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Research Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF *NOTHAPODYTES NIMMONIANA* EXTRACTS

Senthil Rajan Dharmalingam [1]*, Murugananthan Gopal [1], Kumarappan Chidambaram [2], Subashini Rajaram [1]

[1] Swamy Vivekanandha College of Pharmacy, Tiruchengode, Namakkal, Tamil Nadu, India

[2] College of Pharmacy, King Khalid University, Saudi Arabia

*Corresponding Author Email: dsrajansvcp@gmail.com

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ABSTRACT

Nothapodytes nimmoniana (J. Graham) belonging to family Icacinaceae is commonly known as Amruta and found in Maharashtra, Goa, Kerala, Karnataka, Assam, Tamil Nadu in India. The aim of this study is to evaluate the free radical scavenging activity of *Nothapodytes nimmoniana* extracts belonging to the family Icacinaceae. Three successive heartwood and barks extracts (petroleum ether, ethanol and water) of *Nothapodytes nimmoniana* were prepared. The extract were screened for their *in vitro* antioxidant activity using 2,2'-diphenyl-2-picryl hydrazyl (DPPH), 2,2'-azino-bis (3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide, nitric oxide, superoxide, p-nitroso dimethyl aniline (p-NDA) and deoxyribose assays. The amount of total phenolic compounds was further estimated. The total phenol content in methanol and water extracts was found to be 24.34 mg/g and 14.55 mg/g, respectively. Among the three extracts tested, the ethanolic extract showed maximum activity with IC_{50} values 33.85 ± 0.97,16.36 ± 0.74, 125.12 ± 2.31, 185.44 ± 2.62, 282.62 ± 4.68 µg/ml, for ABTS, DPPH, hydrogen peroxide, lipid peroxidation, nitric oxide, hydroxyl and superoxide radical inhibition assays, respectively. The water extract showed moderate activity than ethanolic extract when tested for ABTS, DPPH, hydrogen peroxide, lipid peroxidation, nitric oxide, hydroxyl and superoxide radical inhibition assays. The petroleum ether extract showed less activity at high concentrations when tested for compared with other extracts. All the three extracts showed less activity at high concentrations when tested for p-NDA assays. The successive ethanolic extract exhibited significant antioxidant activity and merits further investigations in animal models and isolation of its active constituents.

Keywords: *Nothapodytes nimmoniana*, DPPH, ABTS free radical scavenging activity

INTRODUCTION

Nothapodytes nimmoniana (J. Graham) Mabberly (Icacinaceae) [formerly, *Mappia foetida* Miers] belonging to family Icacinaceae, is an endangered medicinal tree is a shrubby small tree, 3-8 m tall, smooth, grey, wrinkled bark, about 5 mm thick with broad dark green leaves and flowers. It is a rich source of the potent alkaloid Camptothecin, 9-methoxy camptothecin and mappicine[1]. It is the endanger medicinal tree from Western Ghat (Karnataka) and vulnerable in Kerala, Tamil Nadu, Himalayan foothills in north India, Myanmar and Thailand which produce Camptothecin[2,3].

Nothapodytes nimmoniana contains camptothecin as its active constituent which is used in the treatment of cancer. In 21st century, Camptothecin, a monoterpeneindole alkaloid, is regarded as one of the most promising anticancer drug[4]. It is an excellent source of quinoline alkaloids, camptothecin and 9-methoxy camptothecin which are used clinically or after derivatization as anti-cancer agents for the treatment of solid tumors[5,6].

Recently, Food and Drug Administration (FDA) has approved Irinotecan and topotecan are two water-soluble derivatives of CPT for treating colorectal and ovarian cancer[6-8].

Due to unavailability of synthetic CPT, demand of the CPT is immensely high. Although, incredible research works are being made to enhance the synthetic production of camptothecin, regrettably none of the methods has come out of the laboratory research for a commercially viable scale production.

According to 2006 survey, *Nothapodytes nimmoniana* Graham considered as primary alternate source of CPT[3]. Efforts are further intensified on prospecting for optimization of media composition for enhanced *in vitro* response in terms of multiplication as well as high yield of CTP[9].

The aim of present investigation was to establish comparative baseline data on the total phenolic content and free radical scavenging activity of Different extract of heartwood and barks of *Nothapodytes nimmoniana* (J. Graham) Mabberly.

MATERIAL AND METHODS

Collection of Plant Material

The heartwood and barks of *Nothapodytes nimmoniana* (Graham) were collected from the botanical garden at the Campus of Swamy Vivekanandha College of Pharmacy, Tiruchengode. The tree shade dried heartwood and barks were pulverized to get coarse powder and sieved through mesh sieve No.22 and stored in an airtight plastic container. The coarsely powdered heartwood and barks of *Nothapodytes nimmoniana* (Graham) were used for further extraction.

Preparation of Aqueous Extract

Powdered plant material (100 g) was dipped into 20 volumes of distilled water and kept at room temperature for 2 h and then homogenized and rotary evaporator at 40°C under reduced pressure for two hours. After cooling, the resulting material was centrifuged 10,000 rpm for 15 min. The supernatant was collected and centrifuged again at 8000 rpm for 15 min to obtain a clarified mixture and stored at 4°C in a refrigerator for further use.

Preparation of ethanolic extract

Briefly, 150 gm of ground plant material was soaked in sufficient quantity of 70% ethanol at room temperature for 72 hours by cold maceration after which the filtrate was collected through a piece of muslin cloth and then followed by filter paper. The ground plant material was re soaked for twice before filtration. The filtrate was concentrated in a rotary evaporator under reduced pressure at 40°C to yield crude extract. This extract was stored at 4°C in refrigerator until use.

Preparation of Petroleum ether extract

The collected plant material was dried (30 ± 2ºC) for 14 days, ground and sieved to get fine powder from which the extracts were prepared by subjecting to the successive extraction, by using a hot continuous percolation method in Soxhlet apparatus with petroleum ether (60-80ºC). The powdered whole plant was extracted with petroleum ether (1 L). After complete extraction (18 hours), the solvent was removed by distillation under reduced pressure. The resulting extract was dried using a water bath to get semisolid[10].

Physicochemical analysis

Extractive values were performed according to the methods prescribed[11].

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedures[12].

Preparation of Test and Standard Solutions

All the three extracts of *Nothapodytes nimmoniana* and the standard antioxidants (ascorbic acid, rutin and butylated hydroxy anisole) were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for the *in-vitro* antioxidant assays using six different methods. As DMSO interferes in the case of hydrogen peroxide method the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain lower dilutions.

Total Phenolic Compounds Estimation

Antioxidant compounds generally contain phenolic group(s) and hence, the number of phenolic compounds in all the three extracts of the whole plant was determined using Foline-Ciocalteu reagent[13]. In a series of test tubes, 0.4 ml of the extract in methanol was taken, mixed with 2 ml of Folin-Ciocalteu reagent and 1.6 ml of sodium carbonate. After shaking, it was kept for 2 h and the absorbance was measured at 750 nm using a Shimadzu-UV-160 spectrophotometer. Using Gallic acid monohydrate, a standard curve was prepared. The linearity obtained was in the range of 1-10 µg/ml; using the standard curve, the total phenolic compounds content was calculated and expressed as gallic acid equivalent in mg/g of extracts.

Free radical scavenging activity

The three extracts were tested for their *in-vitro* antioxidant activity using standard methods. In all these methods, a fixed concentration of the standards or extracts solution was used to prepare final concentration of 1000 µg/ml to 0.45 µg/ml after all the reagents were added. Absorbance was estimated against a blank solution without the reagents containing the standards or extracts. A control test was performed without the standards or extracts. Percentage scavenging and IC_{50} values ± S.E.M were calculated.

DPPH Radical Scavenging Method

A 10 µl aliquot of the different concentrations of extracts and standards were added to 200 µl of DPPH in methanol solution (100 µM) in a 96-well microtitre plate (Tarson Products (P) Ltd., Kolkata, India). After incubation at 37°C for 20 min, the absorbance of each solution was determined at 490 nm using ELISA reader (Bio-Rad Laboratories Inc, California, USA, Model 550)[14].

Scavenging of ABTS Radical Cation

To 0.2 ml of various concentrations of the extracts and standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution were added and incubated for 20 min. Absorbance of these solutions were measured spectrophotometrically at 734 nm[15].

Scavenging of Hydrogen Peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS at pH 7.4). Various concentrations of the extracts and standards in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min, the absorbance was measured at 230 nm[16].

Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory activity of three extracts, the compound and standard were carried out. Egg lectin (3 mg/mL, phosphate buffer, pH 7.4) was sonicated. The test sample of different concentrations was added to 1 mL of liposome mixture, control was without test sample. Lipid peroxidation was initiated by adding 10 µL ferric chloride (400 mM) and 10 µL L-ascorbic acid (200 mM). After incubation for 1 h at 37◦C, the reaction was stopped by adding 2 mL of 0.25N HCl containing 15% trichloro acetic acid and 0.375% thiobarbituric acid and the reaction mixture was boiled for 15 min then cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm.

Nitric Oxide Radical Inhibition Assay

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml) and the extracts and standard solutions (1 ml) were incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of diazotization reaction and then 1 ml of NEDD was added, mixed and allowed to stand for another 30 min in diffused light. The absorbance was measured at 540 nm against the corresponding blank solutions in a 96-well microtitre plate (Tarson Product (P) Ltd., Kolkata, India) using ELISA reader (Bio Rad Laboratories Inc, California, USA, Model 550)[17].

Scavenging of Hydroxyl Radical by Deoxyribose Method

Various concentrations of the extracts and standards in DMSO (0.2 ml) were added to the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH, 7.4, 20 mM) to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37°C. After incubation, ice-cold trichloro acetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v) in 0.25 N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm[18].

Scavenging of Hydroxyl Radical by p-NDA Method

Various concentrations of the extracts and standards in distilled DMSO (0.5 ml) were added to a solution mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and p-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM), to produce a final volume of 3 ml. Absorbance was measured at 440 nm[19].

Table 1: *In vitro* antioxidant activity of *Nothapodytes nimmoniana* extracts

Extracts	$^{+}IC_{50}$values ± SEM* (µg/mL)							
	ABTS	DPPH	Hydrogen Peroxide	Lipid peroxidation	Nitric Oxide	Deoxyribose	p-NDA	Superoxide
NNP	792.42 ± 2.68	722.27 ± 1.41	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
MNE	33.85 ± 0.97	16.36 ± 0.74	125.12 ±2.31	185.44 ± 2.62	282.62 ± 4.68	462.43 ± 3.62	> 1000	895.24 ± 3.72
MNW	57.95 ± 0.38	256.21 ± 3.96	273.46 ±4.66	472.52 ± 1.52	522.19 ± 2.34	> 1000	> 1000	> 1000
Standards								
Ascorbic Acid	10.37 ± 0.74	4.68 ± 0.85	181.19 ±3.82	-	-	-	> 1000	-
Rutin	0.72 ± 0.62	8.46 ± 0.77	37.92 ± 0.83	-	83.17 ± 2.49	-	234.52 ±2.94	-
BHA	-	-	22.69 ± 0.92	-	-	71.22 ± 1.68	-	-
α-Tocopherol	-	-	-	90.51 ± 1.22	-	-	-	-

* Average of three independent determinations, three replicates and values are mean ± SEM.
$^{+}$ IC_{50} = Concentration of the sample/standard required to inhibit 50% of free radicals.

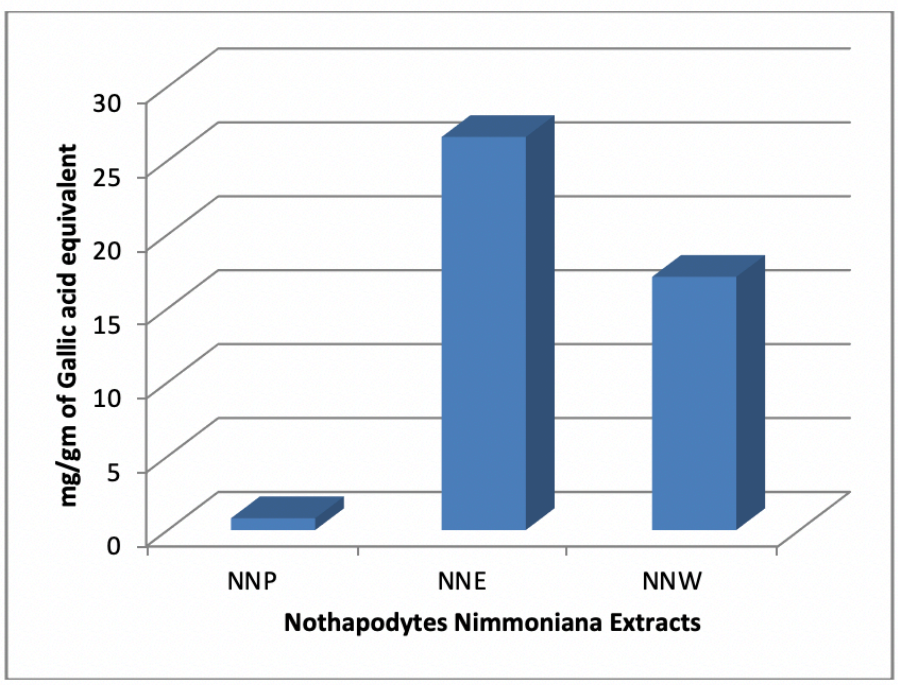

Figure 1: Total Phenol estimation of successive extracts of *Nothapodytes nimmoniana*

Scavenging of Super Oxide Radical by Alkaline DMSO Method

To the reaction mixture containing 0.1 ml of NBT (1 mg /ml solution in DMSO) and 0.3 ml of the extracts and standard in DMSO, 1 ml of alkaline DMSO (1 ml DMSO containing, 5 mM NaOH in 0.1 ml water) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm[20].

RESULT AND DISCUSSION

Extractive Values of heartwood and barks of *N. nimmoniana* gave different range of yields in various solvents. The drug was extracted with various solvents with the help of maceration process. Percentage of aqueous extract (0.07) was higher than ethanolic (0.04) and petroleum ether (0.01) extracts.

Phytochemical Screening showed the presence of terpenoids, alkaloids, tannins, flavonoid, carbohydrates, saponins, steroids.
The total phenolic compounds of two successive extracts were expressed as gallic acid equivalent in mg/g of extracts (Figure 1). The ethanolic extract has the highest phenolics content (26.59 mg/g) followed by water extract (17.13 mg/g) and petroleum ether extract (0.82 mg/g). The petroleum ether extract contains very less amount of phenolic compounds. The Among the three extracts tested for *in vitro* antioxidant activity using six methods, the successive ethanolic extract (NNE) exhibited good antioxidant activity in ABTS, DPPH, hydrogen peroxide, lipid peroxidation and nitric oxide radical scavenging methods with IC_{50} values of 33.85 ± 0.97,16.36 ± 0.74, 125.12 ± 2.31, 185.44 ± 2.62, 282.62 ± 4.68 µg/ml respectively. The successive aqueous extract (NNW) showed moderate activity in ABTS, DPPH, hydrogen peroxide, lipid peroxidation and nitric oxide radical scavenging methods with IC_{50} values of 57.95 ± 0.38, 256.21 ± 3.96, 273.46 ± 4.66, 472.52 ± 1.52, 522.19 ± 2.34 µg/ml, respectively (Table 1). The petroleum ether extract (NNP) showed low activity in ABTS and DPPH methods with IC_{50} values of 792.42 ± 2.68 and 722.27 ± 1.41 µg/ml respectively. The petroleum ether extract failed to exhibit antioxidant in the scavenging of hydrogen peroxide, lipid peroxidation, nitric oxide radical, superoxide radical and hydroxyl radical. All the three extracts failed to exhibit antioxidant activity in the scavenging of superoxide radical by alkaline DMSO method and hydroxyl radical by p-NDA method. The IC_{50} values obtained, however, for all the extracts in all the methods were found to be higher than the standards used, indicating their low activity compared to the standards.

The preliminary phytochemical investigations of the three extracts revealed the presence of steroids, terpenoids, alkaloids, and carbohydrate and phenolic compounds such as tannins, flavonoid etc., and several such constituents were known to possess potent antioxidant activity[21-23].

CONCLUSION

In conclusion, the successive ethanolic extract of *Nothapodytes nimmoniana* was found to possess significant free radical scavenging activity. Good correlation between total phenolic content and antioxidant activity was also observed. This observation clearly shows the relationship between the phenolic compounds and its antioxidant potential. However, a large number of phytocompound groups are implicated for antioxidant activity. The observed antioxidant activity of the successive ethanolic extract of *Nothapodytes nimmoniana* may be due to the presence of any of these constituents. The constituents responsible for the anti-oxidative activity of *Nothapodytes nimmoniana*, however, are currently not clear. Further work is, therefore, under progress to identify and isolate the antioxidative constituents and to establish the activity in animal models.

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