ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACTS OF *NOTHAPODYTES NIMMONIANA* (J. GRAHAM) MABBERLY

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Summary

The objective of the present study was to establish comparative baseline data on the total phenolic content and antioxidant activity of methanolic extract of different parts of *Nothapodytes nimmoniana* (J. Graham) Mabberly (Family Icacinaceae). Antioxidant activity of methanolic extracts were determined using various *in vitro* models like Diphenylpicrylhydrazyl (DPPH) radical, nitric oxide radical, superoxide radical and peroxide radical scavenging activity. The reducing power, anti-lipid peroxidation potential and total phenolic content of methanolic extracts were also determined. Preliminary phytochemical screening of drug indicates the presence of alkaloids, tannins, steroid, terpenoids, phenolics, coumarins and fixed oil. Among the various plant parts phenolic content ranged from 281.0 to 454.0 mg gallic acid equivalent (GAE) /100 g dry sample with maximum content found in methanolic extract of fruits (454.0 mg GAE/gm DW). Fruits shown maximum antioxidant activity with an IC_{50} value of 0.177 ± 0.2 mg/mL for DPPH radical, 0.177 mg/mL for H_2O_2 radical, 0.167 mg/mL for superoxide radical and 0.175 mg/mL for nitric oxide radical. Fruits showed maximum anti-lipid peroxidation effect (0.362 mg/mL) with higher reducing potential 3.65. The results were significant as compared to standard antioxidants such as l-ascorbic acid and α-tocopherol. On the basis of above results it was concluded that the methanolic extracts of different parts of *N. nimmoniana* showed significant antioxidant activity.

Keywords: *Nothapodytes nimmoniana*, Icacinaceae, Camptothecin, Anti-cancer, Free radicals.

Introduction

Uncontrolled generation of free radicals together with the reduction in antioxidant vitamins and enzymes is considered to be the main contributor to oxidative stress (1). Free radicals attack membrane lipids, protein and DNA, which is believed to be involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative and inflammatory diseases (2). The addition of antioxidants to food products has therefore become popular as a means of increasing shelf life and to reduce wastage and nutrition losses by inhibiting and delaying oxidation (3). Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are widely used in the food industry. However, there are serious concerns about the carcinogenic potential of these substances and there has been a general desire to replace synthetic antioxidants (4, 5). Therefore, intensive research is being carried out on the extraction, characterization and utilization of natural antioxidants that may serve as potent barks. Plant phenolics are multifunctional and can act as reducing agents (free radical candidates in combating carcinogenesis and aging processes. Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of the plants such as fruits, vegetables, nuts, seeds, leaves, roots and terminators), metal chelators and singlet oxygen quenchers. Studies have shown that consumption of foods and beverages rich in phenolic content is correlated with reduced incidence of heart disease (6).

Camptothecin (CPT), a monoterpene indole alkaloid, is regarded as one of the most promising anticancer drug of the twenty first century (7, 8). The cellular target of camptothecin is DNA topoisomerase I and numerous analogs have been synthesized as potential therapeutic agents (9). CPT inhibits the replication of Human Immuno Deficiency Virus (HIV) *in vitro* and is also shown to be effective in the complete remission of lung, breast, uterine and cervical cancer (10-12). Several water soluble derivatives of camptothecin (topotecan and irinotecan) are

currently being used for the treatment of colorectal and ovarian cancer (13-15). The molecular and cytotoxic effects of camptothecin on *Plasmodium falciparum* proven that CPT is an interesting target for new anti-malarial drug development (16). CPT was first discovered in the Chinese deciduous tree, *Camptotheca acuminata* (17). The highest concentration of CPT (0.3% w/w) was found in *Nothapodytes nimmoniana* (*N. nimmoniana*) (18).

N. nimmoniana (J. Graham) Mabberly (Family Icacinaceae), formerly known as *Nothapodytes foetida* Sleumer and *Mappia foetida* Miers, is a small tree naturally distributed in many parts of Western Ghats of India, some parts of Assam, Himalayan foothills, Ceylon, Burma and Thailand. In the absence of synthetic sources, the global demand for CPT (US$ 4045 million in 2002) is being met by the extraction of naturally existing populations of *N. nimmoniana* from the Western Ghats, India (19). Consequently in the last decade alone, over 20% of the population of the species has been lost from the Western Ghats (20, 21). The aim of present study was to evaluate antioxidant activity of *N. nimmoniana* by using various *in vitro* models.

Methods

Collection of plant material

Plant material of *N. nimmoniana* was collected from Mahabaleshwar region of Maharashtra, India, in the month of November. The herbarium was authenticated by Chief Botanist, Botanical Survey of India (BSI) and voucher specimen (NNASP1) was kept at departmental herbarium of BSI.

Drugs and Chemicals

The chemicals used in the experiments were of analytical grade. Tocopherol, Sodium nitropruside (SNP), sulphanilamide, N-(1-naphthyl) ethylene diamine dihydrochloride, potassium nitrite, ethylene diamine tetra acetic acid (EDTA), potassium ferricyanide, ascorbic acid, and ferric chloride were procured from Sigma Chemicals, Mumbai, India. DPPH, nitro blue tetrazolium (NBT), riboflavin and deoxyribose were purchased from Hi Media Chemicals Ltd. Mumbai, India.

Preparation of extract

Plant material of *N. nimmoniana* was collected, thoroughly washed, segregated into different parts (roots, stems, leaves and fruits) and dried at 55ºC in an air dryer for 48 h. Dried materials were powdered separately with a Wiley mill (Model 4276-M, Thomas Scientific, USA) to pass a 20 mesh sieve and stored in sealed plastic bags. About 500 mg of the various powders were taken in 5 mL volumetric flask, mixed with 5 mL of MeOH and vortexed for 2 min followed by sonication (33 MHz, Roop Telesonic, India) at room temperature for 5 min. The process was repeated thrice for complete extraction. After sonication, methanolic extracts were combined and evaporated to dryness *in vacuo*. Dried extract was dissolved in methanol to prepare dilutions in a range of 100-500 µg/mL.

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedures (22, 23).

Determination of total phenolic content

The total phenolic content (TPC) of the methanolic extract of *N. nimmoniana* was determined by the method of Folin-Ciocalteu reaction using gallic acid as standard (24). To 100 µL of extract (100 µg/mL), add 500 µL of (50%) Folin-Ciocalteu reagent followed by the addition of 1 mL of 20% Na_2CO_3 solution. A mixture was incubated at room temperature for 20 min. and the absorbance was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram samples.

Anti-oxidant studies

Determination of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity

The antioxidant activity, based on scavenging of stable DPPH free radical, was determined (25). Different concentrations of test sample were added to 3 mL of 0.004% methanol solution of DPPH. Absorbance at 517 nm was measured after 30 min and the percent inhibition activity was calculated as

DPPH Scavenged (%) = (A cont. - A test) / (A cont.) x 100

Where, A cont. = Absorbance of control reaction

A test = Absorbance of test reaction

The antioxidant activity of the extract was expressed as IC_{50}.

Determination of nitric oxide (NO) radical scavenging activity

Nitric oxide (NO) was generated from sodium nitropruside (SNP) and measured by Greiss reaction. SNP in aqueous solution at physiological pH instinctively generates NO, which intermingles with oxygen to generate nitrite ions that can be anticipated by Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). Scavengers of NO with oxygen leading to reduced production of NO. SNP (5 mM) in phosphate buffer saline (PBS) was mixed with different concentration of (100-500 µg/mL) drug dissolved in suitable solvent and incubated at 25°C for 150 min. The above samples were reacted with Greiss reagent. The absorbance of chromophore created during diazotization of nitrite with sulphanilamide and following coupling with naphthyl ethylene diamine was read at 546 nm and referred to the absorbance of standard solution of potassium nitrite treated in the same way with Greiss reagent (26).

Superoxide anion (O_2^-) radical scavenging activity

Measurement of superoxide anion (O^{2-}) scavenging activity of extracts was based on the method described with slight modification (27, 28). O^{2-} radicals are generated non-enzymatically in Phenazine methosulphate–Nicotinamide adenine dinucleotide phosphate (PMS–NADH) systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 mL of Tris-HCl buffer (16 mM, pH 8.0) containing NBT (50 µM) solution and NADH (78 µM) solution. The reaction was started by adding PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$(\%)\ I = (A0 - A1) / (A0) \times 100$$

where A0 was the absorbance of the control and A1 was the absorbance of extract and the standard compound.

Determination of reducing power

Sample solutions containing 100–500 µg extracts were prepared from the stock solution. To a 1 mL aliquot of the extract, 1 mL of 0.2 M phosphate buffer pH 6.6 and 1 mL of 1% (w/v) $K_3Fe\ (CN)_6$ were added. The mixture was incubated at 50°C for 20 min. 10% w/v of trichloroacetic acid (1 mL) was added and the resulting mixture was centrifuged at 3000 rpm for 10 min. 1 mL of the supernatant was taken to which was added 1 mL of distilled water and 0.2 mL of 0.1% (w/v) $FeCl_3$ solution. The absorbance was measured at 700 nm using UV–Vis spectrophotometer (Perkin-Elmer, USA.). The reducing power of α-tocopherol was also determined. Increased absorbance of the reaction mixture indicated increase reducing power (29).

Determination of hydroxyl radical (OH^-) scavenging activity

Non-site-specific hydroxyl radical (OH^-) scavenging activity assay was evaluated by a previously reported method (30). The mixture containing $FeCl_3$ (100 µM), ascorbic acid (100 µM), ethylene diamine tetra acetic acid (EDTA, 100 µM), H_2O_2 (10 mM), deoxyribose (2.8 mM), and test sample in 500 µL phosphate buffered saline (PBS, 20 mM, pH 7.4) was incubated for 1 h at 37°C. After adding 250 µL of trichloroacetic acid (10% w/v) and 250 µL of thiobarbituric acid (1% w/v), the reaction mixture was boiled for 15 min in a water bath. The colour development was measured at 532 nm and the scavenging activity of test sample was expressed as the percentage inhibition of the deoxyribose degradation to malonaldehyde. The site specific assay for hydroxyl radical scavenging activity was performed in a similar manner to that of the non site specific assay, except that EDTA was discarded.

Determination of H_2O_2 radical scavenging activity

The ability of extract to scavenge H_2O_2 was determined. A solution of H_2O_2 was prepared in PBS (pH 7.4). H_2O_2 concentration was determined spectrophotometrically, by measuring absorption with extinction coefficient for H_2O_2 of 81 m^{-1} cm^{-1}. Extracts (100-500 µg/mL) in distilled water were added to H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing PBS without H_2O_2 (31).

Lipid peroxidation assay

Thuong et al. (2007) reported that inhibitory activity of kudingcha against mitochondrial lipid peroxidation was measured by the thiobarbituric acid reactive substance (TBARS) method. They also reported the preparation of mitochondria and measurement of lipid peroxidation (LPO) was reported previously (32).

Statistical analysis

Experimental results were mean ±SEM of three parallel measurements. Analysis of variance was performed by ANOVA followed by Newmans-Keul multiple comparison test. $p < 0.05$ was considered as significant.

Results and Discussion

Preliminary phytochemical screening

Characteristic phytochemical tests showed the presence of alkaloids, steroids, terpenoids, tannins, phenolics, coumarins and fixed oil (Table 1).

Total phenolic content

Antioxidant activity of the plant extract is often associated with the phenolic compounds present in them. Hydrogen donating property of polyphenolic compounds is responsible for the inhibition of free radical induced LPO (33). The total phenolic content of methanolic extract of *N. nimmonian*a fruits is more than the other extracts (454 mg GAE /gm DW) (Table 2).

DPPH radical scavenging activity

The methanolic extracts of *N. nimmoniana* significantly ($p< 0.05$) scavenge DPPH radical in a concentration dependent manner. The methanolic extract of fruits shown more scavenging activity than methanolic extracts of stem, root, leaf and flower with IC_{50} value of 0.177 mg/mL, 0.178 mg/mL, 0.234 mg/mL, 0.253 mg/mL and 0.413 mg/mL respectively which is significant as compare to control and α-tocopherol (Vit E) 0.191 mg/mL (Table 3). DPPH, is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It has characteristic absorbance maxima at 517 nm, widely used to evaluate the free radical scavenging effect of natural antioxidants (34). Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compound. DPPH radical scavenging activity increased with the increase of phenolic compound content (35-37).

Nitric oxide radical scavenging activity

The different fractions quenched NO released by Sodium nitropruside (nitric oxide donor). The fractions significantly and dose dependently decreased the release of NO. ROS like O^{2-} may react with NO results in reactive nitrogen species (RNS) such as NO_2, N_2O_4. Both ROS and RNS causing cellular damage (38). Fruits have maximum scavenging activity with IC_{50} value 0.175 mg/mL. All plant materials shown significant scavenging activity as compare to control (Table 3).

Superoxide radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals (39). From the investigations, it was found that the methanolic extracts inhibit superoxide radicals in a dose dependent manner. In the PMS/NADH-NBT system, superoxide anions derived from dissolved oxygen by PMS/NADH coupling reaction reduce NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anions in the reaction mixture. The methanol extract of *N. nimmoniana* fruits exhibited maximum superoxide scavenging activity (IC_{50} value 0.167) (Table 3).

Reducing power activity

The reducing power of the methanolic extracts of *N. nimmoniana* and the reference compound, ascorbic acid increased steadily with increasing concentration. The reducing powers (absorbance at 700 nm) of methanolic extracts of *N. nimmoniana* fruits and α-tocopherol were 3.65 and 4.02 at a dose of 0.5 mg/mL. This indicate that methanolic extracts can act as electron donors and can

react with free radicals to convert them to more stable products and thereby terminate radical chain reactions. The results were significant as compare to control (Figure 1).

H_2O_2 radical scavenging activity

H_2O_2 radical itself not very reactive but it can sometimes be toxic to cell because it generates OH^- radical in the cell (40). Fruits significantly inhibit peroxide radical with IC_{50} value 0.384 mg/mL (Table 3).

Lipid peroxidation assay

It is known that a cleavage product of lipid peroxidation accumulates in nervous system, cardiovascular system and muscle fibers (41). Prevention of lipid peroxidation in rat liver and brain homogenate confirmed that drug is active against free radicals in biological membranes. The methanolic extract of *N. nimmoniana* root showed maximum anti-lipid peroxidation effect in liver homogenate with IC_{50} value of 0.684 mg/mL (Table 3). The result shows that inhibition of TBARS formation in rat liver homogenate increased with increasing concentration.

Table 1: Phytochemical analysis of methanolic extracts of *N. nimmoniana*.

Chemical Test	Root	Stem	Leaf	Fruit
Alkaloids	+	+	+	+
Terpenoids	+	+	+	+
Fixed oil	-	-	-	+
Steroids	+	+	+	+
Phenol	+	+	+	+
Saponins	-	-	-	-
Gums	-	-	-	-
Coumarins	+	+	+	+
Carbohydrates	-	-	-	-
Amino acids	-	-	-	-
Glycosides	-	-	-	-
Resins	-	-	-	-
Tannins	+	+	+	+

(+ Present, - Absent)

Table 2: Total phenolic content in different samples of *N. nimmoniana*.

Samples	Phenolic content (mg GAE/100 gm sample)
Root	349.0 ± 0.05*
Stem	309.0 ± 0.01**
Leaves	281.0 ± 0.01**
Flower	316.0 ± 0.003***
Fruits	454.0 ± 0.001***

All values are expressed as mean ± SEM (n=3);

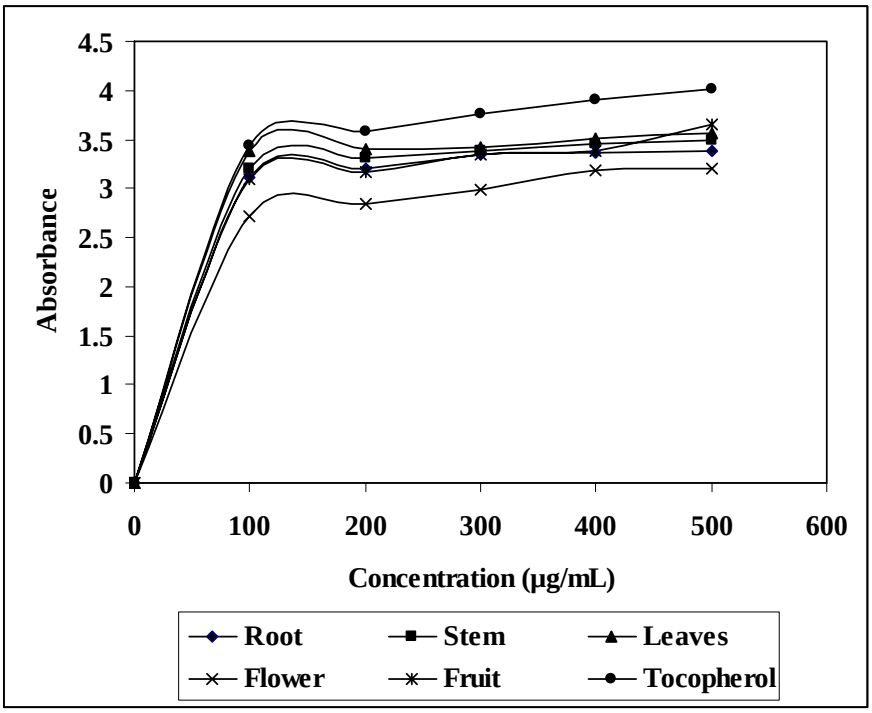
*p<0.05 considered significant as compare to control.

Table 3: Free radical scavenging activity of methanolic extract of *N. nimmoniana*.

Free radical	IC_{50} value (mg/mL)					
	Root	Stem	Leaves	Flower	Fruits	Vit-E
DPPH	0.234 ± 0.05*	0.178 ± 0.02**	0.253 ± 0.001***	0.413 ± 0.02**	0.177 ± 0.05*	0.191 ± 0.05*
H_2O_2	0.217 ± 0.05*	0.251 ± 0.001***	0.220 ± 0.05*	0.384 ± 0.02**	0.204 ± 0.01**	0.201 ± 0.001***
Super oxide	0.477 ± 0.001***	0.180 ± 0.001***	0.218 ± 0.01**	0.241 ± 0.01**	0.167 ± 0.001***	0.577 ± 0.02**
Nitric oxide	0.183 ± 0.01**	0.178 ± 0.05*	0.202 ± 0.01**	0.179 ± 0.01**	0.175 ± 0.001***	0.621 ± 0.03*
LPO (liver)	0.684 ± 0.01**	0.397 ± 0.01**	0.511 ± 0.05*	0.518 ± 0.02**	0.362 ± 0.01**	0.295 ± 0.01**

All values are expressed as mean ± SEM (n=3);
*p<0.05 considered significant as compare to control.

Figure 1: Reducing power activity of methanolic extracts of *N. nimmoniana*.



All values are expressed as mean ± SEM (n=3);
*p<0.05 considered significant as compare to control.

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