risk of free radical mediated disorders. Inflammation is the normal response of a living tissue to injury caused by physical or noxious chemical stimuli or microbiological toxins. Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing many pro-inflammatory molecules such as nitric oxide (NO), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and of cytokines, like interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (4).

Among these, NO is an important molecule for host defense response against various pathogens such as bacteria, viruses, fungi, and parasites (5). Under normal physiological conditions, NO plays an important role in the regulation of various pathophysiological processes such as neuronal communication, vasodilatation,

and neurotoxicity (6). However, overproduction of NO has been concerned in the development of various inflammatory diseases, such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and atherosclerosis (7). Accordingly, the regulation of these pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated macrophage cell line is an effective therapeutic strategy for the development of novel anti-inflammatory agents.

Plants are important source of therapeutic drugs and play a significant role in the survival of the tribal and ethnic communities. Plants products are known to possess a variety of secondary metabolites which have various biological activities (8). A number of Indian plants have been investigated for their beneficial use as antioxidants using presently available experimental techniques. Recently, several experimental studies have contributed scientific evidence for the pharmacological effects of various medicinal plants observed in folk medicine. During the ethnobotanical interview, the traditional healers of Melur of Bodha hills are using the bark of *Dolichandroneatrovirens* (Roth) Sprague (Family: Bignoniaceae) to successfully treat inflammations. But there is no information available on pharmacological evaluation of *D. atrovirens*. Hence, the stem bark of *D. atrovirens* was rightly chosen in the present study for understanding its anti-inflammatory effect. Based on the above knowledge, the present investigation was

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undertaken to evaluate the antioxidant and anti-inflammatory (through the inhibition of NO production in RAW 264.7 cells activated with lipopolysaccharide) of *D. atrovirens* bark.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of extracts

The bark sample of *D. atrovirens* was collected from Bodha hills, Melur, Southern Eastern Ghats, Tamil Nadu, India. The freshly collected bark sample was washed thoroughly in tap water, shade dried at room temperature (25°C), powdered, and used for solvent extraction. The plant material was successively extracted with n-hexane, chloroform, ethyl acetate and methanol using Soxhlet apparatus and the air dried residues were further extracted with hot water by the method of maceration for 24 h. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. The solvents were evaporated using a rotary vacuum-evaporator and air dried. The extract recovery in different solvents was expressed as percent of the plant sample dry matter.

### 2.2. Determination of total phenolic, tannin, and flavonoid contents

The total phenolic contents of the bark were determined by Folin-Ciocalteu method. The amount of total phenolics was calculated as gallic acid equivalents (GAE) as described by Siddhuraju and Becker (9). The total flavonoid content was determined by the method described previously by Zhishen et al. (10) and expressed as gram of rutin equivalent (RE)/100 g of extract.

### 2.3. In vitro antioxidant activity

#### 2.3.1. Antioxidant activity by ABTS and DPPH assays

The total antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method of Re et al. (11). The DPPH radical scavenging activity of different extracts of *D. atrovirens* bark was measured according to the method of Blois (12). IC<sub>50</sub> values of the extract i.e., concentration of the extract necessary to decrease the initial concentration of DPPH or ABTS by 50% was calculated.

### 2.4. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells

Based on the results of antioxidant and cytotoxicity studies, methanol extract of bark was selected for further studies.

### 2.4.1. Cell culture

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 µg/L streptomycin, and 100 IU/mL penicillin at 37°C in a 5% CO<sub>2</sub> atmosphere (HERAcell 150, Thermo Electron Corp. Waltham, MA, USA).

### 2.4.2. Cell viability assay

The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability. Briefly, RAW 264.7 cells were seeded in 96-well plates at the density of 5 × 10<sup>4</sup> cells/well. After 24 h of incubation, the adhered cells were treated with various concentrations of the extracts. Twenty four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h at 37°C and 5% CO<sub>2</sub>. The medium was then removed and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 550 nm on a microplate reader (Biotek, Winooski, VT, USA).

### 2.4.3. Inhibition of nitric oxide production

The RAW 264.7 cells were seeded at a density of 5 × 10<sup>5</sup> cells/well in 24 well plates and incubated for 12 h at 37°C and 5% CO<sub>2</sub>. Then media of each well were aspirated and fresh FBS-free DMEM media were replaced. Different concentrations of *D. atrovirens* extract were prepared in FBS-free DMEM to give a total volume of 500 µL in each well of a microtiter plate. After 1 h treatment, cells were stimulated with 1 µg/mL of LPS for 24 h (13).

The presence of nitrite was determined in cell culture media using commercial nitric oxide detection kit. Protocols supplied with assay kit used for the application of assay procedure. Briefly, 100 µL of cell culture medium with an equal volume of Griess reagent in a 96-well plate was incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of nitrite in the media was calculated from sodium nitrite (NaNO<sub>2</sub>) standard curve.

### 2.5. Statistical analysis

The values expressed are means of three replicate determinations ± standard deviation. The statistical analysis was carried out by analysis of variance (ANOVA) followed by Tukey's test. The data were evaluated with SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

### 3. RESULTS AND DISCUSSION

#### 3.1. Preliminary phytochemical studies of different extracts of *D. atrovirens* stem bark

The yield percent, total phenolic, and flavonoid contents of the extracts obtained from *D. atrovirens* bark powder using hexane, chloroform, ethyl acetate, methanol and water are presented in Table 1. The maximum extract yield was obtained in the hot water extract (13.2%) followed by methanol extract (11.3%). The results of total phenolic and tannin contents are expressed as gallic acid equivalents, whereas the flavonoid content is expressed as rutin equivalent. The extractable total phenolics (476.2 mg GAE/g extract) and flavonoids (129.0 mg RE/g extract) were found to be higher in the methanol extract of *D. atrovirens* bark. On the other hand, among the different sample extracts, the lowest concentrations of phenolics (65.7 mg GAE/g extract) and flavonoids (18.2 mg RE/g extract) were observed in the hexane extract.

**Table 1. Extraction yield and total phenolic, tannin and flavonoid contents of different extracts of *D. atrovirens* bark.**

Sample	Extract yield (%)	Total	
		phenolics (mg GAE/g extract)	Flavonoid (mg RE/g extract)
Hexane	0.2	65.7 ± 8.9 <sup>e</sup> 127.3 ±	18.2 ± 0.8 <sup>e</sup>
Chloroform	0.8	11.8 <sup>d</sup>	48.6 ± 1.9 <sup>d</sup>
Ethyl acetate	1.7	361.4 ± 20.2 <sup>c</sup>	87.2 ± 2.7 <sup>c</sup>
Methanol	11.3	476.2 ± 19.1 <sup>a</sup>	129.0 ± 3.7 <sup>a</sup>
Water	13.2	398.8 ± 18.7 <sup>b</sup>	97.6 ± 2.6 <sup>b</sup>

Total phenolic content is expressed as gallic acid equivalent (GAE)

Flavonoid content is expressed as rutin equivalent (RE).

Values are mean of three replicate determinations (n = 3) ± standard deviation.

Mean values followed by different superscripts in a column are significantly different (P < 0.05).

Plant phenolics have received considerable attention because of their potential biological activity. Phenolic compounds such as flavonoids, phenolic acid, and tannins possess diverse biological activities including anti-inflammatory, anti-carcinogenic, and antiatherosclerotic activities. These activities might be related to their antioxidant activity (14). The higher amount of phenolics in the methanol extract of *D. atrovirens* could be due to higher solubility of phenolic compounds. Phenolics are powerful antioxidants and act in a structure-dependent

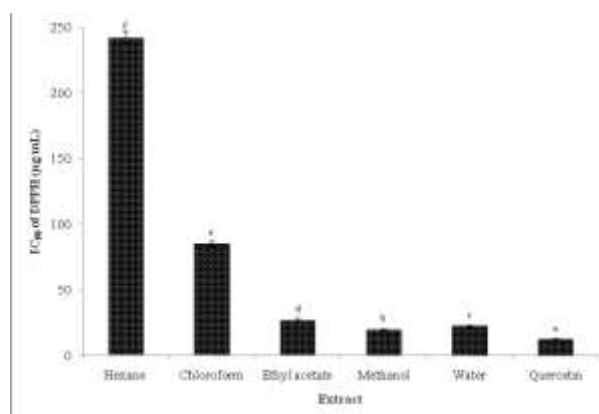
manner; they can scavenge reactive oxygen species, and chelate transition metals which play vital roles in the initiation of deleterious free radical reactions (15). Obviously, total phenolic content could be regarded as an important indication of antioxidant properties of plant extracts. Crude extracts of fruits, vegetables, and other plant materials are rich in phenolics (16). Since the extracts of *D. atrovirens* bark possess appreciable phenolic, and flavonoid contents, it can be taken as a good indication for its higher antioxidant capacity. There is increasing evidence that consumption of a variety of phenolic compounds present in natural foods may lower the risk of serious health disorders because of the antioxidant activity of these compounds (17).

#### 3.2. In vitro antioxidant assays

##### 3.2.1. Free radical scavenging activity on DPPH

The scavenging abilities of different solvent extracts of *D. atrovirens* bark were concentration-dependent and expressed as IC<sub>50</sub> values (Figure 1). Concentration of the sample necessary to decrease the initial concentration of DPPH by 50% (IC<sub>50</sub>) under the experimental condition was calculated. All the extracts exhibited appreciable DPPH radical scavenging activity ranging from IC<sub>50</sub> 19.52 µg/mL (methanol extract) to IC<sub>50</sub> 241.99 µg/mL (hexane extract). DPPH free radical scavenging effect of *D.*

*atrovirens* bark extracts and quercetin was in this order: Quercetin > methanol > water > ethyl acetate > chloroform > hexane.



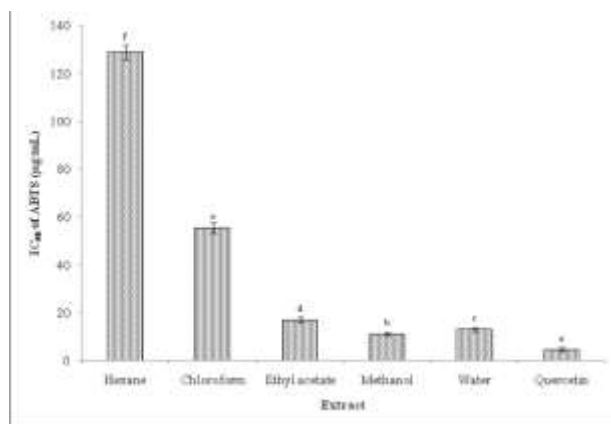
**Fig. 1. DPPH radical scavenging activity of different extracts of *D. atrovirens* bark.**

The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by antioxidant. DPPH scavenging assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds (18). The principle of this assay is

DPPH• on accepting a hydrogen (H) atom from the scavenger molecule i.e. antioxidants; the purple color of the DPPH thus changes to yellow which indicates that scavenging reaction (19). The antiradical scavenging activities of different extracts of *D. atrovirens* bark were also in agreement with the above reports and would be related to the nature of phenolics, thus contributing to their electron transfer/ hydrogen donating ability (Figure 1). Antioxidants with DPPH radical scavenging activity could donate hydrogen to free radicals, particularly to the lipid peroxides or hydroperoxide radicals that are the major propagators of the chain autoxidation of lipids, and to form nonradical species, resulting in the inhibition of propagating phase of lipid peroxidation (20). Previous literatures have also expressed the positive correlation between the polyphenolic compounds and DPPH radical scavenging activity (21,22).

### 3.2.2. Antioxidant activity by the ABTS<sup>•+</sup> assay

The different extracts from the *D. atrovirens* bark were fast and effective scavengers of the ABTS radical (Figure 2). In ABTS<sup>•+</sup> scavenging activity, the IC<sub>50</sub> values varied significantly (P < 0.05) and ranged from 10.82 to 128.6 µg/mL. Similar to DPPH radical scavenging activity, methanol extract showed the highest ABTS radical scavenging activity than other extracts (IC<sub>50</sub> at 10.82 µg/mL). The lowest ABTS radical scavenging activity was found in the hexane extract with the IC<sub>50</sub> value of 128.6 µg/mL.



**Fig. 2. ABTS radical scavenging activity of different extracts of *D. atrovirens* bark.**

Scavenging of ABTS<sup>•+</sup> is a simple and inexpensive method used to evaluate the radical scavenging ability of the plant extracts. Generation of ABTS<sup>•+</sup> is from oxidation of ABTS by potassium persulfate and determines the antioxidant activity of electron donating antioxidants (scavengers of aqueous phase radicals) and chain breaking

antioxidants (scavengers of lipid peroxy radicals) (23). ABTS<sup>•+</sup> also involves an electron transfer process. In the present study, the obtained results clearly indicate that all the tested extracts effectively inhibited and scavenged the radicals. Actually, the ABTS radical cation scavenging activity also reflects hydrogen-donating ability. The high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS<sup>•+</sup>) (24). Since, the extracts from *D. atrovirens* bark sample have the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction, they could serve as potential nutraceuticals when ingested along with nutrient.

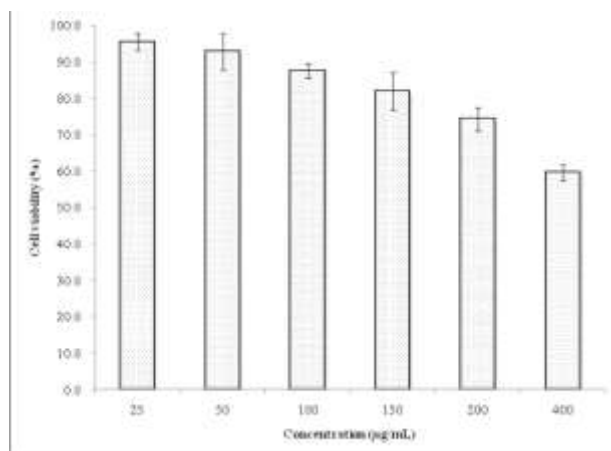
The results of *in vitro* antioxidant tests have shown that the methanol extract of *D. atrovirens* as good antioxidant and free radical scavenger. The highest amount of total phenolics and antioxidant activity was shown by the methanol extract of *D. atrovirens*. So this extract was chosen for the further investigations.

### 3.4. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells

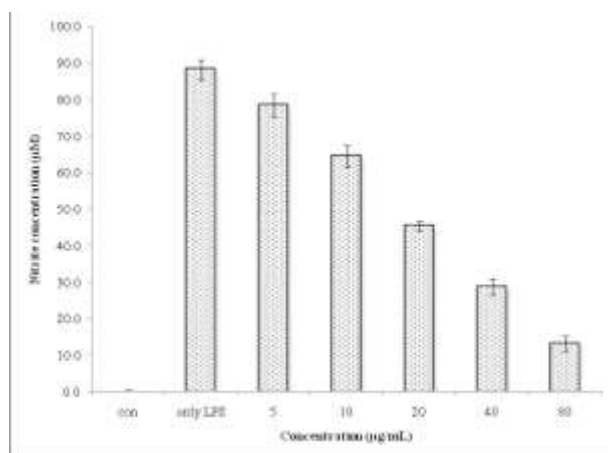
In the present study, methanol extract of *D. atrovirens* bark was evaluated for the inhibition of NO production in the LPS-stimulated RAW264.7 cells. The nitrite accumulation in the cells increased due to the LPS treatment. When compared to the untreated control, the pre-treated cells induced with LPS released a lower level of NO in the medium measured as its stable non-volatile breakdown product nitrite. The methanol extract remarkably inhibited (P < 0.05) the nitrite accumulation in LPS-stimulated RAW 264.7 cells in a concentration dependent manner. The methanol extract exhibited the reduction of nitrite level to 13.1 µM at the concentration of 80 µg/mL. The result of MTT cell viability assay revealed that the inhibitory effect of methanol extract was not due to cell damage (viability >90%) (Figures 3 and 4).

Macrophages play important roles in inflammation through the production of several pro-inflammatory molecules, including NO. Production of excessive NO has been associated with a range of inflammatory diseases including arteriosclerosis, ischemic reperfusion, hypertension and septic shock (25,26). Recent studies have demonstrated that the plant foods including fruits, vegetables and medicinal herbs are an excellent source of antioxidant molecules that effectively inhibit the inflammatory process by affecting different molecular targets (27,28). In this study, methanol extract of *D. atrovirens* bark exhibited a higher level of total phenolics when compared with other extracts. In addition, phenolic

compounds are known to be potent for inhibiting NO and peroxy nitrite productions (29). Higher level of polyphenol content with strong antioxidant potential of plant samples are a good target for examining the inhibitory activity against NO production.



**Fig. 3. Cell viability of methanol extract of *D. atrovirens* bark against RAW 264.7 cells.**



**Figure 4. Effect of methanol extract of *D. atrovirens* bark on nitric oxide production in LPS-induced RAW 264.7 cells.**

RAW 264.7, a murine macrophage cell line has been frequently used for the screening of anti-inflammatory drugs. The results of the present study demonstrated that the methanol extract significantly decreased the nitrite accumulation in LPS-stimulated RAW 264.7 cells in a concentration dependent manner. NO is a multifunctional signaling molecule, thus the impact of the extract or compound on NO production likely has further effects on signaling pathways in many cell types (30). Epidemiological studies have shown a positive correlation between consumption of plant foods, which are rich sources of antioxidants, and reduction in risk of diseases mediated by reactive oxygen species. Previous studies have also

suggested that plant secondary metabolites act as excellent anti-inflammatory agents and they play an important role in oxidative stress and inflammation (31-33).

#### 4. CONCLUSION

The extractable total phenolics, and flavonoids were found to be higher in the methanol extract of *D. atrovirens* bark and the methanol extract of *D. atrovirens* bark manifested the strongest radical scavenging activities. The methanol extract of *D. atrovirens* bark also significantly decreased nitrite accumulation in LPS-stimulated RAW 264.7 cells indicating that they potentially inhibited the NO production in a concentration dependent manner. Further studies are warranted in relation to the mechanism of action of bioactive components from the methanol extract of *D. atrovirens* bark.

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