Pharmacogn. Res.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogres.com | www.phcog.net

ORIGINAL ARTICLE

High-Performance Liquid Chromatography Analysis and Antioxidant Activities of Extract of *Azadirachta indica* (Neem) Leaves

Emmanuel Ekow Biney[1], Matthew Nkoom[2], Williams Kweku Darkwah[1,2], Joshua Buer Puplampu[1]

[1]Department of Biochemistry, School of Biological Sciences, University of Cape Coast, Cape Coast, Ghana, [2]Department of Environmental Engineering, Key Laboratory of Integrated Regulation and Resource Development on Shallow Lakes, Ministry of Education, College of Environment, Hohai University, Nanjing, China

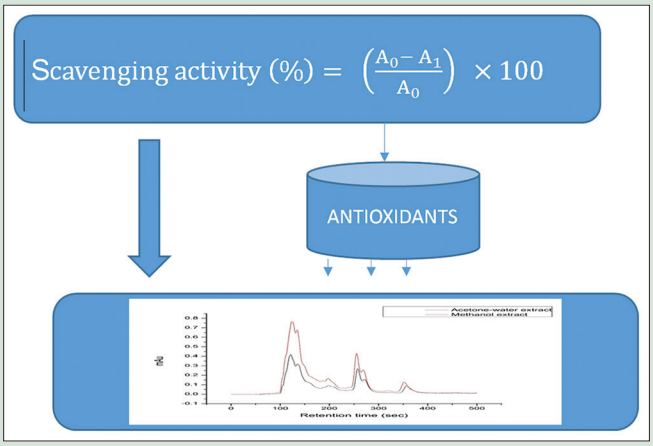
ABSTRACT

Background: Extracts from *Azadirachta indica* (*A. indica*) tend to provide numerous health benefits including antioxidant activity. The main objectives for this research were to use standard procedures to determine total phenols, total alkaloids, total flavonoid, 1,1-diphenyl-2-picrylhydrazine (DPPH)-scavenging activity, ferric reducing power, and total antioxidant capacity and analyze active components of the extracts using high-performance liquid chromatography (HPLC). **Materials and Methods:** *In vitro* antioxidant potential of the *A. indica* extract was evaluated using DPPH and total antioxidant ability assays. Ferric reducing power ability of the extract was also examined using tannic acid and ascorbic acid as standard. Concentrations of plant extracts ranging from 0.02 to 0.10 mg/ml were prepared and mixed with appropriate volumes of reagents. **Results:** Methanol extract of *A. indica* exhibited higher content of phytochemical compounds (alkaloid = 1.9 mg QE/g; flavonoids = 3.5 mg QE/g; and phenols = 4.9 mg QE/g) at concentration 0.1 mg/ml compared to the acetone/water extract (alkaloid = 1.7 mg QE/g; flavonoids = 1.4 mg QE/g; and phenols = 2.6 mg QE/g). An overall trend found in the present study highlights the fact that the methanol extracts have better antioxidant capacities (DPPH, total antioxidant ability, and ferric reducing antioxidant property) than the acetone/water extract. HPLC analysis conducted also reveals seven peaks for methanol extract and six for acetone–water extract with different heights. **Conclusion:** HPLC analysis of *A. indica* extracts exhibited the presence of azadirachtin compound. The study showed that the extracts can competently protect the body against oxidative stress and therefore can be used as a source of potent natural antioxidant compounds.

Key words: Antioxidant activity, *Azadirachta indica*, high-performance liquid chromatography analysis, leaf extracts, phytochemicals

SUMMARY

- This research reports the use of standard procedures to determine total phytochemicals and antioxidant capacity and analyze active components of the extracts using high-performance liquid chromatography
- The present study highlights the fact that the methanol extracts have better antioxidant capacities than the acetone/water extract
- The study revealed that the extracts can expertly shield the body against oxidative stress and thus can be used as a source of potent natural antioxidant compounds.



Abbreviations Used: HPLC: High-performance liquid chromatography, GAEs: Gallic acid equivalents, TCA: Trichloroacetic acid, DPPH: 1,1-Diphenyl-2-picrylhydrazine, FRAP assay: Ferric reducing antioxidant property.

Correspondence:
Williams Kweku Darkwah,
Department of Environmental Engineering, College of Environment, Hohai University, Nanjing, China.
E-mail: williamsdarkwakwaku@yahoo.com
Dr. Joshua Buer Puplampu,
Department of Biochemistry, School of Biological Sciences, University of Cape Coast, Cape Coast, Ghana.
E-mail: jpuplampu@ucc.edu.gh
DOI: 10.4103/pr.pr_14_19

Access this article online
Website: www.phcogres.com
Quick Response Code:

INTRODUCTION

Plants contain non-nutritive chemicals that have defensive or disease preventive possessions. A particular healing potential of plants is their antioxidant activity against free radicals. In the human body, oxidative damage of biological molecules by free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS), is involved in obesity, aging, lipid metabolism disorders, diabetes mellitus, coronary heart disorders, and cancer.[1] The ROS and RNS include diverse reactive entities, namely superoxide (O^{2-}), hydroxyl (OH^-), peroxyl (ROO^-), peroxynitrite ($ONOO^-$), and nitric oxide (NO^-) radicals as well as nonfree radical species such as hydrogen peroxide (H_2O_2), nitrous acid (HNO_2), and hydrochlorous acid (HOCl).[2] The possible reasons for the existence of free radicals triggering oxidative stress are the occasional leakages from continuous exposure to chemicals, contaminants, and other exogenous factors.[3,4] Phytochemical compounds, such as phenols, have

Cite this article as: Biney EE, Nkoom M, Darkwah WK, Puplampu JB. High-performance liquid chromatography analysis and antioxidant activities of extract of *Azadirachta indica* (Neem) leaves. Phcog Res 2020;12:29-34.

been described to have antioxidant, hypolipidemia, antimutagenic, and anticarcinogenic activities and hence have received much attention.[5,6]

Natural antioxidants are found in foods and also characterized by aerobic organisms by both enzymatic and nonenzymatic defense mechanisms.[7,8] Synthetic antioxidants mostly used have been pronounced to cause greater risks of side effects, such as toxicity cell damage and inflammation. This has, therefore, caused a greater interest in finding antioxidant therapeutic solutions from natural sources.[9]

Conventionally, solvents such as methanol, ethanol and acetone have been routinely used to extract phenolic/antioxidant compounds from fresh plants at different concentrations in the presence of water;[10] plays a substantial character in the precise quantification of antioxidants. Nevertheless, in general, solvents such as methanol, ethanol, and acetone have different polarities, vapor pressures, and viscosities; solvents with low viscosity have low density and high diffusivity, which can allow them to with no trouble diffuse into the pores of the plant materials to leach out the active constituents.[11,12]

Although extensive investigation on the antioxidant properties of most plants, little is known about the many tropical underutilized plants in developing countries, especially Ghana. One such plant is *Azadirachta indica* (*A. indica*) which is a versatile perennial slender creeping herb. *A. indica* also known as neem tree and Indian lilac is a tree in the mahogany family Meliaceae and one of two species in the genus *Azadirachta*. The plant has shown many health remunerations, for instance, exhibiting immunomodulatory antimalarial, antioxidant, antifungal, antibacterial, anticarcinogenic, antiulcer, antimutagenic, and anti-inflammatory properties.[13] Therefore, in this study, the phytochemical constituents, antioxidant property, and high-performance liquid chromatography analysis of methanol and acetone–water extracts of *A. indica* leaf were investigated to assess the potential protective benefits of this plant against degenerative reactions induced by free radicals.

MATERIALS AND METHODS

Plant material

Fresh leaves of *A. indica* (neem) were collected from the University of Cape Coast Botanical Garden, Ghana, during the autumn season. The taxonomic identity of the leaf was determined with voucher number UC/SBS/00134, by a plant taxonomist at the Department of Botany, University of Cape Coast, Ghana. The sample was washed under running tap water to eradicate undesirable dirt and other foreign materials. The sample was air-dried under shade until no moisture left. The dried sample was ground into powder using a blender.

Preparation of plant extracts

Methanol and acetone–water extraction

Using the Soxhlet apparatus, the powdered leaves (45 g) were extracted distinctly using 250 mL methanol and 400 mL acetone–water solvents. The acetone–water solvent was in 1:1 ratio. The extraction process was run for 5 h. The solvents used were afterward recovered under pressure using the rotatory evaporator, thereby concentrating the extracts.

Phytochemical screening

Qualitative analysis

Phytochemical qualitative test of the methanol and acetone–water extracts of *A. indica* (neem) was performed in accordance with standard protocols[14-16] to reveal the existence of reducing sugars, proteins, phenolic compounds, flavonoids, tannins, saponins, alkaloids, terpenoids, and cardiac glycosides.

Quantitative analysis

Determination of total phenolic content

The total phenolic content was determined using the Folin–Ciocalteu assay described by Meda *et al*.[17] Briefly, 0.5 mL of extract (1 mg/mL) and 2.5 mL of 10% Folin–Ciocalteu reagent solution were mixed. After incubation for 2 min at room temperature, 2.5 mL of 7.5% sodium carbonate solution was added. The mixture was incubated at 45°C for 45 min and subsequently photometrically measured at 760 nm. Gallic acid (0–100 μg/mL) was used as standard to produce the calibration curve. The average of three readings was used, and the total phenolic content was articulated in milligrams of gallic acid equivalents (GAEs) per g of the leaf extract.

Determination of total alkaloids

Exactly 1 g of the sample was weighed into a 250 ml beaker, and 40 ml of 10% acetic acid in ethanol was added and covered and permitted to stand for 4 h. This was filtered, and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was through. The whole solution was allowed to resolve, and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The reaction mixture was incubated in the dark for 30 min and the absorbance read at 512 nm. Gallic acid was used as standard to produce the calibration curve. The average of three readings was used, and the total alkaloid content was articulated in milligrams of GAEs per g of the leaf extract. The filtrate is the alkaloid, which was dried and evaluated.[14]

Determination of total flavonoids

Exactly 1 g of the plant sample was extracted repetitively with 10 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The residue was later transported into a container and vaporized into dryness over a water bath and weighed to a constant weight.[18]

Determination of free radical scavenging activity

Ferric reducing antioxidant property (assay)

Ability of the sample extracts to reduce ferric ions was measured according to the modified method defined by Oyaizu, 1986. A mixture containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 mL of different concentrations (0.02–0.10 mg/ml) of extract and standards prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 mL of trichloroacetic acid (10% w/v), which was then centrifuged at 3000 rpm for 10 min. About 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1% w/v). The absorbance was then measured at 700 nm against a blank sample containing a phosphate buffer. Better absorbance of the reaction mixture points out greater reducing power of the plant extract.

Determination of 1,1-diphenyl-2-picrylhydrazine -free radical scavenging activity

1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging activity was measured conferring to the technique of Shimada *et al*. 1992. The reaction mixture contained 1.0 mL of a number of concentrations (0.02–0.10 mg/mL) of extracts and standard (ascorbic acid) and 1.0 mL of DPPH solution (0.135 mM). The mixture was shaken robustly and left in an obscure environment for 30 min. The absorbance was measured at 517 nm against a reagent blank containing only methanol. All experiments were performed in triplicates. The inhibition percentage for scavenging DPPH radical was calculated according to the equation:

$$\text{DPPH scavenging activity (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

Where A_0 was the blank absorbance and A_1 was the mixture containing the extract absorbance or the standard absorbance.

Total antioxidant ability assays

The antioxidant activity of the extracts was assessed by the phosphomolybdenum method bestowing to the technique of Prior *et al.*[19] A reagent solution prepared contained ammonium molybdate (4 mM), sodium phosphate (28 mM), and sulfuric acid (0.6 M) and mixed in 1:1:1 ratio, respectively. Accurately, 0.3 mL of the various concentrations of the extracts (20–200 µg/mL) was mixed with 3 mL of the reagent solution. The tubes containing reaction solution were incubated at 95°C for 60–90 min. Then, the absorbance of the solution was measured at 695 nm using spectrometer against blank after cooling to room temperature. For standard (ascorbic acid), concentrations of 20–200 µg/mL were used. Each concentration was prepared in triplicates. A mixture containing 0.3 mL methanol and 3 mL reagent solution was used as a blank.

High-performance liquid chromatography analysis

Serial dilutions of both solvents extracts (acetone–water and methanol) thus, the mobile phase, were prepared. About 1 mL of the various concentrations were pipetted into the sample collectors and placed on the autosampler. The high-performance liquid chromatography (HPLC) was then allowed to carry out the analysis in an isocratic condition using a C_8 reverse phase column at 35°C. Each sample was run for 20 min. The flow rate for the analytical separation was 1 mL/min. Origin 8.1 data analysis tool was then used to analyze the results obtained.

Statistical analysis

All tests were conducted in triplicate. Data are reported as mean ± standard error. Results were evaluated statically using Microsoft Excel 2010 and Origin 8.1 data analysis tool.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical screening piloted discovered the existence of steroids, flavonoids, saponins, tannins, alkaloids, and glycosides in both neem leaf extracts. On the other hand, anthraquinones were lacking in both solvent extracts and terpenoids only absent in the acetone–water extract [Table 1]. The presence of these chemical compounds indicated their solubility in the extraction solvents. These results are seen to be in agreement with a comparative report of diverse extraction solvents by Cowan.[20]

Total phenolic content

Phenol, an important phytochemical constituent, shows an essential role in reducing free radicals found in the body. This can be associated with the methanol extract having more phenolic compounds than that of acetone–water extract. The methanolic extract (4.9 mg QE/g) showed relatively high amount of total phenol content as compared to acetone–water extract (2.6 mg QE/g) of neem leaf, and this can be attributed to their solubility levels in the extraction solvents [Table 2 and Figure 1]. Redox potentials of the phenolic composites allow them to perform as reducing agents, hydrogen donators, and singlet oxygen quenchers and also tend to have metal chelation potentials.[21]

Total flavonoids

The two different extracts contain different amount of flavonoids in methanol extract (3.539 mg QE/g) and acetone–water extract (1.4 mg QE/g) [Table 2 and Figure 2]. Total flavonoid content determined was found to be higher in the methanol extract than the acetone–water. This result then confirms the correlation between antioxidant activity, total phenol, and total flavonoids contents. Thus, the antioxidant capacity of the two solvents can be anticipated on the basis of its total phenols and total flavonoids contents. Difference in quantity of total flavonoids seen can be as a result of the poor water solubility attributable to their phenolic nature [Figure 2]. Findings by Ghimeray and group.[22] reported high total polyphenol and total flavonoid substances in methanol crude neem leaf extract than in water neem leaf extract which supports the drift found in our study. The enzyme polyphenol oxidase which reduces polyphenols in aqueous media can be credited to the level of polyphenols in aqueous extract.[22,23] As a result of the high amount of flavonoids in methanol extract (3.5 mg QE/g) [Table 2 and Figure 2], a number of chronic and degenerative ailments, for example, cancer, cardiovascular diseases, arthritis, aging, cataract, memory loss, and Alzheimer's diseases, whose underlying condition is as a result of deficiency in the level of antioxidant, can be prohibited or hindered. Furthermore, methanol neem leaf extract can be used to protect biological molecules (proteins) which are at risk of damage by the presence of free radicals.[24-26]

Table 1: Phytochemicals screening of methanol and acetone-water neem leaf extract

Phytochemicals	Methanol extract	Acetone-water extract
Phenols	+++	++
Steroids	++	+
Terpenoids	+	–
Flavonoids	+++	++
Saponins	++	+
Anthraquinones	–	–
Tannins	+ +	+
Alkaloids	++	+
Glycosides	+	++

+: Present; –: Absent

Table 2: Quantitative contents of phytochemicals in methanol and acetone-water *Azadirachta indica* (neem) leaf extract (mg/QE/g)

Samples	Flavonoids	Phenols	Alkaloids
Acetone/water	1.4±0.5	2.3±0.09	1.7±0.01
Methanol	3.5±0.38	49±0.084	1.9±0.034

Values represent the mean of three readings±SD of the mean. SD: Standard deviation

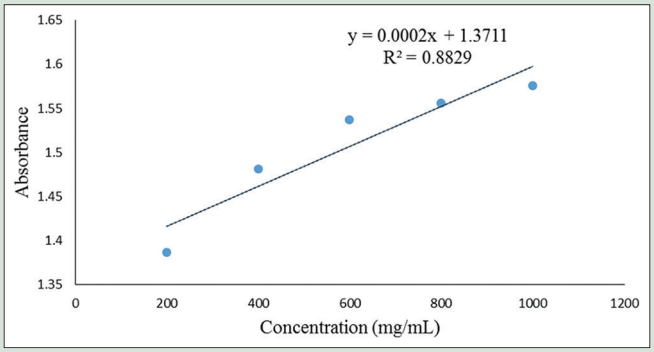


Figure 1: Standard curve for total phenol content estimation at 760 nm

Total alkaloids

There was a statistically significant difference in the levels of alkaloids between the methanol and acetone–water extracts of *A. indica* (neem tree) leaf. Because of the increased quantity of alkaloids in methanol extract (3.5 mg QE/g) [Table 2 and Figure 3], a number of chronic and degenerative ailments such as cancer, cardiovascular diseases, arthritis, aging, cataract, memory loss, and Alzheimer's diseases, whose underlying condition is as a result of deficiency in the level of antioxidant, can be prohibited or delayed.

Antioxidant activity

% Inhibition of 1,1-diphenyl-2-picrylhydrazine assay

The *in vitro* antioxidant assays performed on this plant reveal significant antioxidant potential. DPPH is a stable-free radical commonly used to investigate the scavenging activity of phytochemicals. The outcomes of the DPPH-scavenging activity of the two extracts, alongside with ascorbic acid (reference standards), are presented in Figure 4. Although both extracts of *A. indica* (neem tree) leaf show DPPH-scavenging activity, the activity is lower compared to the standards (tannic acid and ascorbic acid). The methanol extract shows higher activity (48.98%) paralleled to the acetone–water extract which shows 32.29% inhibition at the highest concentration of 0.1 mg/ml. This result agrees well with the result on total phenolic content which is higher in the methanol extract. Usually, high total phenol contents lead to an improved DPPH-scavenging activity.[27,28] Furthermore, this result is in line with other findings[28] where methanol extract showed higher antioxidant activity over aqueous extract.

Ferric reducing power (ferric reducing antioxidant property)

The ferric reducing antioxidant property (FRAP) assay is mostly labored to measure the antioxidant influence of any constituent in the reaction medium, as its reducing ability. Naturally, the present antioxidants are deliberated as reductants, and the inactivation of oxidants by reductants is described as a redox reaction.[29] Figure 5 shows the results of the FRAP assay of methanol and acetone–water extracts of *A. indica* (neem tree) leaf. The methanol extracts of the *A. indica* (neem tree) leaf showed significantly higher FRA*P* values compared to all of the acetone–water extracts. An inclusive trend found in the present study climaxes the point that the methanol extract has better antioxidant abilities than the aqueous extract. A conceivable intention for this dissimilarity is that methanol and acetone–water accumulates different quantities of phytochemicals, which in turn can affect the level of antioxidants present.

Total antioxidant capacity

Antioxidants substituting as reducing agents scavenge free radicals in the body. To work effectively and efficiently, they need to have some level of antioxidant capacity. In a concentration-dependent manner, the *A. indica* (neem tree) leaf extracts were initiated to have lower level of total antioxidant capacity than the standard, ascorbic acid. Furthermore, between the two solvent extracts, methanol extract was found to contain high antioxidant level than the acetone–water [Figure 6]. Phenol, an important phytochemical constituent, shows a significant role in reducing free radicals found in the body. This can be associated with the methanol extract having more phenolic compounds than that of acetone–water extract, thereby reducing more phosphomolybdic acid (Mo^{+6}) to the phosphate/Mo^{+5} complex (green chromogen) than the acetone–water extract.[30]

High-performance liquid chromatography analysis

The HPLC analysis revealed several peaks in excess of seven in the methanol *A. indica* (neem tree) leaf extract and six peaks for acetone–water *A. indica* (neem tree) leaf extract. The six peaks found in the acetone–water *A. indica* (neem tree) leaf extract were common to both extracts with different heights/area (similar retention times

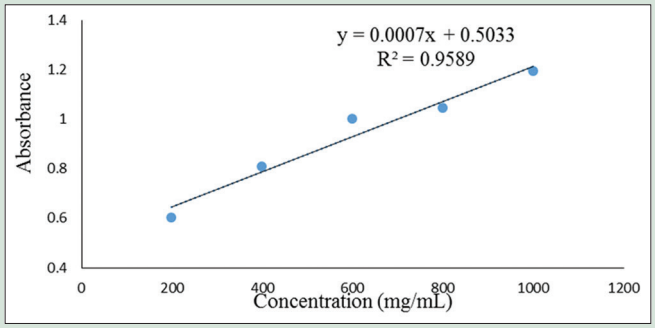


Figure 2: Standard curve for total flavonoid content estimation at 512 nm

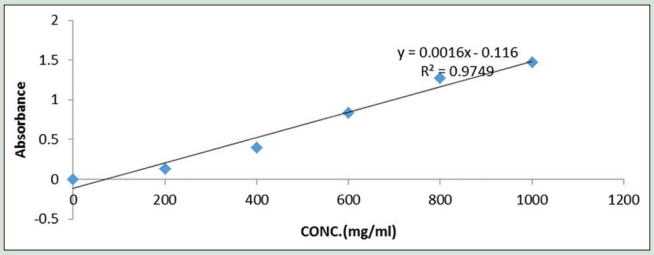


Figure 3: Standard curve for total alkaloid content estimation at 512 nm

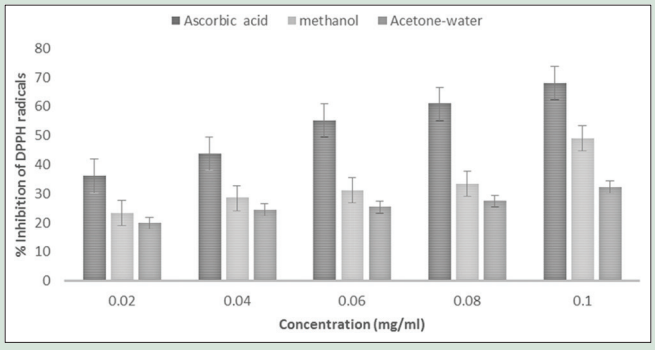


Figure 4: 1,1-Diphenyl-2-picrylhydrazine scavenging activities of methanol and acetone–water extracts from *Azadirachta indica* (neem tree) leaf and standard, ascorbic acid

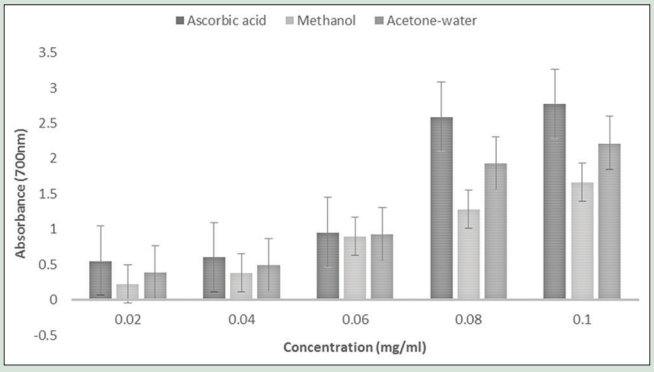


Figure 5: Ferric reducing power of methanol and acetone–water extracts from *Azadirachta indica* (neem tree) leaf and standard, ascorbic acid

between 180 and 360 s) [Figures 7-9]. This suggests that both extracts have similar or same components with different compositions.

Serially diluted samples were introduced into the HPLC using the autosampler. The chromatogram from the methanolic extracts showed increasing peak height/area for the increasing concentrations. Similar trends were observed for acetone–water extracts. There were, however, larger peak heights for the methanolic extracts as paralleled to the acetone–water one. These observed trends are presented in the chromatograms [Figures 7-9].

HPLC results of neem leaf hexane extracts by Ghimeray and coworkers to show azadirachtin and nimbin reported only the presence of nimbin content at a retention time around 29 min. However, the retention time between 0 and 5 min of the neem leaf hexane extract showed similar chromatograms for both the methanol and acetone–water *A. indica* (neem tree) leaf extracts in this study. This, therefore, shows linearity between chromatogram peaks from hexane, methanol, and acetone–water *A. indica* (neem tree) leaf extracts. This similarity in results indicates the extraction of similar active components by different solvent systems and their concentrations related to their level of solubility in the solvent systems used.

CONCLUSION

The outcome of this research points out that extracts of *A. indica* (neem tree) leaf comprise a variety of phytochemical compounds in appreciable quantity that can expertly protect the body against oxidative stress caused by free radicals and might, therefore, be used as a source of potent natural antioxidant compounds. The antioxidant activity of *A. indica* (neem tree) leaf may justify further investigation of its other beneficial biological properties and determine its safety.

Financial support and sponsorship

Nil.

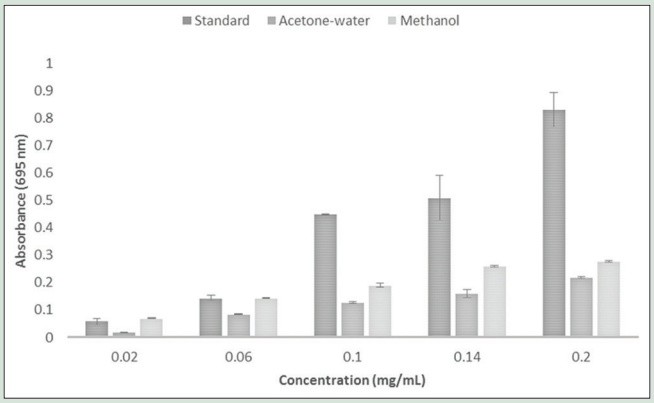


Figure 6: Total antioxidant activities of methanol and acetone–water extracts from *Azadirachta indica* (neem tree) leaf and standard, ascorbic acid

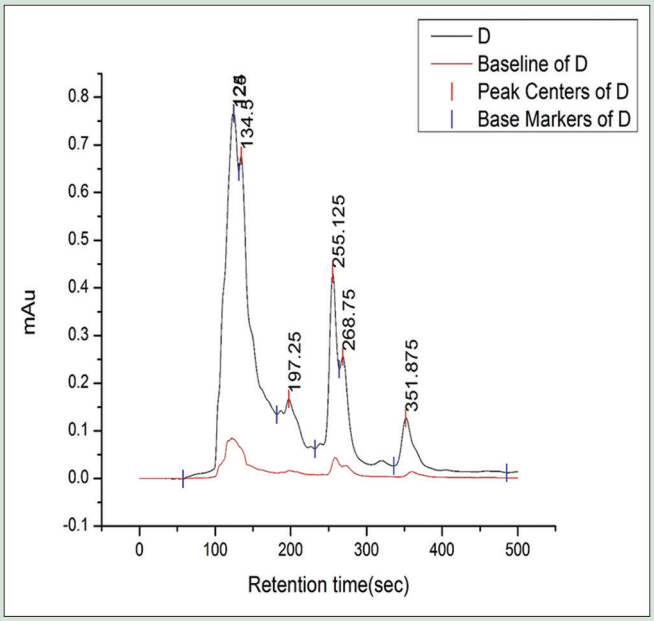


Figure 8: Chromatogram of varying concentrations of methanol neem leaf extract at 254 nm by high-performance liquid chromatography analysis

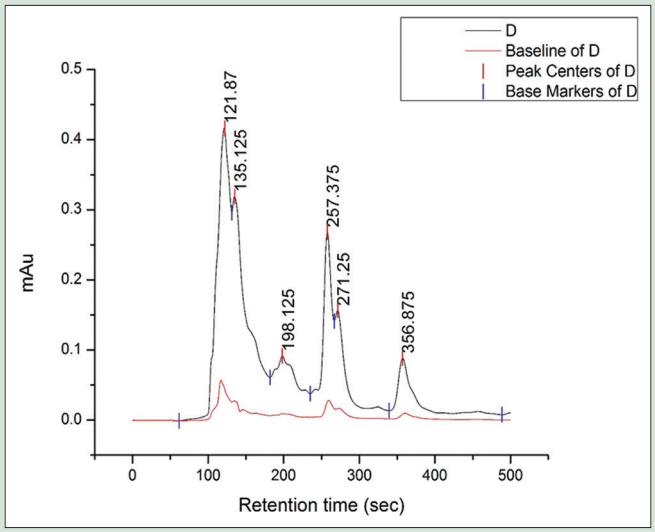


Figure 7: Chromatogram of varying concentrations of acetone–water neem leaf extract at 254 nm by high-performance liquid chromatography analysis

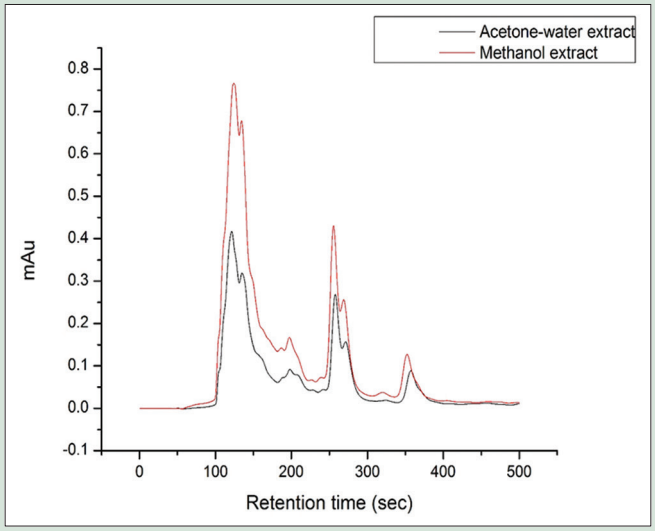


Figure 9: Chromatogram of both spectra for methanol and acetone–water extracts by high-performance liquid chromatography analysis

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Jayaprakasha GK, Rao LJ. Phenolic constituents from the lichen *Parmotrema* stuppeum (Nyl.) hale and their antioxidant activity. Z Naturforsch C 2000;55:1018-22.
2. Mavi A, Terzi Z, Ozgen U, Yildirim A, Coşkun M. Antioxidant properties of some medicinal plants: Prangos ferulacea (*Apiaceae*), Sedum sempervivoides (*Crassulaceae*), *Malva neglecta* (*Malvaceae*), Cruciata taurica (*Rubiaceae*), *Rosa pimpinellifolia* (*Rosaceae*), *Galium verum* subsp. verum (*Rubiaceae*), *Urtica dioica* (Urticaceae). Biol Pharm Bull 2004;27:702-5.
3. Ara N, Nur H. *In vitro* antioxidant activity of methanolic leaves and flowers extracts of Lippa Alba. Res J Med Med Sci 2009;4:107-10.
4. Saha MR, Alam MA, Akter R, Jahangir R. *In vitro* free radical scavenging activity of *Ixora coccinea* L. Bangladesh J Pharmacol 2008;3:90-6.
5. Boham BA, Kocipai-Abyazan R. Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium vaticulatum* and *V. calycinium*. Pacific Sci 1974;48:458-63.
6. Shi J, Gong J, Liu JE, Wu X, Zhang Y. Antioxidant capacity of extract from Edible flowers of *Prunus mume* in China and its active components. LWT Food Sci Technol 2009;42:477-82.
7. Mosquera OM, Correa YM, Buitrago DC, Niño J. Antioxidant activity of twenty five plants from Colombian biodiversity. Mem Inst Oswaldo Cruz 2007;102:631-4.
8. Koomson DA, Kwakye BD, Darkwah WK, Odum B, Asamoah KA, Aidoo G. Phytochemical constituents, total saponins, alkaloids, flavonoids and Vitamin C contents of ethanol extracts of five *Solanum torvum* fruits. Pharmacogn J 2018;10:946-50.
9. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 2010;4:118-26.
10. Alothman M, Bhat R, Karim AA. Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. Food Chem 2009;115:785-8.
11. Darkwah WK, Adinortey M, Weremfo A. Phytochemical constituents and aromatic content of methanol and aqueous extracts of *Dissotis rotundifolia* whole plant. South Asian Res J Natl Prod 2018;1:1-5.
12. Hemwimol S, Pavasant P, Shotipruk A. Ultrasound-assisted extraction of anthraquinones from roots of *Morinda citrifolia*. Ultrason Sonochem 2006;13:543-8.
13. Oyaizu M. Studies on products of browning reaction – Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr 1986;44:307-15.
14. Harborne A. Phytochemical Methods a Guide to Modern Techniques of Plant Analysis. London: Springer Science and Business Media; 1998.
15. Trease G, Evans W. A Text Book of Pharmacognosy. Vol. 198. London: Academic Press; 1989. p. 22-40.
16. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 1992;40:945-8.
17. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem 2005;91:571-7.
18. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem (*Azadirachta indica*). Curr Sci Bangalore 2002;82:1336-45.
19. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005;53:4290-302.
20. Cowan MM. Plant products as antimicrobial agents. Clinical microbiology reviews 1999;12:564-82.
21. Rice-evans CA, Miller NJ, Bolwell PG, BramLey PM, Pridham JB. The Relative antioxidant activities of plant-derived polyphenolic flavonoids. Free radical research 1995;22:375-83.
22. Ghimeray AK, Jin CW, Ghimire BK, Cho DH. Antioxidant activity and Quantitative estimation of azadirachtin and nimbin in *Azadirachta indica* A. Juss grown in foothills Of Nepal. African Journal of Biotechnology, 2009;8:3084-91.
23. Darkwah WK, Ao Y, Adinortey MB, Weremfo A, Abrokwah FK, Afriyie E. Total phenolic, flavonoid and alkaloid contents, oxidative DNA damage protective and antioxidant properties of methanol and aqueous extracts of *Dissotis rotundifolia* whole plant. Free Radicals Antioxidants 2018;8:82-8.
24. Devasagayam TP, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD, *et al.* Free radicals and antioxidants in human health: Current status and future prospects. J Assoc Physicians India 2004;52:794-804.
25. Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. Nutrition 2002;18:872-9.
26. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44-84.
27. Ebrahimzadeh MA, Bahramian F. Antioxidant activity of *Crataegus* pentaegyna subsp. elburensis fruits extracts used in traditional medicine in Iran. Pak J Biol Sci 2009;12:413-9.
28. Nandhakumar E, Indumathi P. *In vitro* antioxidant activities of methanol and aqueous extract of *Annona squamosa* (L.) fruit pulp. J Acupunct Meridian Stud 2013;6:142-8.
29. Siddhuraju P, Becker K. The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. Food Chem 2007;101:10-9.
30. Yeboah S, Darkwah WK. *In vitro* antioxidants and antimicrobial properties of methanol extracts from *Ocimum basilicum, Eucalyptus globulus* and their combination. Int J Res Appl Sci Eng Technol 2017;5:232-6.