Journal of Traditional and Folk Practices
Volume 05(2), December 2017, 3 - 152.
ISSN 2278 - 5906

Assessment of free radical scavenging activity and polyphenolic content in leaves of *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. - a medicinal plant used by Cholanaickan tribe in India

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Received: 25 May 2017 Accepted: 08 July 2017

Abstract

Neurocalyx calycinus (R. Br. ex Benn.) Rob. is an endemic species of the family Rubiaceae, found in Western Ghats of India and Sri Lanka. The leaf paste of *N. calycinus* has been used by Cholanaickan tribe as medicine to heal wounds, burns, inflammation and pain. Many of the human diseases are manifested due to the accumulation of free radicals. Plant derived antioxidants have the power to scavenge these free radicals without any internal complications. Aqueous, n-hexane, chloroform, ethyl acetate and ethanol extracts of the leaves of *N. calycinus* were selected for qualitative phytochemical screening. DPPH, nitric oxide, superoxide and hydroxyl radical scavenging assays were carried out. Total phenolic, flavonoid and antioxidant content of all the extract was measured. Phytochemical screening showed that alkaloids, steroids, phenolic compounds, glycosides and saponins were found in the ethanolic extract. Ethanol extract showed better antioxidant activity than the other solvent extracts. The reducing power of all the extracts was increased dose dependently. Highest amount of phenolic (56.27 mg of gallic acid equivalent /gm of dried extract), flavonoid (36.92 mg of quercetin equivalent /gm of dried extract) and antioxidant (85.68 mg of ascorbic acid equivalent/gm of dried extract) content was found in the ethanolic extract. A positive correlation (R^2) was found between the total phenolic content and free radical scavenging activity of the leaf extract of *N. calycinus.* The results confirmed that the phenolic compounds in the ethanolic extract of the leaves of *N. calycinus* are potent source of natural antioxidants.

Keywords: Antioxidant activity, Cholanaickan tribe, Free radical, *Neurocalyx calycinus*, Rubiaceae

1. Introduction

The free radicals have been well established as the root cause of many human disorders and disease conditions. A free radical is defined as the occurrence of an unpaired electron in an organic molecule or metal ion, which is highly unstable and reactive, seeking to acquire electrons from other substances. Wide ranges of plant based phenolic compounds are recommended for the scavenging of free radicals. They play an excellent role as antioxidants, free radical scavengers and chelators due to the presence of hydroxyl substituent and their aromatic structure which enables them to scavenge free radicals (Kahkonen *et al*., 1999). The use of plants in traditional/tribal medicine provides a large source of natural antioxidants that might serve as leads for the development of novel anti-inflammatory, antiulcerogenic, antinecrotic, neuroprotective and hepatoprotective drugs (Repetto and Llesuy, 2002). Therefore, investigations of herbal

antioxidants and active molecules in traditional medicines and their use in treating certain human diseases have received much attention (Lin *et al*., 2010). The modern drug discovery screening techniques and traditional knowledge base have given clues to the discovery of valuable drugs (Surveswaran *et al*., 2006). It was estimated that in India 70% of the population remains dependent on traditional medicines to help meet their health care needs (WHO, 2005) and nearly 7000 species of plants are recognized as of ethnobotanically important (Anonymous, 1994).

Neurocalyx Hook. is an endemic taxon in the family Rubiaceae, mainly distributed in southern Western Ghats of India and Sri Lanka (Bremer, 1979). *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. is a herbaceous plant restricted to the rocky crevices near the streams in the evergreen forests and grows up to 20 inches (Bremer, 1987; Takhtajan, 2009). The medicinal use of *N. calycinus* was first documented by Dr. S. Rajasekharan and team in 1987 during ethnobiological studies carried out in the Cholanaickan tribal settlement of Nilambur forest in Malappuram district, Kerala, India (unpublished data). According to tribal information (Figure 1A), the fresh leaves of *N. calycinus* (Figure 1B), locally know as 'Pachachedi' is prepared in the form of paste and applied externally to arrest the bleeding due to bear bites, and heals fresh wounds, inflammation and pain (which is used according to the required quantity) and decoction of the leaves (30 mL) administered orally thrice a day for one week to one month depending upon the symptoms (Saradamma *et al*., 1987).

Fig. 1: Tribal information of *Neurocalyx calycinus*: **A.** Kuppamala Kaniyan, a Cholanaickan physician, holding the plant, *N. calycinus* and narrating its therapeutic effects; **B.** Habitat of *N. calycinus.*

As far as literature survey could ascertain, free radical scavenging activities of *N. calycinus* have not previously been published. Hence, the objective of present study was to evaluate the total phenolic content, free radical scavenging and antioxidant activities of the leaf extracts of *N. calycinus* to understand the usefulness of this plant in medicine.

2. Materials and Methods

2.1. Chemicals

Gallic acid, potassium acetate, quercetin, sodium phosphate, 2-deoxy-D-ribose, α, α diphenyl-2-picryl-hydrazyl (DPPH), sulfanilamide, reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside, L-ascorbic acid, phenazine methosulphate (PMS) and N-(1-naphthyl) ethylenediamine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Aluminium trichloride, nitroblue tetrazolium (NBT), trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu's phenol reagent (FCR), sodium hydroxide, hydrogen peroxide and potassium ferricyanide, were purchased from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. All other reagents, chemicals, and solvents were of analytical grade and were purchased from Hi Media Laboratories, Mumbai (India). Phosphate buffered saline (PBS) and other reagents were prepared according to standard protocol.

2.2. Collection plant material

The fresh leaves of *N. calycinus* were collected from the evergreen forest streams of Ezhumadakku (Latitude 8°37'28"N, Longitude 77°12'53"E and Altitude 1200-1600 m asl) in Athirumala, Thiruvananthapuram, Kerala, India in the month of April 2014. The plant material was taxonomically identified by the plant taxonomist of JNTBGRI. A voucher specimen (TBGT 86801 dated 14/12/2015) was certified and deposited at the Institutional Herbarium.

2.3. Preparation of plant extract

The leaves were thoroughly washed, segmented, shade dried at room temperature for one week and powdered to 80 mesh size (Usha Shriram (India), Noida, UMA 29103448). One part of the powdered plant material (500 g) was extracted with water *viz.,* maceration technique upto 24 h. The extract was filtered and concentrated to obtain a solid consistency of NCAQ. The other part of the plant material (500 g) was used for successive solvent extraction using n-hexane, chloroform, ethyl acetate and ethanol (98 % v/v) using Soxhlet apparatus, until the solvent becomes colourless (Harborne, 1998). Each time before extracting with the next solvent, marc was air dried below 35 ± 5 °C. The extract was filtered (Whatman filter paper No. 1), concentrated under reduced pressure at 30 ± 10 mbar in a rotary evaporator at 30 ºC – 60 ºC (Rotavapor R-215, Buchi, Switzerland) to obtain NCHE, NCCE, NCEA and NCET. Colour of each extract was determined using horticultural colour chart (Wilson, 1938). The yields of all the extracts were determined and expressed in percentage, dried in desiccators (Auto Secador 401110, USA) and were stored in air-tight containers in refrigerator at 4ºC.

2.4. Qualitative phytochemical screening

All the leaf extracts were subjected to qualitative phytochemical tests to detect the presence of various phytoconstituents (Sofowora, 1982; Harborne, 1998) such as carbohydrates (Molisch's test, Fehling's test, Barfoed's test and Benedict's test), proteins (Millon's test, Biuret test and Ninhydrin test), alkaloids (Mayer's test, Wagner's test, Hager's test and Dragendorff's test), glycosides (Borntrager's test, Legal's test, Keller Kiliani test and Kedde test), phenolic compounds (ferric chloride test, gelatin test, lead acetate test, alkaline reagent test and Shinoda's test), phytosterols (Libermann–Burchard's test and Salkowski's test), fixed oils and fats (spot test and saponification test), saponins (foam test), gum and

mucilage (alcohol 95% test), volatile oils (steam distillation), anthraquinones (chloroform - 10% ammonia test) and iridoids (Trim- Hill reagent test).

2.5. α, α-diphenyl-β-picryl-hydrazyl radical scavenging activity

The free radical scavenging ability of the plant extracts was estimated by using DPPH assay (Brand-Williams *et al*., 1995). DPPH solution (100 µM) was prepared in chilled methanol (the absorbance value is less than 1). Various concentrations (1mL) of each plant extracts (0.1 – 0.5 mg/mL) and standard L-ascorbic acid (0.01 - 0.08 mg/mL) in 98% (v/v) methanol were allowed to react with 2 mL of DPPH solution. After shaking vigorously, the reaction mixture was set aside in the dark at 30 ºC for 30 min. and absorbance was recorded at 517 nm using a UV-VIS spectrophotometer (G9821A, Cary 100 UV-Vis spectrophotometer, Agilent Technologies, USA). The % reduction of DPPH radical scavenging activity, Q was calculated using the following formula:

$$Q = 100 [(Ao - Ac) / Ao]$$

where Ao is the absorbance of DPPH radical + methanol; Ac is the absorbance of DPPH radical + sample extract/standard. The IC_{50} value (concentration of substrate that causes 50 % loss of the DPPH activity) was determined by using the dose response curve through GraphPad Prism software.

2.6. Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity of the plant extracts was estimated by Griess Illosvoy reaction with slight modification (Marcocci *et al*., 1994). At physiological pH 7.4, sodium nitroprusside generates nitric oxide radicals under aerobic conditions which interact with oxygen to produce stable nitrite ions that can be estimated using Griess reagent. sodium nitroprusside (1 mL, in 10 mM phosphate buffer, pH 7.4) was mixed with 1 mL of various concentrations of plant extracts (0.1 – 0.5 mg/mL) and standard L-ascorbic acid (0.01 - 0.08 mg/mL) in 98% (v/v) methanol and incubated at 29ºC for 2.5 h. An aliquot of 1 mL of the incubated solution was taken out, and one mL of freshly prepared Griess reagent [1% (w/v) sulphanilamide in 2% (v/v) o-phosphoric acid and 0.1 % (W/V) of N-1- napthylethylene diamine dihydrochloride] added to it. The absorbance of the pink coloured chromophore in diffuse light was read at 540 nm using the UV-VIS spectrophotometer against solvent blanks. The % reduction of nitric oxide radical scavenging activity was calculated using the following formula;

$$Q = 100 [(Ao - Ac) / Ao]$$

where, Ao is the absorbance of nitric oxide radical + ethanol; Ac is the absorbance of nitric oxide radical + sample extract/standard. The IC_{50} value was determined using the dose response curve through GraphPad Prism software.

2.7. Hydroxyl radical scavenging assay

The 2-deoxyribose degradation assay was used to determine the scavenging effect of the extracts on the hydroxyl radical (Halliwell *et al*., 1987). Each reaction mixture contained 100 µL of 2.8 mM 2-deoxy-2-ribose, 200 µL of 104 µM EDTA, 200 µL of 100 µM $FeCl_3$, 100 µL of 1 mM H_2O_2, 100 µL of 100 µM L-ascorbic acid in 20 mM potassium phosphate buffer solution (pH 7.4) and different concentrations of (0.1 to 0.5 mg/mL) plant extracts, in a final volume of 2 mL. The mixtures were incubated for 1 h at 37°C, followed by addition of 1 mL of 1% (w/v) TBA

in 0.05 M NaOH and 1mL of 2.8% (w/v) TCA. The resulting mixture was heated for 20 min at 100°C. After cooling on ice, absorbance was measured at 532 nm. Inhibition of 2-deoxyribose degradation expressed in percentage was calculated as per the equation:

$$Q = 100 [(Ao - Ac) / Ao]$$

where, A_0 is the absorbance of the control, and Ac is the absorbance of the tested sample. The IC_{50} value was determined using the dose response curve through GraphPad Prism software.

2.8. Superoxide radical scavenging assay

The ability of plant extract to inhibit the superoxide radicals was determined by the Nitroblue tetrazolium reduction method (Nishikimi *et al*., 1972). NBT (1 mL, 156 µM) in 100 mM phosphate buffer solution (pH 7.4), 1 mL of 468 µM NADH in 100 mM phosphate buffer solution (pH 7.4) and 0.1 mL of different concentrations of the plant extracts were mixed. The reaction was started by adding 100 µL of 60 µM phenazine methosulphate in 100 mM phosphate buffer (pH 7.4) solution to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank samples, containing all the reagents except the PMS. Ascorbic acid was used as the standard. The % reduction of superoxide radical scavenging activity was calculated using the following formula;

$$Q = 100 [(Ao - Ac) / Ao]$$

where Ao is the absorbance of control; Ac is the absorbance of superoxide radical + sample extract/ standard. The IC_{50} value was determined by using the dose response curve through GraphPad Prism software.

2.9. Determination of reducing power activity

The reductive ability (iron (III) to iron (II) transformation) of samples was determined by the method of Fejes *et al*. (2000). Various concentrations (10 – 500 µL) of samples (1 mg/ mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %); the mixture was incubated at 50 °C for 20 min. At the end of incubation, trichloroacetic acid (2.5 mL, 10 % w/v); was added to the mixture and centrifuged at 3000 g for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and freshly prepared ferric chloride (0.5 mL, 0.1%). The absorbance was measured at 700 nm. An increase in absorbance indicated increase in reducing power. Ascorbic acid was used as the reference compound.

2.10. Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phospho molybdenum method according to the procedure described by Prieto *et al*. (1999). The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.2 mL extract was combined with 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm, using a UV-VIS spectrophotometer against blank, which contained 2 mL of reagent solution and the approximate volume of same solvent was used for the sample, after cooling to room temperature. The total antioxidant capacity was expressed as equivalents of ascorbic acid

(mg AE/g of dry extract). The calibration curve was prepared by mixing ascorbic acid (250 to 15.625 µg/mL) with methanol.

2.11. Determination of the total phenolic content

TPC of the extracts was determined by using the F-C reagent colorimetric method (Ainsworth and Gillespie, 2007). F-C reagent (200 µL, 10 % v/v) was added to 100 µL of plant extracts (mg/mL) dissolved in 95% (v/v) methanol and vortexed thoroughly. After 3 min, 800 µL of 700 mM sodium carbonate was added and incubated at 37ºC for 1 h. The absorbance was measured at 765 nm, using a UV-VIS spectrophotometer. The calibration curve was prepared by using the standard known concentrations of gallic acid (serial dilutions covering 8 µM – 6 mM in 95% (v/v) methanol). The TPC of the samples was expressed as mg of GAE per gm of the dried extract.

2.12. Determination of the total flavonoid content

TFC of the extracts was determined by using the aluminium chloride colorimetric method (Chang *et al*., 2002). 5 mL reaction mixture containing 500 µL of plant extracts (mg/mL) dissolved in methanol, 1.5 mL of methanol, 100 µL of 10 % (w/v) aluminium trichloride, 100 µL of 1 M potassium acetate and 2.8 mL of distilled water was gently mixed for 5 min. by vortexing and kept at 37ºC for 30 min. The absorbance was measured at 415 nm using a UV-VIS Spectrophotometer. The calibration curve was prepared by using the standard known concentrations of quercetin (serial dilutions covering 10 µg – 80 µg in 98 % (v/v) methanol). The TFC was expressed as mg of QE per gm of the dried extract.

2.13. Statistical analysis

All experiments were carried out in triplicate. Results were expressed as mean ± standard error of the mean (SEM). The IC_{50} values were calculated by non-linear regression. Correlation analysis was performed using Pearson correlation coefficient by paired t tests. $p< 0.05$ was considered significant. Statistical calculations were performed using the GraphPad Prism 7.01 program (GraphPAD, San Diego, California, USA).

3. Results

3.1. Extractive yield

The extractive value, consistency and colour of leaf extracts of (NCAQ, NCHE, NCCE, NCEA and NCET) *N. calycinus* was determined and tabulated (Table 1). The aqueous extract gave maximum extractive yield (16.23 % w/w), compared to other solvent extracts. Amongst all organic solvents, highest extractive yield was in polar solvent ethanol. The extractive yield of nonpolar solvent, n-hexane the extract was less but more than the semi-polar solvent extracts, chloroform and ethyl acetate.

Table 1: Extractive yield of different solvent extracts of *N. calycinus* leaves

Plant extract	Consistency	Colour	Yield (% w/w)
NCAQ	Solid	Maroon 1030	16.23 ± 0.29
NCHE	Sticky	Willow green 000862/1	1.77 ± 0.20
NCCE	Sticky	Ivy green 0001060	2.41 ± 0.23
NCEA	Sticky	Leek green 000858	3.26 ± 0.27
NCET	Sticky	Leek green 000858/2	8.41 ± 0.37

Each value is the average of three measurements (n=3) ± SE.

3.2. Qualitative phytochemical analysis

Phytochemical screening of various solvent extracts of leaf of *N. calycinus* confirmed the presence of primary metabolites such as carbohydrates, proteins and amino acids and secondary metabolites such as alkaloids, steroids, phenolic compounds, glycosides, saponins and the absence of anthraquinones, gums, mucilage and volatile oils (Table 2). The ethanolic extract (NCET) showed maximum number of phyto constituents followed by ethyl acetate extract.

Table 2: Qualitative phytochemical analysis of *N. calycinus* leaves

Phytoconstituents		NCAQ	NCHE	NCCE	NCEA	NCET
Carbohydrates	Molish's test	+ + +	−	−	+ + +	+ + +
	Fehling's test	+ + +	−	+ +	+ +	+ +
	Barfoed's test	+ + +	−	−	+ + +	+ +
	Benedict's test	+ + +	−	+ +	+ + +	+
Proteins and amino acids	Millon's test	+ +	−	−	+	+
	Biuret test	+ +	−	+	+	+
	Ninhydrin test	+ +	−	+	+	+
Alkaloids	Mayer's reagent	+	−	+	+	+
	Wagner's reagent	+	−	+	+	+ + +
	Hager's reagent	−	−	+	+ +	+ + +
	Dragendorff's reagent	−	−	−	+	+
Glycosides	Borntrager's test	−	−	−	−	−
	Legal's test	−	−	−	−	+
	Keller – Kiliani test	−	−	−	+ +	+ + +
	Kedde test	−	−	−	+	+ +
Phenolic compounds	Ferric chloride test	+ +	−	+ +	+	+ + +
	Gelatin test	+	−	+	+	−
	Lead acetate test	+ +	−	−	−	+ +
	Alkaline reagent test	−	−	+	+ +	+ + +
	Shinoda's test	−	−	−	+	+ +
Phytosterols	Libermann Burchard	−	+ + +	−	−	−
	Salkowski reaction	−	+ +	−	+	+
Fixed oils and fats	Spot test	−	+	−	−	−
	Saponification test	−	+ +	−	−	−
Saponins	Foam test	+	−	−	+	+
Gum and mucilage	Alcohol 95% test	−	−	−	−	−
Volatile oils	Steam distillation	−	−	−	−	−
Iridoids	Trim- Hill reagent test	−	−	−	+	+ + +
Anthraquinones	chloroform - 10% ammonia test	−	−	−	−	−

+ = slightly present, + + = moderately present, + + + = highly present,− = absent. All the tests were carried out three times. Observations were based on the colour intensity and precipitation with appropriate reagents.

3.3. DPPH radical scavenging activity

The scavenging activities of leaf extracts of *N. calycinus* were determined using free radicals of DPPH and compared with positive control, ascorbic acid (Figure 2 and Table 3). The result showed that among the various extracts of *N. calycinus*, NCET (IC_{50} = 0.161 mg/mL) possessed the maximum antioxidant activity while NCHE (IC_{50} = 0.460 mg/mL) had the lowest scavenging effect, as compared to other extracts. It was found that DPPH radical scavenging activities of ascorbic acid were greater than those of NCET.

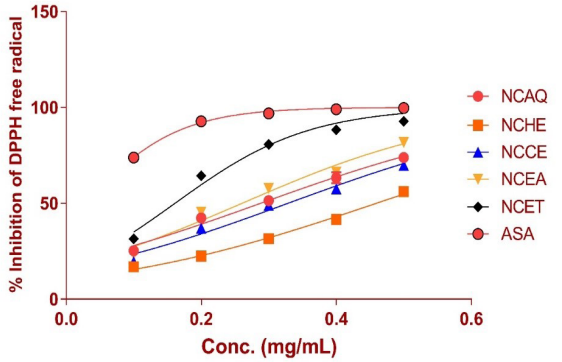

Fig. 2: DPPH radical scavenging activity of various extracts from the leaves of *N. calycinus* at different concentrations. The values are expressed as Mean ± SE, n = 3.

3.4. Nitric oxide radical scavenging activity

Nitric oxide is a reactive free radical generated from sodium nitroprusside in aqueous solution at physiological pH and reacts with oxygen to form nitrite that can be estimated by using Griess reagent. Suppression of released NO may be partially attributed to direct NO scavenging. The NCET (IC_{50} = 0.224mg/mL) showed highest NO scavenging activity (Figure 3 and Table 3) compared to other extracts of *N. calycinus*. The standard ascorbic acid (IC_{50} = 0.068 mg/mL) was more potent than the NCET.

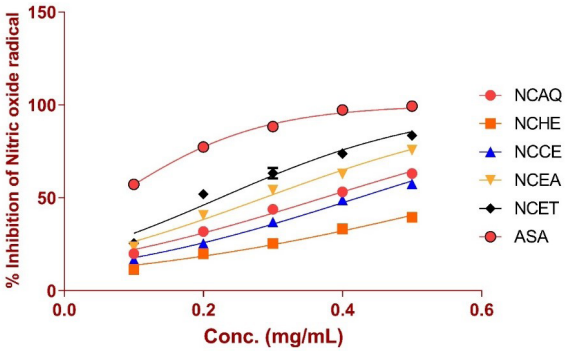

Fig. 3: Nitric oxide radical scavenging activity of various extracts from the leaves of *N. calycinus* at different concentrations. The values are expressed as Mean ± SE, n = 3.

3.5. Hydroxyl radical scavenging activity

The effect of various solvent extracts of leaves of *N. calycinus* on oxidative damage, induced by Fenton reaction was measured by the TBA method (Figure 4 and Table 3). All the extracts have a stronger concentration-dependent inhibition of deoxyribose oxidation. Among them, NCET (IC_{50} = 0.237 mg/mL) had highest hydroxyl radical scavenging capacity, while NCHE (IC_{50} = 0.684 mg/mL) had the lowest. The decreasing order of hydroxyl radical scavenging activity of the extracts was found to be NCET > NCEA > NCCE > NCAQ > NCHE.

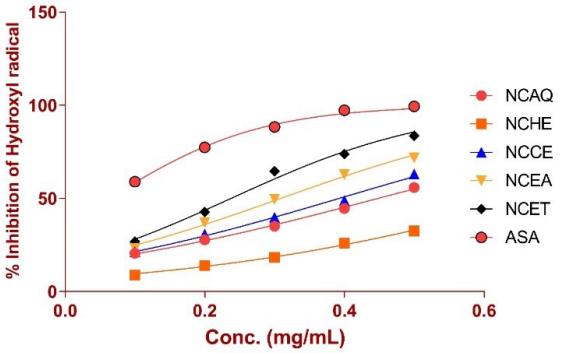

Fig. 4: Hydroxyl radical scavenging activity of various extracts from the leaves of *N. calycinus* at different concentrations. The values are expressed as Mean ± SE, n = 3.

3.6. Super oxide radical scavenging activity

The superoxide anion radicals are derived in PMS, NADH, NBT system, where the decrease in absorbance at 560 nm with the extracts of *N. calycinus* indicates the consumption of superoxide anion in the reaction mixture, thereby exhibiting a dose dependent (0.1 to 0.5 mg/mL) increase in superoxide scavenging activity (Figure 5 and Table 3). Among the various extracts, NCET (IC_{50} = 0.195 mg/mL) showed maximum superoxide radical scavenging activity (87.93% at 0.5 mg/mL).

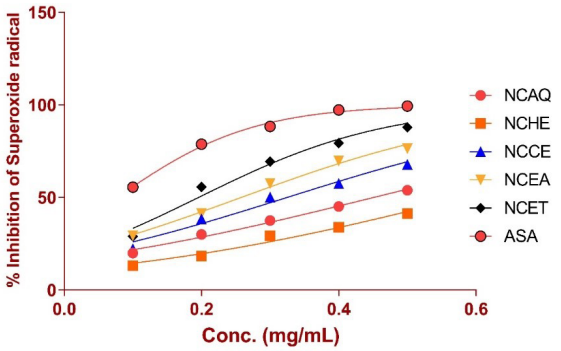

Fig. 5: Superoxide radical scavenging activity of various extracts from the leaves of *N. calycinus* at different concentrations. The values are expressed as Mean ± SE, n = 3.

Table 3: IC_{50} values of different extracts of *N. calycinus* leaves for various antioxidant systems

Plant extracts	IC_{50} value (mg/mL) of radical scavenging activity			
	DPPH	Nitric oxide	Hydroxyl	Super oxide
NCAQ	0.288 ± 0.009	0.372 ± 0.006	0.448 ± 0.010	0.451 ± 0.009
NCHE	0.460 ± 0.007	0.604 ± 0.015	0.684 ± 0.017	0.583 ± 0.018
NCCE	0.329 ± 0.007	0.422 ± 0.006	0.392 ± 0.006	0.324 ± 0.008
NCEA	0.261 ± 0.009	0.287 ± 0.009	0.309 ± 0.005	0.258 ± 0.006
NCET	0.161 ± 0.007	0.224 ± 0.010	0.237 ± 0.007	0.195 ± 0.008
ASA	0.025 ± 0.006	0.068 ± 0.006	0.062 ± 0.007	0.076 ± 0.005

IC_{50} = Half maximal inhibitory concentration; Each value in the table is represented as Mean ± SE (n=3).

3.7. Reducing power activity

The reducing power assay serves as a significant indicator of potential antioxidant activity. The conversion of iron (Fe^{3+}) in ferric chloride to ferrous (Fe^{2+}) is due to an increase in the antioxidant activity of plant extract. Aqueous extract and different solvent extracts of *N. calycinus* showed concentration-dependant reductive effects (Fig. 6). Among them, the ethanolic extracts containing a high amount of reductone fraction showed highest reducing power.

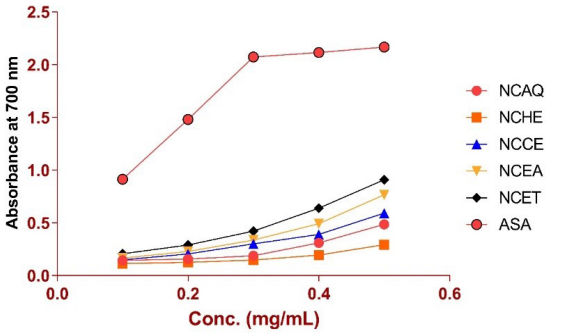

Fig. 6: Reducing power assay of various extracts from the leaves of *N. calycinus* at different concentrations. The values are expressed as Mean ± SE, n = 3.

3.8. Total antioxidant capacity

Total antioxidant capacity was determined spectrophotometrically through phosphor molybdenum method. Antioxidant capacity of ascorbic acid has been used as reference standard by which plant extracts with potential antioxidant activity was compared and expressed as equivalents of ascorbic acid. The total antioxidant activity determination of *N. calycinus* leaf extract showed that ethanolic extract contained highest (85.68 mg of AE/gm of dried extract) antioxidant compounds as equivalents of ascorbic acid, which effectively reduced the oxidant in the reaction matrix (Figure 7).

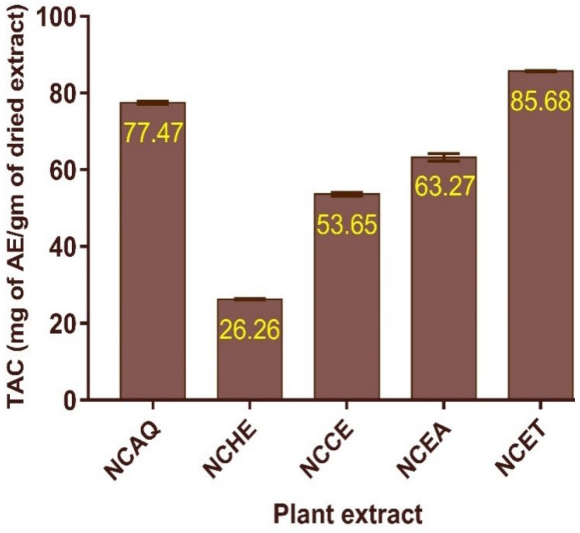

Fig. 7: Total antioxidant capacity of leaf extracts of *N. calycinus*. The values are expressed as Mean ± SE, n = 3.

3.9. Total phenolic content

TPC of *N. calycinus* leaf extract was estimated and represented in terms of gallic acid equivalent (Fig. 8 and Table 4). Among all the extracts, the highest total phenol content was found in the NCET (57.15 mg of GAE/gm of dried extract).

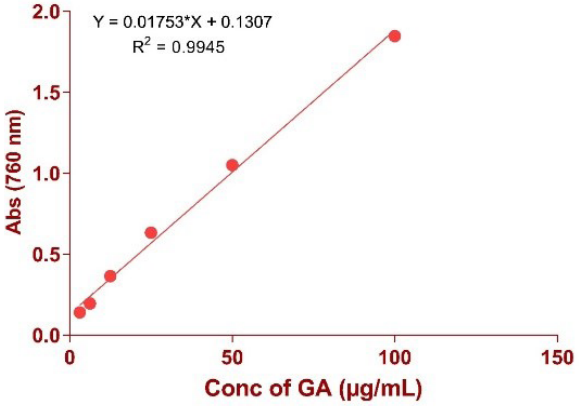

Fig. 8: Total phenolic content estimation of leaf extracts of *N. calycinus*; Absorbance of Gallic acid at 760 nm

3.10. Total Flavonoid content

Total flavonoid content of *N. calycinus* leaf extract was estimated and represented in terms of quercetin equivalent (Fig. 9 and Table 4). Among all the extracts, the highest total flavonoid content was found in the NCET (36.87 mg of QE/gm of dried extract).

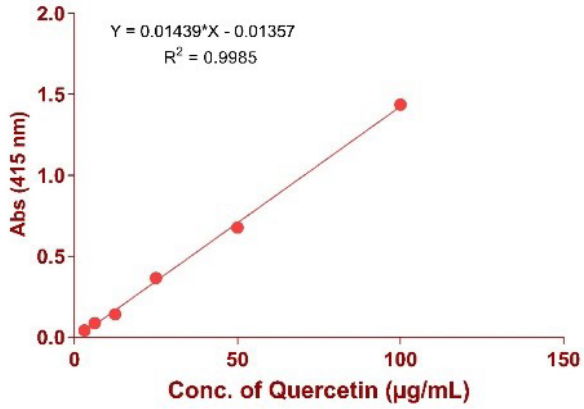

Fig. 9: Total flavonoid content estimation of leaf extracts of *N. calycinus*; Absorbance of quercetin at 415 nm

Table 4: Total phenolic and flavonoid contents in the various solvent extracts of leaves of *N. calycinus*

Plant extracts	TPC (mg of GAE/gm of dried extract)	TFC (mg of QE/gm of dried extract)
NCAQ	38.71 ± 2.86	17.86 ± 1.40
NCHE	9.13 ± 1.33	4.53 ± 0.98
NCCE	36.24 ± 2.08	28.87 ± 1.30
NCEA	41.40 ± 1.10	20.77 ± 1.14
NCET	56.27 ± 2.10	36.92 ± 1.23

The values are Mean (n =3) ± SE.

3.11. Correlation analysis of different antioxidant assays

The IC_{50} values of different antioxidant assays were correlated with TPC and TFC as assessed using linear regression analysis. The TPC (Fig. 10 and Table 5) showed a strong correlation with DPPH radical scavenging assay (R^2 = 0.9754) and NO radical scavenging assay (R^2 = 0.9465) while TFC (Fig. 11 and Table 5) showed a strong correlation with hydroxyl radical scavenging assay (R^2 = 0.8140) and super oxide radical scavenging assay (R^2 = 0.8155).

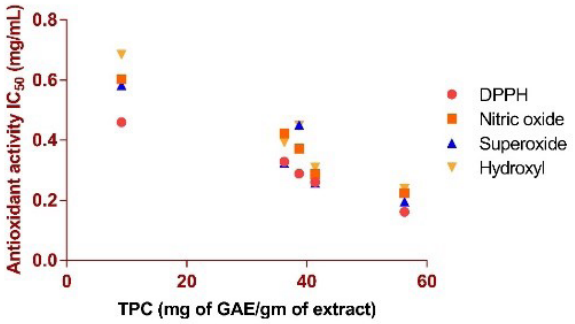

Fig. 10: Correlation between total phenolic content against different free radical scavenging activity of leaf extracts of *N. calycinus*.

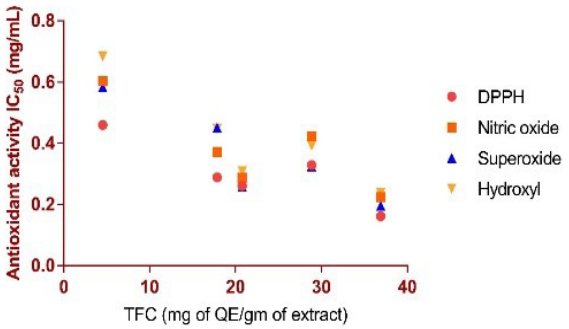

Fig. 11: Correlation between total flavanoid content against different free radical scavenging activity of leaf extracts of *N. calycinus*.

Table 5: Correlation between total phenolic and flavonoid content against different free radical scavenging activity of leaf extracts of *N. calycinus*.

	Pearson Correlation Coefficient (R^2)			
	DPPH	**NO**	**• OH**	**O^2-**
Total phenolics	0.9754** (0.0017)	0.9465** (0.0053)	0.9379** (0.0067)	0.8231* (0.0334)
Total flavonoids	0.7437^{ns} (0.0600)	0.6672^{ns} (0.0915)	0.814* (0.0362)	0.8155* (0.0357)

P value (two-tailed test) is indicated in the parenthesis. ns, Non-significant, $^{**}P < 0.01$, $^{*}P < 0.05$.

4. Discussion

In the recent past, the importance of functional foods, nutraceuticals and other natural health products has been well recognized in relation to health promotion, disease risk reduction and decrease in health care costs (Hasler, 1998). Many of the important biological moieties inside the living system possess paired electrons in the outer orbit, but in the case of free radicals, an unpaired electron is alone (Elochukwu, 2015). Human bodies create free radicals naturally at the time of food processing. Besides, additional free radicals will accumulate when cells are exposed to environmental toxins, pollutants, ultraviolet rays and stressed conditions. These free radicals are highly reactive, relatively unstable and have tremendous potential to damage cells and tissues (Lobo *et al*., 2010). Natural antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Both hydrophilic and lipophilic antioxidants have confirmed their activity against diseases like tumors, heart diseases, atherosclerosis and various types of tissue damage (Halliwell, 1996; Jang *et al*., 2007). Plant derived antioxidants have immense importance in maintenance of health and rejuvenation of tissues. Glutathione, ubiquinol and uric acid are the main antioxidant enzymes that are produced in our body. Vitamin E, β-carotene and vitamin C are the supplementary antioxidants. Both have the capacity to scavenge free radicals (Lone *et al*., 2013). Various free radical scavenging methods used in the present study are simple and have provided reproducible results showing antioxidant properties of *N. calycinus*.

The extractive values make a valuable test to check the quality of drug. Any variation in the chemical constituents may cause a change in the extractive values. Thus, it helps in the determination of adulteration and is an index of the purity of crude extract for clinical studies. The *N. Calycinus* aqueous extract gave the highest extractive value, may be due to the presence of more amounts of polar compounds than nonpolar ones. The variation in the extractive values may be possible due to the presence of specific compound, according to the solubility, soil condition, atmospheric condition and water content of the sample (Jahan *et al*., 2008, Thomas, *et al*., 2008).

Preliminary phytochemical screening is a way to understand the major phyto compounds present in the plant. The presence of alkaloids, steroids, phenolic compounds, glycosides and saponins depends upon the solvent used for the extraction and the part of the plant used for the study (Anonymous, 1985). The key interactions between plant habit and their habitat including biotic and abiotic environment, plays a significant role in the production of metabolites. The main reason for the biological activity of the plants is due to the presence of chemical constituents which are mainly soluble in the polar, semi-polar and nonpolar solvents.

Free radicals of DPPH are commonly used for screening medicinal plants to investigate their antioxidant potential. The purple colored methanolic DPPH solution turn to yellow by the radical scavengers present in the plant extracts (Blois, 1958). The observed antioxidant activity of the extracts may be due to the neutralization of free radicals, either by transfer of hydrogen atom or by transfer of an electron (Naik *et al*., 2003). In

the present study, a gradual increasing in DPPH free radical scavenging activity was observed in concomitance to increasing concentration of the extract. The results existed clearly indicate that in screening of different extracts of *N. calycinus*, ethanolic extract had promising scavenging effect. Plant extract that contain compounds like phenols and flavonoids that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity (Ahmad *et al*., 2010).

Nitric oxide is a potent pleiotropic inhibitor of physiological process, such as smooth muscle relaxation, neural signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays important roles such as effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Hagerman *et al*., 1998). Presence of excessive NO implicated diseases condition, such as cancer and inflammation (Miller *et al*., 1993) and several other diseased conditions (Hepsibha *et al*., 2010). Nitric oxide is an unstable species under the aerobic condition. It reacts with O_2 to produce the stable product, nitrates and nitrite through the intermediates -NO_2, N_2O_4 and N_3O_4 (Patel *et al*., 2010). Phyto constituents have the ability to counteract with oxygen that reacts with nitric oxide and thus inhibited the generation of nitrite, to prevent the human body from the ill effects caused due to excessive nitric oxide generation. The level of nitrous oxide was significantly reduced with the addition of *N. calycinus* leaf extract in crude form. The high activity of NO scavenging exhibited by *N. calycinus* was confirmed its medicinal value and its usage by the tribal peoples as medicine particularly for external uses. Since nitric oxide plays a crucial role in the inflammation during pathogenesis (Moncada *et al*., 1991), the extract can be used for the treatment of inflammation and for wound healing.

The interaction between iron ions and hydrogen peroxide in biological systems can release highly reactive tissue-damaging hydroxyl radicals (Halliwell, 1978). All the extracts of leaves of *N. calycinus* exhibit potent scavenging activity for hydroxyl radical in a concentration-dependent manner (0.1 to 0.5 mg/mL). The scavenging of these radicals may be due to the hydrogen donating ability of phenolic compounds in the extracts. The hydroxyl radical is a highly potent oxidant that reacts with almost all biomolecules found in living cells. When it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxides are produced (Valentao *et al*., 2002).

The superoxide anions damage biomolecules by forming H_2O_2, peroxyl nitrite, or singlet oxygen during pathophysiologic events (Sannigrahi, *et al*., 2010). In the PMS/NADH-NBT system, superoxide anion is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen (Robak and Gryglewski, 1988). According to the results of this study, it is clearly indicated that NCET has significant superoxide anion radical scavenging activity in a dose dependent manner. *N. calycinus* can be used as an easily accessible source of natural antioxidants and as a possible natural drug candidate for pharmaceutical industry.

The reducing capacity of the extract gives a significant indication of its antioxidant activity

also (Oyaizu, 1986). In the reducing power assay, the presence of antioxidants (reducers) in the extract would result in the reduction of Fe^{3+} (ferrocyanide complex) to Fe^{2+} (ferrous) by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's blue at 700 nm. Increasing absorbance indicates an increase in the reductive ability. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts (Benzie and Szeto, 1999; Koleva *et al.,* 2002; Mohamed, *et al.,* 2009). The absorbance records suggest that the alcoholic extract of leaves of *N. calycinus* may act as good electron donors and react with free radicals to convert them into more stable products and subsequently terminate the free radical chain reactions.

The evaluation of TAC is an appropriate tool to determine the additive antioxidant properties of plant extracts (Pellegrini *et al*., 2006). It is evident that all the extracts of leaf of *N. calycinus,* NCET had high total antioxidant capacity while hexane extract showed the least.

Phenolics are diverse group of metabolites present in plant kingdom. The NCET extract showed the highest phenolic content compared to aqueous and ethyl acetate extract. Preliminary phytochemical screening has already shown that *N. calycinus* ethanolic extract contained phenolic compounds. The phenolics possess a wide spectrum of biochemical activities such as antioxidant, anti-mutagenic, anti- carcinogenic as well as the ability to modify gene expression (Nakamura *et al*., 2003). In addition, Moure *et al*. (2001) had demonstrated that high polarity of solvent yields high amounts of polyphenolics. Therefore, differences in TPC recorded in the present study may be attributed to the difference in solvent and the presence of polyphenolics.

The role of flavonoids as antioxidants or health promoting compounds is due to the presence of anion radicals (Havsteen, 1983; Das and Pereira, 1990). The ethanolic extract of *N. calycinus* showed the highest flavonoid content. The presence of high flavonoid content may be the reason for its NO scavenging activity and reducing power. Numerous epidemiological studies confirmed that flavonoids will reduce the risk of cardiovascular and carcinogenic effects (Cook and Samman, 1996). It has been acknowledged that flavonoids show significant antioxidant action on human health and fitness. However, further studies are required, especially in identification and quantification of individual flavonoids and phenolic acids.

Phenolic compounds are important plant antioxidants with considerable scavenging activity against radicals. The polyphenols contained in the leaf extracts of *N. calycinus* may act by donating electrons and reacting with free radicals to convert them into more stable products as well as to terminate the chain reactions (Diouf *et al*., 2009). Thus, antioxidant capacity of a sample can be attributed mainly to its phenolic compounds (Zheng and Wang, 2003; Chinnici *et al*., 2004; Huang *et al*., 2009). It has been reported that the antioxidant activity of phenols is mainly due to their redox properties as hydrogen donors and singlet oxygen quenchers (Evans *et al*., 1997; Aiyegoro and Okoh, 2010). Thus, we can conclude that the antioxidant potential of the leaf extract of *N. calycinus* may possibly be attributed to the presence of synergistic action of phenolic compounds in it (Graversen, *et al*., 2008; Liu *et al*., 2008; Altunkaya *et al*., 2009).

Excellent linear correlations between antioxidant activity tests and total phenolic content were reported by Sultana *et al*. (2007) and de Oliveira *et al*. (2009).

5. Conclusion

The present study confirmed that among all the tested extracts, *N. calycinus* ethanolic extract exhibited most potent radical scavenging effect which showed a strong correlation with the total phenolic content. This radical scavenging property of *N. calycinus* helps to control the oxidative and non-oxidative damage caused by reactive oxygen and nitrogen species. Further investigations are to be conducted for isolation of bioactive compounds and *in vivo* testing of antioxidant property of *N. calycinus*, which leads to the development of drug molecules for combating human diseases resulting from oxidative stresses.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors are thankful to the Director, JNTBGRI for constant encouragement, support, and providing necessary facilities. This work was financially supported by the Kerala State Council for Science, Technology and Environment (KSCSTE), Government of Kerala.

Author contributions

S. Rajasekharan performed the field studies and documented the tribal information on *Neurocalyx calycinus*; S R Suja and E A Siril designed the concept; A L Aneesh Kumar performed the study and acquired the data; A L Aneesh kumar, S R Suja and E A Siril wrote the manuscript with inputs from other S. Rajasekharan.

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