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ORIGINAL ARTICLE

Total phenolic contents and free-radical scavenging activities from methanolic extracts of *Nauclea subdita* (Korth) Steud. heartwood

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Fatin Ruzanna Jamaluddin, Razak Wahab, Jamaluddin M. Daud and Shafiqur Rahman; Total phenolic contents and free-radical scavenging activities from methanolic extracts of *Nauclea subdita* (Korth) Steud. heartwood

ABSTRACT

Investigation on the total phenolic content (TPC) and free-radical scavenging activity of *Nauclea subdita* (Korth) Steud. were carried out. Information on these activities might reflect the potency of the plant extract as an antioxidant. Young and matured trees of *N. subdita* were harvested and cut into three heartwood parts (top, middle and bottom) and grounded into powdered forms. Soxhlet extraction of the heartwood samples using polar (methanol) and non-polar (hexane) solvents, and phytochemical screening tests were carried out. The total phenolic contents of the methanolic crude extracts from heartwood parts were determined by colorimetry at 760 nm using Folin-Ciocalteau's reagent, and gallic acid was used as standard. The methanolic extracts were also checked for primary antioxidant activities using an *in vitro* method, measured by free-radical scavenging activity of 2, 2'-diphenyl-1- picrylhydrazyl (DPPH) radical. The absorbances were measured at 517 nm by UV/VIS spectrophotometer and compared with standards Trolox and butylated hydroxyanisole (BHA). The results from this study suggest that *N. subdita* heartwood extract may serve as a potential source of natural antioxidant for future development in food and nutraceutical applications.

Key words: *Nauclea subdita*, antioxidant activity, TPC, 2, 2', diphenyl-1-picrylhydrazyl (DPPH) radical, methanolic extract.

Introduction

Nauclea subdita is a plant from the *Rubiaceae* family that composes many species. It is a tropical plant that is indigenous to most regions in Asia and Africa. Generally, the plant grows in lowland to hill forests, also in swampy areas and habitually along streams and rivers and normally planted to stabilize slopes and river banks. It is a timber species (Lim *et al.,* 2004). Young leaves are edible, and a source of traditional medicine based on the great experience of people in different countries, especially the Sabahan ethnic in Semporna, Sabah, Malaysia. The plant is normally used for treating stomach ache, blood pressure, diabetes and skin problems.

The information on the traditional usage of the plant was a useful basis for further research and development. Medicinal tall trees, as we know play an important role in our forest structure, biodiversity, eco-balance and usually as timber production in wood product industries (Lim *et al.,* 2004). An important case, as this forest product can use as medicinal materials for human. Many medicinal materials are destructively obtained from plants such as roots, bark, sapwood, heartwood and other plant parts in the forest (Kaviarasan *et al.,* 2007). More sustainable ways of harvesting the medicinal materials from the forest should be introduced.

Since there are not many studies on *Nauclea subdita* has been carried out, this paper reports the results of antioxidant activities on heartwood of the plant. These studies were carried out at the School of International Tropical Forestry, Universiti Malaysia Sabah, and Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, Kuantan Campus, from July 2010 to Jan. 2012.

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Materials and Methods

Chemicals and Reagents:

All the chemicals and reagent used in this experiment were of analytical grade. The 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu and other chemicals and solvents such as methanol and hexane. All the chemicals were obtained from Sigma or Merck.

Sample Preparation:

Collection and identification of *N. subdita* plant materials with different types, young and a mature tree were collected fresh from forest sources in Pitas, Sabah in March 2010 and have been identified by experts at the Forest Research Institute Malaysia (FRIM), Kepong. The young and matured trees of *N. subdita* were cut into three portions (top, middle and bottom). The tree portions of wood were chopped into small part, grinded into particles and dried in the room temperature with air-conditioner for two weeks, and the samples was dried in freeze dryer before extraction. The dry powdered of wood materials were blended using a laboratory blender after took weight of the powdered samples.

Extraction Analysis and Phytochemical Screening Method:

Ground samples in the form of dried fine powder (100 g each placed in cellulose timble) were extracted in soxhlet extractors using non-polar solvent (hexane) and polar solvent (methanol) separately for 72 hours, following the method (Sengul *et al.,* 2009). The extracts were concentrated under vacuum at 60ºC using a rotary evaporator. The solid residues obtained after the rotary evaporation process were dried, kept in glass vials and stored in a refrigerator. The residues were taken from the refrigerator the subsequent experiments. The yields for methanol extract from each sample were the top, middle and bottom from portions from both maturity groups of trees in heartwood parts.

Phytochemical screening were performed in accordance to the standard procedures. A qualitative phytochemical test was performed to detect the presence of phenolic compounds (ferric chloride and gelatin tests), flavanoids, tannins, phytostrols (Libermann-Burchard's test), terpenoids (Salkowski test), steroids, alkaloids, cardiac glycoside (Keller-Killiani test) and saponins are carried out using standard procedures as described by (Abubakar *et al.,* 2008) and (Kumar *et al.,* 2007).

Total Phenolic Content:

The total phenolic content of the extracts were determined by the Folin-Ciocalteau method with some modifications (Azlim *et al.,* 2010, Rankovic *et al.,* 2011). Gallic acid was used as standard. 0.5 mg/mL stock standard solution of Gallic acid was prepared by dissolving 250 mg of dry Gallic acid in 1mL of 70 % of methanol and then diluted into 500 mL of distilled water; the stock solution was stored at 4°C. Working standards of between 0.02 to 0.10 mg/mL were prepared by diluting the stock solution with distilled water. The extracts were prepared, 100 µL of each extract was transferred into a test tube and 0.75 mL of Folin-Ciocalteu reagent was added and mixed (previously diluted 10 times with distilled water). The above solution was then kept for incubation at room temperature for 5 min, then 0.75 mL of 6 % (w/v) sodium carbonate was added to the mixture and mixed gently, vortex thoroughly. After standing at room temperature for 90 minutes, the absorbance was measured at 760 nm using a perkin-elmer UV-VIS lambda 25 spectrophotometer (Rankovic *et al.,* 2011). Gallic acid was used to produce standard calibration curve was plotted in the range of 0.02 to 0.10 mg/mL. The total phenolic content was expressed in g of Gallic acid equivalents (GAE) / 100 g of extract.

Free-Radical Scavenging Activity by DPPH Method:

The antioxidant activity between the samples of the sapwood, heartwood and bark extracts of *N. subdita* was determined using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay by the referred method (Ludmila *et al.,* 2003, Guno *et al.,* 2010). The radical scavenging activity was determined by a modified method described by (Ayoola *et al.,* 2008). The crude extracts of heartwood from *N. subdita* were mixed with 95% methanol to prepare the stock solution (300 mg / 3000 mL). The test samples will prepare from stock solution. The extracts were prepared at concentrations of 0.2, 0.3, 0.6, 1.3, 2.5, and 5.0 mg/mL in methanol (Analar grade). DPPH solution will prepare in methanol. Freshly prepare DPPH solution were added in each of this test tube containing *N. subdita* extracts and allowed to stand for 30 min in the dark. The absorbance were taken at 517 nm using a spectrophotometer. BHA and Trolox acid were use as standard (Nurliyana *et al.,* 2010,

Paduch *et al.,* 2008). The DPPH solution without sample solution was use as negative control. 95 % methanol was use as blank. Percent scavenging of the DPPH free radical will measure using the following equation;

$$\text{Scavenging capacity (\%)} = 1 - \frac{\text{absorbance of test sample}}{\text{absorbance of the control}} \times 100$$

An IC_{50}, the amount of samples extracted into 1 mL solution necessary to decrease by 50 % the initial DPPH concentration was derived from the % disappearance vs. concentration plot (Concentration here means mg of crude extracted into 1 ml solution) (Manjunatha *et al*., 2011). IC_{50} values were calculated in mg dried material equivalents/ml for sample extracts. BHA and Trolox were used as positive control. All the antioxidant measurements were performed in triplicate, and the data were expressed as average ± standard deviations (SD).

Statistical Analysis:

All the antioxidant measurements were performed in triplicate, and the data were expressed as average ± standard deviations (SD). Two-way analysis of variance (ANOVA), Duncan's tests was carried out to test any significant differences between treatments. A $P < 0.05$ was considered as statistically significant.

Result and Discussion

Phytochemical Screening and Extraction Analysis:

The presence of natural product compounds as indicated during phytochemical screening are summarized in Table 1 and Table 2. Screening tests of the methanolic crude extracts of *N. subdita* revealed the presence of phenolic compound, flavanoids, terpenoids, alkaloids, phytosterols and saponins in the heartwood of young and mature plants, whereas only phytosterols were detected in hexane extracts.

Table 1: Phytochemical screening of young tree.

Plant 1	Phenolic compound	Flavanoids	Tannins	Phytosterols	Steroids	Terpenoids	Alkaloids	Saponin	Cardiac Glycoside
					Hexane				
P1TW	-	-	-	+	-	-	-	-	-
P1MW	-	-	-	+	+	-	-	-	-
P1BW	-	-	-	+	-	-	-	-	-
					Methanol				
P1TW	+	+	-	+	-	+	+	+	-
P1MW	+	+	-	+	-	+	+	+	-
P1BW	+	+	-	+	-	+	+	+	-

Note : + = present, - = absent, W = Wood, P1 = Young Tree, T = Top, M = Middle, B = Bottom

Table 2: Phytochemical screening of mature tree.

Plant 2	Phenolic compound	Flavanoids	Tannins	Phytosterols	Steroids	Terpenoids	Alkaloids	Saponin	Cardiac Glycoside
					Hexane				
P1TW	-	-	-	+	-	-	-	-	-
P1MW	-	-	-	+	+	-	-	-	-
P1BW	-	-	-	+	-	-	-	-	-
					Methanol				
P1TW	+	+	-	+	-	+	+	+	-
P1MW	+	+	-	+	-	+	+	+	-
P1BW	+	+	-	+	-	+	+	+	-

Note : + = present, - = absent, W = Wood, P2 = Mature Tree, T = Top, M = Middle, B = Bottom

Based on the results of the screening tests, only the crude extracts of *N. subdita* were further studied for antioxidant properties. The yields for methanol extract from each sample young were; P1T (0.740 g), P1M (0.638 g) and P1B (0.761 g) (see Table 3). A second extraction cycle was done to obtain more samples for the tests. Second extraction yielded for P1T (0.910 g), P1M (0.434 g) and P1B (0.820 g) (see Table 3). The yields for methanol extract from each sample mature were; P2T (0.138 g), P2M (0.144 g) and P2B (0.265 g) (see Table

4). A second extraction cycle was done to obtain more samples for the tests. Second extraction yielded for P2T (0.238 g), P2M (0.232 g) and P2B (0.289 g) (see Table 4).

Table 3: Extraction yields for the young tree.

Extracted Parts	First extraction	Second extraction
P1T	0.740 g	0.910 g
P1M	0.638 g	0.434 g
P1B	0.761 g	0.820 g

P1 = Young Tree, W = Wood, Top = Top, M = Middle, B = Bottom

Table 4: Extraction yields for the mature tree.

Extracted Parts	First extraction	Second extraction
P2T	0.138 g	0.238 g
P2M	0.144 g	0.232 g
P2B	0.265 g	0.289 g

P2 = Mature Tree, W = Wood, Top = Top, M = Middle, B = Bottom

Hexane extraction has yielded negligible weight of residue, which indicated the low non-polar content of the sample, and the relatively high presence of polar compounds. The study continued with methanol extracts only, and hexane extracts were eliminated.

Total Phenolic Content (TPC) Assay:

The TPCs (in g gallic acid equivalent (GAE)/100 g extract) of young and mature tree samples of *N. subdita* were investigated as shown in Fig. 1. The middle and bottom parts of heartwood samples for young tree had almost two times higher TPCs when compared to samples of the mature trees. Whereas TPCs of the top part of both young and mature heartwoods were almost equal, but still lower than top and bottom parts of the young heartwoods. The bottom part of young heartwood has the highest TPC (5.359 ± 0.000 g GAE) and the lowest TPC (3.206 ± 0.005 g GAE) for the bottom part of mature heartwood.

Comparison between maturity groups in heartwood parts of TPC:

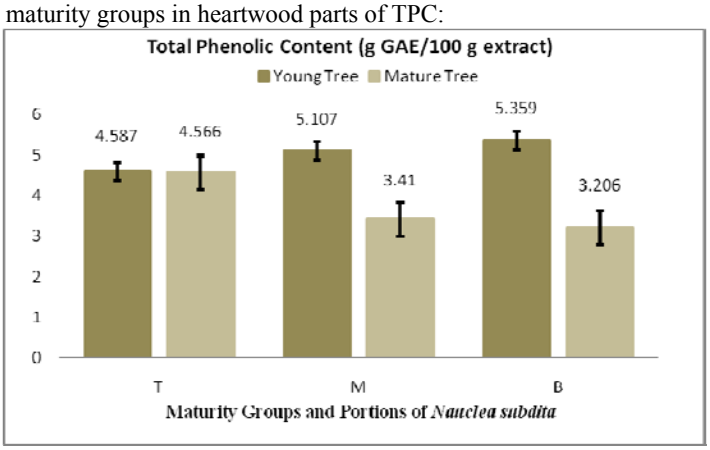

Fig. 1: Level of TPCs in heartwoods of young and mature tree of *N. subdita* in g GAE/100 g extract.

All phenolic compounds of plant origin have the structural requirements as free-radical scavengers and have potential as antioxidants (Jayathilakan *et al.*, 2007). In this study, gallic acid was used as reference standard for TPC and the antioxidant level is expressed in g gallic acid equivalent (GAE) per 100 g of the plant extracts (Lim *et al.*, 2007, Norshazila *et al.*, 2010), since gallic acid is one of the major polyphenolic compounds in plants.

The mean and standard deviation result of phenolic content of all part of samples extracts which is by using methanol is summarized in Fig. 1. The oxidation-reduction reaction of antioxidant compounds with the Folin-Ciocalteau reagent is the basis of the TPC measurement (Verzelloni *et al.*, 2007). A purple-coloured solution was produced when *N. subdita* extracts reacted with Folin-Ciocalteau reagent and sodium carbonate. The intensity of the purple colour of phosphomolybdic-phosphotungstic-phenolic complex increases as the TPCs of the extract increased (Nurliyana *et al.*, 2010). The results indicated that young heartwood of *N. subdita* trees contains higher TPC than the mature heartwood.

Excess free-radicals such as superoxide anion radicals and peroxy radicals in the human body can be scavenged by gallic acid or any equivalents compounds and protect human tissues against oxidative stress

(Rankadilok *et al.*, 2006). The plants with high potential of phenolic compounds to scavenge radicals such as singlet oxygen, superoxide and hydroxyl radicals may be explained by their phenolic hydroxyl groups (Rankovic *et al.*, 2011).

Free-Radical Scavenging Activity:

The primary antioxidant activities of *N. subdita* parts in methanol extracts were measured using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH). The DPPH radical scavenging activities were determined through 517 nm absorbance measurement. Scavenging activity of BHA and Trolox was used as the standard. The mean of measurement of absorbance was calculated using formulation stated earlier (Scavenging capacity formulae). The percentages of inhibition of free radical by both BHA and Trolox as standards are depicted in Fig. 2, whereas for young and mature trees are shown in Fig. 3 and Fig. 4. According to Fig. 3 and Fig. 4 showed the DPPH radical scavenging activity of maturity groups between young and mature trees from different portions were top (T), middle (M) and bottom (B). The Fig. 3 and Fig. 4 showed the mean and SD in P1T, P1M and P1B where six concentration are tested in these studies. All samples showed the trend increase when inhibit from lower to the higher concentration. The IC_{50} of Trolox and BHA are 0.11 mg/mL and 0.09 mg/mL respectively (Fig. 2). The antioxidant standards used in this study (Trolox and BHA) exhibited good free radical scavenging activity from 96 % of scavenging activity. The Fig. 3 showed IC_{50} of PIT (> 5 mg/mL), PIM (3.5 mg/mL) and P1B (1.71 mg/mL) in young trees of heartwood extracts, which indicates the remarkable antioxidant activity of the extracts. The Fig. 4 showed the P2T (>5 mg/mL), P2M (> 5 mg/mL) and P2B (4.91 mg/mL) of mature trees in heartwood extracts. From the graph, it was shown that the strength of the scavenging activity followed the order of BHA > Trolox > methanol. In the present of study, methanolic extracts of samples showed the potential free-radical scavenging activity.

Comparison of heartwood parts between portions and maturity groups in radical scavenging activity:

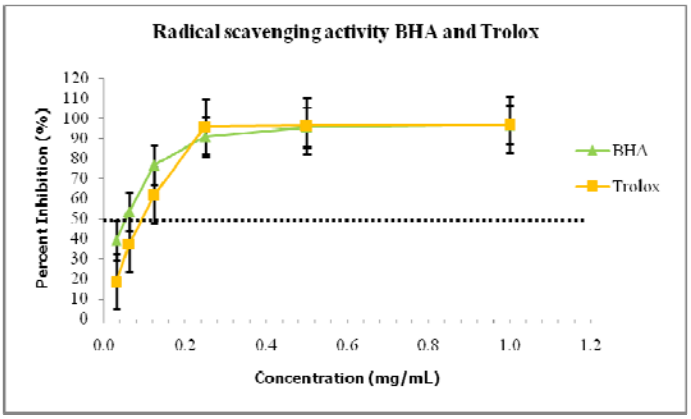

Fig. 2: Mean ± SD and IC_{50} of BHA and Trolox.

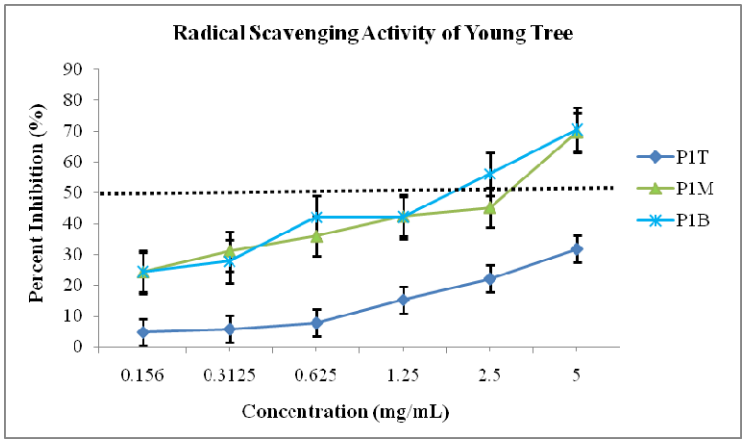

Fig. 3: The radical scavenging activity between portions in young trees showing mean ± SD and IC_{50}

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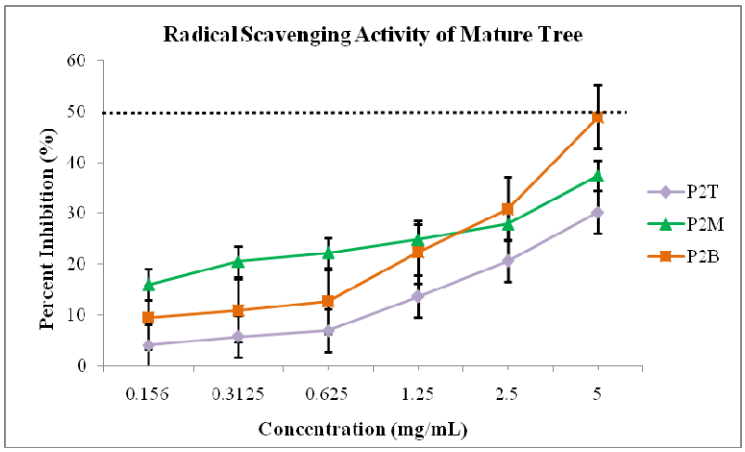

Fig. 4: The radical scavenging activity between portions in mature trees showing mean ± SD and IC_{50}

The TPC of *N. subdita* heartwood parts were found higher in young trees compared to the mature tree's extracts. As we can see every portion show the varieties of the result where some parts are higher and lower from IC_{50} level. The free-radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic DPPH. DPPH has been widely employed to evaluate the free-radical scavenging activity of natural antioxidants. This plant can be higher than standard from the isolation pure compound. Research also indicates that the DPPH test is particularly suitable for the evaluation of antioxidant activity of crude extracts (Nurliyana *et al.,* 2010). DPPH is the paramagnetic compound with an odd electron shows strong absorption band in methanol. DPPH shows a strong absorption band at 760 nm in the visible spectrum (deep purple color) (Norshazila *et al.,* 2010). As the electron became paired off in the presence of free-radical scavenging, the absorption vanishes and the resulting discoloration stoichiometrically coincides with respects to the number of electrons taken up (Rankovic *et al.,* 2011). The scavenging activity of antioxidants by hydrogen donation decreases the absorbance as the color changes from purple to yellow. It is strongly recommended that a fresh DPPH is prepared prior to assay (Rankovic *et al.,* 2011).

The *N. subdita* heartwood parts extract showed promising free radical scavenging effect of DPPH in a concentration dependent manner. The reference standard BHA and Trolox also demonstrated a significant radical scavenging potential in the concentration used (Norshazila *et al.,* 2010). The extracts of *N. subdita* were considered to play important roles in the prevention the diseases and may lower the risk of the diseases.

The Table 5 and Table 6 showed all results of heartwood extracts between three portions in mean and SD in six concentration used. These graphs showed that inhibition percentage of BHA and Trolox with six different concentrations. The result presented that P1T showed a maximum inhibition with 31.767 ± 0.281 of maximum inhibition and 4.767 ± 0.281 of minimum inhibition as minimum at 0.3125 concentrations. The result presented that P1M showed a maximum inhibition of 69.557 ± 0.489 at maximum concentration used, and 24.237 ± 0.091 at the least concentration. The result presented that for sample P1B had 70.517 ± 0.368 maximum inhibition and 24.267 ± 0.983 of minimum. The result presented for sample P2T had 30.250 ± 0.632 maximum inhibitions and 4.0167 ± 0.013 of minimum. The result presented for sample P2M had 37.427 ± 1.173 maximum inhibitions and 15.990 ± 0.476 of minimum. The result presented for sample P2B had 48.970 ± 0.598 maximum inhibitions and 9.537 ± 0.394 of minimum.

All analyses were the mean of triplicate measurements ± standard deviation and results are expressed in percent of free radical inhibition. All samples are significant between portions but not significant between maturity groups.

Tables 5 and 6 shows the ANOVA on percent inhibition of the young and mature trees measured by DPPH with values of mean ± SD, IC_{50} and F values. There were significant differences between young or mature tree and between concentration of the samples. The interaction between trees and concentration of young and mature trees are F is equal to 49.649 and 97.323 respectively.

Usually, antioxidant activities are expected in presence of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features (Bhuiyan *et al*., 2010). DPPH assay is to measure the primary antioxidant activity of each sample in the present study. The antioxidant shows the ability to remove the color changes from purple to yellow due to the ability of hydrogen donating. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The absorbance show decreases shortly that will show the highest potent of primary anti-oxidant activity (Nurliyana *et al.*, 2010).

Table 5: Percent inhibition of young trees measured by DPPH, Three replicate, significant value $p < 0.05$, Duncan's Multiple Test, two-way ANOVA.

	Young Tree		
Tree portions	Concentration (mg/L)	Means ±SD	IC_{50}
Top	0.156	4.767 ±0.281	
	0.313	5.780 ±0.303	
	0.625	7.780 ±0.225	> 5.0 mg/mL
	1.250	15.277 ±0.247	
	2.500	22.097 ±0.671	
	5.000	31.767 ±0.281	
Middle	0.156	24.237 ±0.091	
	0.313	31.037 ±0.048	
	0.625	35.827 ±1.096	3.5 mg/mL
	1.250	42.300 ±1.048	
	2.500	45.110 ±0.684	
	5.000	69.557 ±0.489	
Bottom	0.156	24.267 ±0.983	
	0.313	27.710 ±0.693	
	0.625	42.027 ±0.074	1.71 mg/mL
	1.250	42.180 ±0.000	
	2.500	56.050 ±0.130	
	5.000	70.517 ±0.368	
Young Tree			F = 842.898*
Concentration			F = 105.476*
Interaction			F = 49.649*

IC_{50} = Inhibition concentration

Table 6: Percent inhibition of mature tree measured by DPPH**,** Three replicate, significant value $p < 0.05$, Duncan's Multiple Test, two-way ANOVA.

	Mature Tree		
Tree portions	Concentration (mg/L)	Means ±SD	IC_{50}
Top	0.156	4.0167 ±0.013	
	0.313	5.7767 ±0.394	
	0.625	6.927 ±0.134	> 5.0 mg/mL
	1.250	13.677 ±0.152	
	2.500	20.677 ±0.394	
	5.000	-	
Middle	0.156	15.990 ±0.476	
	0.313	20.517 ±0.992	
	0.625	22.220 ±0.684	>5.0 mg/mL
	1.250	24.890 ±0.182	
	2.500	27.857 ±0.585	
	5.000	37.427 ±1.173	
Bottom	0.156	9.537 ±0.394	
	0.313	10.937 ±0.524	
	0.625	12.667 ±0.082	4.91 mg/mL
	1.250	22.397 ±0.983	
	2.500	30.887 ±0.212	
	5.000	48.970 ±0.598	
Mature Tree			F = 771.464*
Concentration			F = 1.219*
Interaction			F = 97.323*

IC_{50} = Inhibition concentration

Conclusion:

The methanolic crude extracts of *N. subdita* show the presence of phenolic, flavanoids, terpenoids, alkaloids, phytosterols and saponins compounds in the heartwood of young and mature trees. The phytosterols were detected in the hexane extracts. The extracted hexane yielded negligible weight of residue indicating the low non-polar content and the relatively high presence of polar compounds. The middle and bottom parts of heartwood of the young *N. subdita* possess TPCs which are twice higher when compared to the mature trees. Equal amounts of TPCs were obtained from the top part of both young and mature heartwoods, but lower than that of the top and bottom parts of the young heartwoods.

Purple-coloured solution was produced when *N. subdita* extracts reacted with Folin-Ciocalteau reagent and sodium carbonate. The intensity of the purple colour of phosphomolybdic-phosphotungstic-phenolic complex increases as the TPCs of the extract increased, which indicate that the young heartwood of *N. subdita* trees contains higher TPC than the mature heartwood.

The scavenging activity of the samples showed the trend increase when inhibit from lower to the higher concentration. The antioxidant standards used in this study (Trolox and BHA) exhibited good free radical

scavenging activity from 96 % of scavenging activity. The methanolic extracts of samples showed the potential free-radical scavenging activity.

The TPC of *N. subdita* heartwood parts was found higher in young compared to the mature trees. The free-radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic DPPH. This plant can be higher than standard from the isolation pure compound. DPPH shows a strong absorption band at 760 nm in the visible spectrum (deep purple color). The scavenging activity of antioxidants by hydrogen donation decreases the absorbance as the color changes from purple to yellow. It is strongly recommended that a fresh DPPH is prepared prior to assay.

The *N. subdita* heartwood parts extract showed promising free radical scavenging effect of DPPH in a concentration dependent manner. The extracts of *N. subdita* were considered to play important roles in the prevention the diseases and may lower the risk of the diseases.

The antioxidant activity observed with the presence of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features. The absorbance ability decreases shows high potent of the primary antioxidant activity.

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