INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

EXTRACTION, ISOLATION AND CHARACTERISATION OF FREE AND BOUND POLYPHENOLS FROM NEEM (*Azadirachta indica* Juss) SEED

Awolu, Olugbenga Olufemi [1], Ojo, Deborah Oluwabukola *[1], Igbe Festus Omotere [2], Onyemechara Linda [1] and Olulana Ibironke [1]
[1]Department of Food Science and Technology, Federal University of Technology, Akure, Ondo State, Nigeria
[2]Department of Biochemistry, Federal University of Technology, Akure, Ondo State, Nigeria
*Corresponding Author Email: ojobukky85@gmail.com

Article Received on: 13/10/17 Approved for publication: 20/12/17

DOI: 10.7897/2230-8407.0812246

ABSTRACT

Free and bound polyphenols were extracted from raw and deffated neem seed and then characterized inorder to determine the effect of defatting on the quantity of polyphenols in neem seeds. The total phenolic content, total flavonoid content, total condensed tannin content and DPPH free radical scavenging activities (FRSA) of the raw and defatted neem seed were evaluated. The characterization of the polyphenols was carried out using gas chromatography. The chromatogram revealed the presence of a substantial amount of polyphenols in neem seed; the raw neem seed had higher contents of tannic acid, azidiradione, nimbin, nimbdin, salamin andazadirachtin. The DPPH free radical scavenging activities and quercetin content of the defatted neem seed were retained while other bioactive components were reduced.

Key words: Polyphenols, antioxidant, neem seed, defatted, biologically active, flavonoid.

INTRODUCTION

The neem (*Azadirachta indica* A. Juss*)* is widely found in the seasonally dry, tropical woodlands of north-east India, Africa and part of Asia. All parts of the neem tree have been used traditionally for the treatment of inflammation and several diseases[1,2]; they possess antifungal and anti-inflammatory potentials[2].

Neem seeds are very rich in fatty acids (oleic acid, stearic acid, palmitic acid and its oil contains about 63% unsaturated fatty acid[3]. Neem oil has been characterized and its efficiency in the production of biodiesel has been evaluated[4]. Neem seed contain tignic acid (5-methyl-2-butanic acid) which is responsible for the distinctive odour of the oil; the bitter taste of the oil is attributed to the presence of a compound called meliacin. Azadirachtin and nimbin are some of the biologically active compounds derived from neem[5].

Polyphenols have been effective in the prevention of diseases, hence its immense health benefits. Increasing experimental evidences have suggested that these compounds can affect a wide range of cell biological function by virtue of their radical scavenging properties. In addition, polyphenols contribute to the organoleptic characteristics of many foods of vegetable origin[6] and are most commonly introduced to the body through the consumption of fruits and vegetables. The biological properties exhibited by polyphenols can however be exploited in the production of nutraceuticals and functional foods enriched with these compounds.

This work was aimed at determining the presence and quantity of polyphenolic compounds in raw and defatted neem seed in order to access its viability for use in nutraceuticals and functional foods.

MATERIALS AND METHODS

Materials and reagents

Neem seeds were obtained from Kano, Nigeria. Materials used include Soxlet apparatus, hammer mill (Alpine Augsburg universal hammer mill, Augsburg, Germany) obtained from Department of Food Science and Technology, Federal University of Technology, Akure, Nigeria;Reagents used (Chloroform, acetone, methanol, sodium nitrate, 1, 1-diphenyl-2-picryl hydrazyl (DPPH), tannic acid, sodium carbonate, vanillin, folin-ciocalteu reagent, aluminium chloride, sodium hydroxide, rutin, gallic acid, potassium acetate) were of analytical grade.

Seed collection and preparation

Dried mature neem seeds were cleansed and sieved to remove foreign matters like matters. The seeds were milled into powder using a hammer mill (Alpine Augsburg universal, Augsburg, Germany).

Defatting of neem seed

The defatting of the neem seeds were carried out using soxhlet extraction method. The dried ground neem (*Azadirachta indica*) seed was placed inside a thimble made from thick filter paper which was loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor was placed onto the flask containing the extraction solvent (chlorofoam) and equipped with a condenser. The extractor was then heated to reflux for 3h at 70°C until there was no oil present in the sample.

Extraction of free soluble polyphenols

The extraction of free polyphenol was carried out by the method reported by [7]. About 39 g of the defatted ground seeds were extracted with 80% acetone and filtered using filter paper (Whatman no.2). The filtrate was then evaporated in a liebig condenser to separate the solvent from the extract. The mixture was distilled at a temperature of 68°C until about 90% of the solvent were evaporated. The phenolic extract was frozen while the residues were kept for the extraction of bound phenolics. Free soluble polyphenols were obtained from the undefatted neem seeds using the same procedure.

Extraction of bound polyphenols

The residue from free soluble extract above was allowed to dry and hydrolyzed with NaOH solution to form slurry at room temperature with stirring. Then the pH of the mixture was adjusted to pH 2.5 with concentrated HCL and the bound polyphenols were extracted with ethyl acetate by filtering the mixture using a filter paper (Whatman no. 2) in separating funnel. The mixture was then evaporated in a liebig condenser to remove the solvent from the extract. The mixture was distilled at a temperature of 68°C until about 90% of the solvent were evaporated. This same procedure was carried out to extract bound phenolics from undefatted neem seeds.

Determination of total phenol content

The total phenol content (TPC) was determined according to the method of [8]. Appropriate dilutions of the extract were oxidized with 0.5ml 10% FolinCiocalteu's reagent (v/v) and 2.5ml of 7.5% sodium carbonate was added. Standard solutions of gallic acid were prepared with concentration 20, 40, 60, 80 and 100ppm. The reaction mixture was incubated for 40min at 45°C and the absorbance was measured at 700nm in the US-VIS spectrophotometer (UV-160A) against the blank solvent. The total phenols were subsequently calculated and expressed as mg GAE/ml.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined using a method reported by [9]. An aliquot of diluted sample or standard solution of (+)-catechin was added to 75mL of $NaNO_2$ solution (5%) and mixed for 6 min before addition of 0.15mL of NaOH were added. The final volume was adjusted to 2.5mL with distilled water and mixed thoroughly. Absorbance was determined at 510 nm against a blank. The total flavonoids content is expressed as milligrams of catechin per gram of dry weight (mg CE/g DW) against the calibration curve of (+)-catechin, from 0 to 400 mg/ml.

Determination of total condensed tannin content

The analysis of total condensed tannin (TCT) was carried out according to the method of [10]. To the suitably diluted sample, 3ml of 4% methanolic vanillin solution and 1.5ml concentrated hydrochloric acid were added. The mixture was allowed to stand for 15min, and then absorption was measured at 500nm against methanol as blank. The amount of condensed tannin was expressed as mg (+)- catechin/g

Determination of 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2 picylhydrazyl) free radical was evaluated as described by [11]. Appropriate dilutions of the extract were mixed with 1ml, 0.4m M methanolic solution. A concentration (0.6ml) of the methanolic free radical extract was used. The mixture was left in the dark for 30 min and the absorbance was determined with a UV-160A spectrophotometer against methanol as blank at 516nm. A negative control of DPPH was taken. The percentage concentration of DPPH in the reaction medium was subsequently calculated using the following formula:

Where $A_{control}$ is the absorbance of the control gave an absorbance value and A_{sample} is the absorbance of the sample

Gas chromatography analyses

This was carried out to characterize polyphenols from raw and defatted neem seeds using gas chromatography (HP 6890 powered with HP Chem Station Rev. A 09.01 [1206] Software), by the described by [3].

Statistical analysis

The data obtained were analyzed using a one-way analysis of variance and the means separated by Duncan New Multiple Range Tests (DMNRT) at 5% significance level (SPSS version 19 computer software).

RESULTS

The total phenolic content (TPC) of extract from defatted and raw neem (*Azadirachta indica)* seed is presented in Table 1. Phenol content varied from 0.45mgGAE/ml in bound phenolic extract of defatted neem seed to 2.03mgGAE/ml in free phenolic extract of raw neem seed.

The results of the total flavonoid content (TFC) of the defatted and raw seed extracts are presented in Table 2. TFC ranged from 0.20 to 0.41mgCE/ml; the least flavonoid content of 0.20mgCE/ml was found in the bound polyphenolic extract of defatted neem seed.

Results of the total condensed tannin content (TCT) of the defatted and raw seed extracts are presented in Table 3. Substantial quantities of TCT than TPC and TFC were found in the defatted and raw neem seeds.TCT content of the defatted and raw neem seeds ranged from 6.46 to 15.9 mgCE/ml. Free phenolic extract of raw neem seed gave the highest total condensed tannin of 15.9mgCE/ml thus a higher degree of potency while the bound polyphenolic neem extract of defatted neem seed gave the lowest total condensed tannin content of 5.51 mgCE/ml.

The results of the DPPH free radical scavenging property of the defatted and raw neem seeds are presented in Table 4. DPPH content of the defatted and raw neem seed ranged from 65.7mg/ml in the bound Phenolic extract of raw neem seed to 83.7mg/ml in the bound Phenolic extract of defatted neem seed.

Polyphenolic compounds present in the defatted and raw neem seed are presented in Table 5. Gas chromatographic analyses reveal the presence of Tannic acid, quercetin, azidiradione, gedunin, meliantriol, nimbin, nimbidin, nimbidol, salamin, azadirachtin, azadirachtin B in the defatted and raw neem seeds. Slight differences in the quercetin content of defatted (4.14mg/100g) and raw neem seed (4.97mg/100g) suggests that quercetin was retained in the defatted neem seed.

Table 1: Total Phenolic Content of Defatted and Raw Neem Seed Extract(mgGAE/ml)

Total Phenolic content (mgGAE/ml)	Defatted Neem Seed	Raw Neem Seed
Free phenolic extract	1.08 ± 0.02^{b}	2.03 ± 0.20^{a}
Bound phenolic extract	0.45 ± 0.00^{b}	1.56 ± 0.05^{a}

Values marked by the different letters within same row are significantly different ($p>0.05$).
Data are expressed as mean ± standard deviation (n=3).
*GAE= Gallic acid equivalents.

Table 2: Total Flavonoid Content of Defatted and Raw Neem Seed Extract(mg CE/ml)

Total Flavonoid Content (mg CE/ml)	Defatted Neem Seed	Raw Neem Seed
Free phenolic extract	0.41 ± 0.02^{a}	0.37 ± 0.02^{b}
Bound phenolic extract	0.20 ± 0.01^{b}	0.40 ± 0.01^{a}

Values marked by the different letters within same row are significantly different ($p>0.05$).
Data are expressed as mean ± standard deviation (n=3).
* CE = Catechin Equivalent

Table 3: Total Condensed Tannin of Defatted and Raw Neem Seed Extract(mg CE/ml)

Total Condensed Tannin (mg CE/ml)	Defatted Neem Seed	Raw Neem Seed
Free phenolic extract	7.18 ± 0.02^{b}	15.9 ± 1.87^{a}
Bound phenolic extract	5.51 ± 0.40^{b}	6.46 ± 0.47^{a}

Values marked by the different letters within same row are significantly different ($p>0.05$).
Data are expressed as mean ± standard deviation (n=3).
* CE = Catechin Equivalent

Table 4: Free Radical Scavenging Activity of Defatted and Raw Neem Seed Extracts (mg/ml)

DPPH(mg/ml)	Defatted Neem Seed	Raw Neem Seed
Free phenolic extract	69.4 ± 2.37^{a}	69.6 ± 1.36^{a}
Bound phenolic extract	83.7 ± 2.46^{a}	65.7 ± 0.11^{b}

Values marked by the different letters within same row are significantly different ($p<0.05$).
Data are expressed as means ± standard deviation (n=3)

Table 5: Gas Chromatogram of Defatted and Raw Neem Seed (mg/100g)

Polyphenol (mg/100g)	Deffatted Neem Seed	Raw Neem Seed
Tannic acid	989.43	1211.99
Quercetin	4.14	4.97
Azidiradione	2.42	208.52
Gedunin	0.15	5.28
Meliantriol	0.59	13.24
Nimbin	1.77	1269.22
Nimbidin	0.48	945.52
Nimbidol	0.18	5.06
Salamin	0.12	710.06
Azadirachtin	0.36	306.23
Azadirachtin B	0.48	16.34

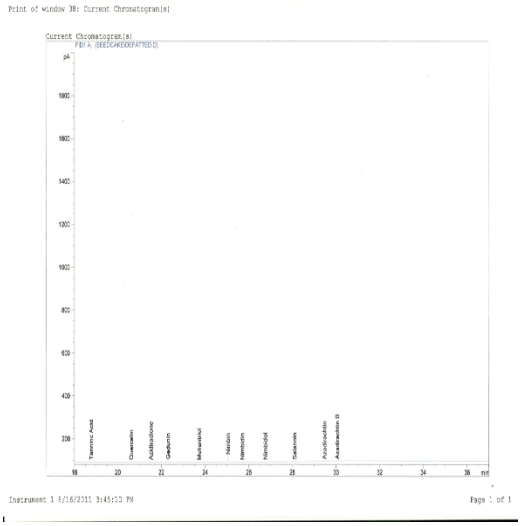

Figure 1: chromatogram of defatted neem seed

DISCUSSION

Analysis of variance on the data showed that the levels of phenol were significantly different ($p<0.05$) in the free soluble and bound phenolic extracts of defatted and raw neem seeds; Phenols are an important component in the maintenance of health due to their preventive activity against infectious and degenerative diseases, inflammation and allergies by their antioxidant, antimicrobial, enzyme modulation mechanisms[12].

There was no significant($p>0.05$) difference in the flavonoid content of free phenolic extract of defatted and raw neem seed. Flavonoids possess anti-bacterial, anti-viral, anti-inflammatory, anti-cancer and hepato-protective activities[13].

Analysis of variance on the data showed that the levels of condensed tannin were significantly different ($p<0.05$) in the free soluble and bound phenolic extracts of defatted and raw neem seeds. Tannins possess anti-inflammatory, anti-carcinogenic, anti-atherosclerotic properties among others[14].

Significant quantities of DPPH were observed in both defatted and raw neem seeds. The results showed that the defatted neem seed contain a higher level of DPPH in the bound phenolic compound than in the free phenolic extracts of defatted neem seeds; Analysis of variance on the data showed significant differences ($p>0.05$) in the DPPH value of the bound phenolic extract of defatted and raw neem seeds while no significant difference exist in the DPPH value of free phenolic extract of raw and defatted neem seeds.

The gas chromatographic analyses show that tannic acid is the most abundant in both defatted and raw neem seeds. The raw neem seed had higher contents of tannic acid, azidiradione, nimbin, nimbidin, salamin and azadirachtin than the defatted neem seed. Quercetin which was slightly retained in the defatted neem seed is well known for its ability to scavenge reactive oxygen species[15]. According to [16], gedunin is a valuable constituent of neem seed while azadirachtin (abundant in the raw neem seed in 306.23mg/100g) is the most important active constituent. Nimbin and Nimbidin highly abundant in the neem seed are known for their role in disease management through modulation of various genetic pathway and other activities

REFERENCES

1. Karonen M, Loponen J, Ossipor V, Pihlaja K. Analysis of procyanidins in pine bark with reversed-phase and normal-phase high performance liquid chromatography-electrospray ionization mass spectrometry. Anal chimActa 2004; 552:105-112.
2. Kausik B, Ishita C,Ranajit BK,Uday B. Biological activities and medicinal properties of neem (*Azadirachta indica*). Current Sci 2002; 82:1336-45.
3. Awolu OO, Obafaye RO, Ayodele BS. Optimization of solvent extraction of oil from neem (*Azadirachta indica*) and its characterizations. JournSci Res & Report 2013; 2(1):304-14.
4. Awolu OO, Layokun, SK. Optimization of two-step transesterification production of biodiesel from neem (*Azadirachta indica*) oil. Int Journ Energy and EnvironEng 2013; 4(1): 39.
5. Sindhu OP, Kumar V, Behl HM. Variability in tritrpenoids (nimbin and salanin) composition of neem among different provinces of India. Industrial Crops and Products 2004; 19(1):69-75 .
6. Kim DH, Jung EA, Sohng IS, Han JA, Kim TH, Han MJ. Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. Arch Pharm Res 1998; 21:17-23.
7. Chu Y, Wu X, Lui RH. Antioxidant and antiproliferative activity of common vegetables. J Agric FoodChem 2002; 50(23):6910-16.
8. Singleton VI, Lamuella-Raventos RM.Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-ciocalteu reagent. Methods in Enzymology 1999; 299:152-78.
9. Dewanto, X., Wu, K., Adom, K., Liu, R.H. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric FoodChem 2002; 50: 3010-14.
10. Sun B, Richardo-Da Silva JM, Spranger I. Critical factors of vanillin assay for catechins and proanthocyanidins. J Agric Food Chem 1998; 46:4267-74.
11. Gyamfi MA, Yonamine M, Aniya Y. Free radical scavenging action of medicinal herbs from Ghana: *Thoningia Sanguinea* on experimentally induced liver injuries. Gen Pharmacol 1999; 32:661-7.
12. Ozcan T, Akpinar-Bayizit A, Yilmaz-Ersan L, Delikanli B. Phenolics in human health. IntJourn Chem Eng &Applic 2014; 5:5.
13. Tiwari SC, Husain N. Biological activities and role of flavonoids in human health – a review. Indian JournSci Res 2017; 12(2):193-196.
14. Wong CC, Li HB, Cheng KW, Chen F. A systematic survey of antioxidant activity of 30 chinese medicinal plants using the ferric reducing antioxidant power assay. Food Chem. 2006; 97:705-711.
15. Liu CL, Chen YS, Yang H, Chiang BH.antioxidant activity of tartary (*Fagopyrum tataricum* (L.) Gaertn.) and common (*Fagopyrum esculentum* Moench) buckwheat sprouts. J Agric FoodChem 2008; 56:173-78.
16. Alzohairu MA. Therapeutics role of *Azadirachta indica* (Neem) and their active constituents in diseases prevention and treatment. EvidBased Complem Altern Med 2016; 7382506.

Cite this article as:

Awolu, Olugbenga Olufemi *et al*. Extraction, isolation and characterisation of free and bound polyphenols from neem (*Azadirachta indica* Juss) seed. Int. Res. J. Pharm. 2017;8(12):30-33 http://dx.doi.org/10.7897/2230-8407.0812246

Source of support: Nil, Conflict of interest: None Declared