Research Journal of Chemical Sciences _____ ISSN 2231-606X
Vol. **5(7),** 52-58, July **(2015)** *Res. J. Chem. Sci.*

Physical characteristics, Chemical composition and Distribution of constituents of the Neem seeds (*Azadirachta indica* A. Juss) collected in Senegal

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Available online at: www.isca.in, www.isca.me
Received 8th July 2015, revised 13th July 2015, accepted 18th July 2015

Abstract

Neem seeds (Azadirachta indica A. Juss) collected in southwest Senegal were characterized. The physical characterization revealed an average seed weight of 0.28 g with 50.89% of kernel and 49.11% of hull. These seeds contain 29.27±0.06% of lipids, 12.10±0.32% of proteins and 43.98±2.67% of parietal constituents (celluloses, hemicelluloses and lignins) with 30.33±1.12% of cellulose containing 68.96% of fibers. The study of the constituent(s) distribution showed that 96.82% of the lipids and 92.20% of the proteins are localized in the kernel, while 92.22% of the parietal constituents are localized in the hull. The azadirachtin is localized in the kernel (99.35%). Neem seeds also contain 14.99±0.37% of hydrosoluble, 0.11±0.05% of polyphenols and 0.76‰ of essential oil. The composition of the proteins revealed 17 amino acids with the predominant compound being glutamic acid (23.65%). The oil fatty acids are oleic acid (41.91±0.69%), linoleic acid (19.59±0.44%), stearic acid (18.71±0.46%) and palmitic acid (15.59 ± 0.27%). The oil is predominantly composed of unsaturated fatty acids (63% in the fatty acids composition). The oil is mainly composed of triglycerides (97.69%). These are mostly made up of SOL (52.93%) and POL (36.61%). The sterols being present at 2.04 g.kg^{-1} in the oil are mainly composed of β-sitosterol, which represents 61.08% of the total sterols. The total tocopherol content is 33.87 mg. 100g^{-1} and the γ-tocopherol is the major compound with 68.69% of total tocopherols.

Keywords: *Azadirachta indica*, neem seeds, oil, proteins, azadirachtin, parietal constituents.

Introduction

Neem (*Azadirachta indica* A. Juss) is a plant of the Meliaceae family belonging to the Indian subcontinent[1]. It was later introduced into many tropical countries of America and Africa including Senegal with a population of 18 to 30 million trees[2]. Different parts or extracts of neem have been used since a long time, particularly in traditional medicine[3]. Nevertheless, in Senegal, their properties and composition are still poorly understood and their potential is under-exploited. From the point of view of the added value, the neem seed is the most important part of the plant given its content in oil and its many active molecules. That is why special attention is paid to the seed. However, research on the seeds has increased since the isolation of azadirachtin as a natural insecticide[4]. Thus, many works have been carried out notably on their characterization. Studies have shown that the lipids content of the neem seed varies from 20 to 32% and the lipids content of the kernel varies from 30 to 52%[5,6]. Unfit for human consumption, it has multiple uses mainly for the soap, pesticide and pharmaceutical[7]. It also has antibacterial, antifungal and medicinal properties[8]. Its fatty acids composition and sterols have been reported[9,10]. In addition to its oil composition, neem seeds contain more than 100 active compounds which are together called triterpenoids or limonoids, including azadirachtin that would be one of the most important biopesticides[10-12]. The average azadirachtin content of neem seed kernels can vary from 2.05 to 6.10 g.kg^{-1}[13]. Several studies concerning its extraction, its purification, its efficacy, its toxicity, etc. were conducted[14-17]. The proteins content of the seeds and its amino acids composition have also been reported[9].

The aim of the present study is to carry out the physical characterization of seeds and to determine their chemical composition and the distribution of the components between the kernel and the hull. It will also determine the physico-chemical characteristics of the oil and the composition of its fatty acids, glycerides, sterols and tocopherols. Thus, knowing the chemical constituents, their locations, their structures and physicochemical properties will provide support to guide the processes of their extraction, separation and purification for their development.

Material and Methods

Plant material: The plant material consisted of some dry neem seeds (*Azadirachta indica*). These seeds coming from mature fruits, collected insouthwest Senegal (12° 33' 40 North, 16° 17' 00'' West) in August 2012, were cleaned and dried whit sun exposure at the open air and then in an oven at 40°C for 7 days.

Kernals and hulls were obtained by shelling seeds. The physical properties (average weight of a seed, a kernal and a hull) were determined with a sampling a minimum of 50 seeds.

Solvents and reagents: All chemical reagents, standards and solvents were of analytical type (HPLC grade), from Sigma-Aldrich, France.

Dry matter: The dry matter content was determined according to French standard NF V 03-903. It corresponds to the mass loss undergone by a sample of about 1 g after drying in an oven at 103°C until a constant mass.

Minerals content: The ash content was determined by mass the loss of the dry matter trough its incineration in a muffle oven, electrically heated at 550°C for 3 hours (NF V 03-922). The sample was then cooled in a desiccator and weighed as it reached room temperature.

Extraction, purification and analysis of azadirachtin: Azadirachtin content in the neem seeds, kernels and hull was determined after its extraction, purification and analysis.

Azadirachtin was extracted in a batch reactor equipped with a mechanical stirrer from 80 g of crushed seeds with a volume of 400 mL of methanol 4 hours (in three successive extractions). The methanolic extract was defatted using hexane and the azadirachtin was extracted with dichloromethane. These operations were performed 3 times.The dichloromethane extract was dry concentrated in a rotary evaporator at 35°C. The solid was taken up in acetonitrile and filtered with a PTFE filter of 0.22 µm. The analysis was performed whit a Dionex type Ultimate 3000 HPLC equipped with a C18 column (100 × 3 mm 3 Omnispher C18), maintained at 30°C and a UV-visible detector (λ = 215 nm). The mobile phase consisted of acetonitrile/water at a flow rate of 0.8 mL/min. The injection volume was 20 µL. The mobile phase flow rate gradient programming was: 20% acetonitrile from 0 to 5 min, increased from 20 to 65% acetonirile from 5 to15 min and maintained at 65% for 5 min more.

The oil extraction: The oil contents were determined by using the standardized soxhlet method (NF ISO 734-1) that consists in extracting the lipids contained in the matter with cyclohexane for minimum 6 hours. An amount of about 30 g of seeds was used. The soxhlet extractor was equipped at its base with a 250 mL flask in which 200 mL of solvent are introduced. The oil used for the analysis of tocopherols was extracted by cold centrifugation.

Physicochemical characteristics: The physicochemical characteristics were determined according to standardized methods: the density (AFNOR T60-214), the viscosity (ASTM D 445), the flash point (ASTM D 93), the freezing point, the pour point (ASTM D 97), the acid value (AFNOR T60-204), the saponification value (AFNOR T60-206), the iodine value (AFNOR T60-203), the peroxyde (AFNOR T60-220), cetane number (ASTM D 976), ash content (ASTM D 482), the carbon residue content (ASTM D 189), the sulphur content (ASTM D 4294), the sediment content (ASTM D 4052), the water content (ASTM D 4052). However, the refractive index was measured at 25°C by direct reading with a refract meter ABBE RMT model (EXACTA + OPTECH France 77646 CHELLES France) while the calorific value was estimated using the following empirical relationship : Calorific = 11380 – Iodine value – 9.15 × saponification value[18].

Gas Chromatography (GC) fatty acids analysis: The fatty acid profile was determined by analysis of its fatty acids methyl esters (FAME) in gas chromatography (GC) according to the NF ISO 5508 standard. The esterification was carried out in two steps, solubilization of the oil by TBME (tert-butyl methyl ether) and uploading TMSH (trimethyl sulphonium hydroxide 0.5 M in methanol). The analysis was performed in type GC 3800 equipped with a Varian CP-select column for FAME fused silica WCOT (length 50 m, internal diameter 0.25 mm, film thickness 0.25 µm) coupled with a flame-ionization detector (FID) heating the components at 250°C. The carrier gas was helium (flow rate of 1 mL/min). The injection was Split (1 : 100. µL 1. 250°C for 55 min). The temperature programming was 185°C for 40 min and then rise from 185°C to 250°C at 15°C/min and finally 250°C for 10.68 min (analysis time 55.01 min). The standard used was the MGFA (SI) and the data was processed whitVarian Star software.

GC glycerids and triglycerids analysis: Analysis of glycerides and triglycerides were carried out after the glycerides silylation by 50 µL of methyl imidazole with 1 mL MSHFBA (N-Methyl-N-trimethyl silyl-Hepta Fluoro butyramide). The analysis are performed by gas chromatography (Perkin Elmer) equipped with a CP Sil column 8CB Low Bleed MS Varian, length 15 m, internal diameter 0.32 mm, film thickness 0.25 µm. The injection was on column type 1 µL. The temperature program was 55°C for 0.5 min, then 200°C/min to 340°C, 340°C for 40 min. Helium was the carrier gas (column head pressure 15 psi). The injection to the oven was performed under the following conditions : 55°C for 0.5 min, 45°C/min to 80°C, 10 °C/min. up to 360°C and 360°C for 16 min. FID carried out detection at 365°C.The compounds were identified by retention time with standards reference and the quantification was carried out by external calibration.

GC Sterols analysis: Sterols were analyzed on the unsaponifiable fraction after silylation by MSHFBA (Methyl trimethylsilyl heptafluorobutyramide + 50 µL 1methyl imidazole). The analysis was performed by GPC (Perkin Elmer lane 2) coupled to an FID (365°C) and equipped with a column CPSil 8 CB (Varian) of length 30 m, diameter 0.25 mm and film thickness 0.25 µm. The injection was on column type (1µL), the carrier gas was helium and the column head pressure was 100 kPa.

The injector temperature programming was 55°C for 0,5min, then raised from 55 to 340°C at 200°C/min and stabilized to

340°C for 30 min. The temperature of the oven was 160°C for 0.5 min, then rise from 160 to 260°C at 20°C/min and stabilized to 260°C for 5.5 min then rise from 260 to 300°C at 2°C/min then maintaining the temperature for 10 min at 300°C finally a rise from 300 to 350°C at 45°C/min and stabilized to 350°C for 3 min.

HPLC tocopherols analysis: The analysis of the tocopherols of neem oil, using the standards of α, γ and δ-tocopherols by external calibration was performed according to EN ISO 9936. Exactly 10 mg of oil were diluted with 1 mL of cyclohexane. The sample was analyzed by HPLC Dionex type equipped with a Kromasil 100 column SIL 5 µ (250 × 4 mm) and a fluorescence detector (λex = 290 nm and λem = 317 nm). The eluent was composed of mixture isooctane/isopropanol (99.5%/0.5%) at a flow rate of 1.1 mL/min.

Proteins content: The protein concentrations were determined by the Kjeldahl method according to French standard NF V 18-100. It consists in determining the total nitrogen content in the sample to obtain an ammonium salt. This analysis was performed using an automatic device consisting of a Kjeltec 8400 Analyzer and Kjeltec 8420 Treader and consists of transformation by mineralization of organic nitrogen in the treated sample (400 mg) and also consists of an acid-base determination of inorganic nitrogen (ammonia).

Amino acids composition: The amino acids dosage of the proteins of the neem seeds was performed according to the European Standard. The analysis was performed by ion-exchange liquid chromatography using Biochrom 30^+ equipped with a filled PEEK column of cation exchange resin according to the following conditions : column temperature : 20 to 99°C, use of pressure 24-150 bars, injection volume 1 to 5000 µL, detection by photometer 440-570 nm.

Parietal constituent's content: The method of Van Soest and Wine also known as ADF-NDF assay makes it possible to determine lignins, celluloses and hemicelluloses[19]. It is based on the difference in solubility of the components. The NDF (Neutral Detergent Fiber) attacks and solubilizes all the compounds except the cellulose, hemicellulose and lignin. The first ADF (Acid Detergent Fiber) permanganate attack solubilized the compounds except cellulose and lignin. The second ADF attack left only cellulose. These attacks are carried out in a device called a Tecator Fibertec M1017.

Results and Discussion

Physical characteristics: The physical characterization of neem seeds reveals that the average mass of a seed is 0.28 g. It is composed at 50.89% by the kernel and at 49.11% by the hull (table-1). These results agree with those published before[20].

Table-1
Physical characteristics of neem seeds

Seed (g)	0.28
Kernel (g)	0.15
Hull (g)	0.13
Kernel (%)	50.89
Hull (%)	49.11

Average results determined by weighing

Compostion and distribution of neem seed constituents: The composition shows an oil content of 29.27±0.06%, a proteins content of 12.10±0.32% and a parietals constituents (fibers) of 43.98±2.67%. The composition of parietals constituents reveals a cellulose content, which represents 30.33±1.12% of the seed composition (Table-2). These values are comparable to those of previous studies[14]. It contains 14.99±0.37% water soluble and 0.11±0.05% of total polyphenols. Polyphenols are known for their many therapeutic and antioxidant properties[21]. The azadirachtin content of the neem seed (2.24 $g.kg^{-1}$) is in the range of literature values[5].

The distribution of the constituents of the seed shows that almost all of the oil is found in the kernel, at 48.98±0.34%, which represent 96.82% of the oil contained in the seed. This is also the case for proteins which are located at 92.20% in the kernel. In contrast with lipids and proteins, parietal constituents are in very low concentrations in the kernel, they constitute the bulk of the hull, whit a quantity of 80.23±3.94% in the hull, representing 92.22% of the total parietal constituents of the seed. These results are comparable with those described in the literature[6,9].

Physico-chemical characteristics of the oil: Several physico-chemical characteristics were determined (table-3). The small amount of free fatty acids, resultedin a low index of acidity (10.2 $mg.g^{-1}$) and high index of saponification (200 $g.mg^{-1}$) showed a high proportion of saponifiable, which confirms its good quality of the need oil seed for the production of soap. The INS factor of 127, gives neem oil a good ability to produce soap, even without mixing with other fats. The iodine value is 74.70 $g.100g^{-1}$and reflects a high proportion of unsaturated fatty acids, of about 60%.The relatively high calorific value (39.61 $MJ.kg^{-1}$) is similar to that of diesel which is 43.8 $MJ.kg^{-1}$ [22]. The high flash point (227°C) confirms the absence of risk of fire during handling or storage.

Fatty acids composition: The fatty acid composition (table-4) shows the presence of nine fatty acids, including four major ones. These are oleic acid (41.91±0.69%), followed by linoleic acid (19.59±0.44%), stearic acid (18.71±0.46%) and palmitic acid (15.59±0.27%). This high linoleic and oleic acid proportion is noted in Jatropha oil[23]. The fatty acid profile is comparable to those presented in the literature[20,9]. Indeed, the ratio unsaturated/saturated fatty acids, being at about 60%, confirms the unsaturated behavour of the oil.

Table-2
Composition and distribution of neem seeds constituents

		Whole seed	Kernel	Hull
Dry Matter(%)		95.73	97.13	95.33
Lipids (DM %)		29.27±0.06	48.98±0.34	1.61±0.05
Proteins (DM %)		12.10±0.32	24.55±0.04	2.08±0.02
Parietal constituents (DM %)	Cellulose	30.33±1.12	4.15±0.20	54.97±0.71
	Hemicelluloses	9.47±0.85	2.38±0.01	13.35±1.28
	Lignins	4.18±0.70	0.23±0.20	11.91±1.95
	Total	43.98±2.67	6.76±0.41	80.23±3.94
Minerals (DM %)		4.72±0.07	4.09±0.01	5.13±0.15
Polyphenol (DM %)		0.11±0.05	-	-
Hydrosoluble components (DM %)		14.99±0.37	23.88±0.13	4.80±0.19
Azadirachtin ($g.kg^{-1}$ DM)		2.24±0.13	4.62±0.18	0.03

DM : Dry Matter

Table-3
Physico-chemical characteristics of the neem seeds oil

Characteristics	Value	Methods
Acidvalue ($mg.g^{-1}$)	10.2	AFNOR T60-204
Saponification value ($mg.g^{-1}$)	200	AFNOR T60-206
Iodine value ($g.100g^{-1}$)	72.82	AFNOR T60-203
INS	127	-
Cetane number	82.22	NF 07013
Peroxyde value (meq $O_2.kg^{-1}$)	1.49	AFNOR T60-220
Density at 25°C	0.919	AFNOR T60-214
Refraction index at 25°C	1.465	Direct reading
Viscosity at 37,8°C ($mm^2.s^{-1}$)	49.79	NF ISO 30104
Calorific value ($MJ.kg^{-1}$)	39.61	-
Flash point (°C)	227	NF EN ISO 2719
Freezing point (°C)	10	ASTM D 97
Pour point (°C)	12	ASTM D 97
Ash (wt%)	0.023	ASTM D 482
Carbon residue (wt%)	1.45	ASTM D 189
Sulfur (wt%)	0.11	ASTM D 4294
Sediments (wt%)	0.01	ASTM D 4052
Water content (%v)	0.2	ASTM D95-90

Table-4
Fatty acids composition of neem seeds oil

Composition	%
C16 :0Palmitic acid	15.59±0.27
C16 :1Palmitoleic acid	0.12±0.00
C18 :0Stearic acid	18.71±0.46
C18 :1Oleic acid	41.91±0.69
C18 :2Linoleic acid	19.59±0.44
C20 :0Arachidic acid	1.33±0.01
C18 :3Linolenic acid	0.44±0.01
C20 :1Gadoleic acid	0.08±0.00
C22 :0Behenic acid	0.86±0.38
Saturated fatty acids	37.00
Unsaturated fatty acids	63.00

Glycerides and triglycrides composition: Determining the glycerides profile neem oil, reveals the predominance of triglycerides, at 97.69% of the glycerides content in the needoil (table-5). This content is in the range of vegetable oils values and confirms the lipids storage function in the seed. Diglycerides (16 and C18) are instead in very small proportion (1.45%) while free fatty acids (FFA) are in negligible proportion (0.76%). These results reflect a significant proportion of the saponifiable fraction of this oil. The saponification fraction represents 96.83% of the total glycerides, resulting in an unsaponifiable fraction of 3.17%. This unsaponifiable content is slightly higher than that obtained by Djenonthin *et al*.[24].

Table-5
Proportion of neem seeds oil glycerides

Composition	%
Triglycerides	97.69
Diglycerides (C16 and C18)	1.45
Monoglycerides C18	0.10
FFA (C16 and C18)	0.76

The oil consists of two major triglycerides: SOL by a proportion of 52.93% and POL by a proportion of 36.61% (table-6). Both triglycerides represent 89.54% of the total triglycerides. This composition perfectly agrees with the fatty acid composition because it consists mainly of triglycerides of oleic acid, linoleic acid, palmitic acid and stearic acid which are the four major fatty acids in neem oil.

Table-6
Neem seeds oiltriglycerides composition

Composition	%
PPPl	0.46
PPlL	7.1
POL	36.61
SOL	52.93
SAO	2.9

P: Palmitic acid C16: 0; Pl: Palmitoleic acid C16: 1; L: Linoleic acid C18: 1; O: Oleic acid C18: 1; S: Stearic acid C18: 0; Arachidic acid C20: 0

Sterols composition: With a grade of 3.34±0.07 g.kg^{-1}in oil, the sterols contribute 10.54% of the unsaponifiable fraction of the oil (Table-7). The β-Sito sterol, with 2.04±0.02 g.kg^{-1}, or 61.08% of total sterols, is the major compound. This is followed far behind by stigmasterol, 0.43±0.01 g/kg (12.87%), Δ^5-Avenasterol, 0.28±0.01 g.kg^{-1} (8.38%) and campesterol, 0.21±0.01g.kg^{-1} (6.92%). These sterols are the most abundant in most vegetable oils[25].

High levels of β-sitosterol are found in most vegetable oils such as olive oil, peanut oil, sunflower oil, *Lophira lanceolata* and *Carapa procera* seed oils with 84.3%, 62.3%, 61.9%, 63.74% and 47.04% respectively, in percentage of the total sterols[26,27]. The β-sitosterol is the most intensely studied sterol because of its importance and its physiological effects on health. Several clinical studies have demonstrated the effectiveness of β-sitosterol on lower cholesterol and its anti tumor effects[28].

Table-7
Neem seeds oil sterols composition

Composition	g.kg^{-1}	%
24-met.cholesterol	0.08±0.01	2.4
Campesterol	0.21±0.01	6.29
Stigmasterol	0.43±0.01	12.87
β-Sitosterol	2.04±0.02	61.08
Δ^5-Avenasterol	0.28±0.01	8.38
Cycloarterol	0.07±0.00	2.1
Mét. Cycloarterol	0.08±0.01	2.39
Citrostadienol	0.2±0,00	0.6
Unknow	0.02±0.00	0.6
Unknow	0.11±0,00	3.29
Total	3.34±0,07	100

Tocopherols composition: The oil has a tocopherol content of 338.70 mg.kg^{-1}, or 1.06% of the unsaponifiable fraction of the oil (table-8). The γ-tocopherol is the major component, representing 68.88% of total tocopherols 233.30±0.39 mg.kg^{-1}. Due to their antioxidant activity, they play an important role in the oxidative stability of the oil during its conservation[29]. Tocopherols are also known for their positive effects on health. Indeed, they prevent the oxidation of polyunsaturated fatty acids in the blood system and protect low density lipoproteins (LDL) from oxidation induced by free radicals causing the development of atherosclerotic lesions. Other studies showed that tocopherols reduce cardiovascular diseases and have some anti-cancer properties[30].

Table-8
Tocopherols composition of neem seeds oil

Composition	mg.kg^{-1}	%
α-tocopherol	65.70±0.12	19.38±0.06
β-tocopherol	2±0.01	0.59±0.02
γ-tocopherol	233.30±0.39	68.88±0.15
δ-tocopherol	37.60±0.09	11.13±0.10
Total	338.70±0.59	100

Amino acids composition of neem seeds proteins: The protein composition of neem seeds reveals the presence of seventeen amino acids (table-9). The major compounds are glutamic acid (23.65±0.2%), aspartic acid (9.62±0.04%), glycine (8.64±0.09%), leucine (8.09±0.11%), serine (7.19±0.24%) and alanine (7.14±0.06%). It is possible to notice the presence of all the essential amino acids leucine, valine, glycine and threonine in significant proportion. This could justify their nutritional quality. Also, this amino acid composition is comparable to those obtained in other geographical neem seeds[29,15].

Table-9
Amino acids composition of the neem seeds

Aminoacids	%
Aspartic acid (Asp)	9.62±0.04
Threonine (Thr)	4.15±0.02
Serine (Ser)	7.19±0.24
Glutamic acid (Glu)	23.65±0.2
Glycine (Gly)	8.64±0.09
Alanine (Ala)	7.14±0.06
Cysteine (Cys)	2.67±0.04
Valine (Val)	6.48±0.06
Méthionine (Met)	0.08±0.05
Isoleucine (Ile)	3.62±0.02
Leucine (Leu)	8.09±0.11
Tyrosine (Tyr)	1.47±0.54
Phenylalanine (Phe)	3.36±0.06
Histidine (His)	2.23±0.02
Triptophane (Trp)	1.06±0.04
Lysine (Lys)	4.14±0.15
Arginine (Agr)	6.41±0.07

Average values obtained from three tests

Conclusion

The study revealed that neem seed weighs an average of 0.28 g, of which 50.89% is kernel and 49.11% is hull. It contains 29.27% of lipids, 12.10% of proteins and 43.28% of parietal constituents. The azadirachtin content is at 2.24 g.kg^{-1}. The study of the distribution of the components shows that 96.82% of the lipids, 92.20% of the proteins and almost all of azadirachtin are located in the kernel, while 92,22% of the parietal constituents are located in the hull. The composition of the proteins revealed the presence of all essential amino acids. The physico-chemical characteristics of the oil showed that apart from its traditional uses, it has a good potential for biofuel production and soaps. The fatty acid profile showed four majority compounds, oleic acid, linoleic acid, stearic acid and palmitic acid. The glyceride composition revealed that triglycerides constitute 97.69%. The majority of the oil triglycerides are SOL and POL. The analysis of the unsaponifiable fraction showed that the β-sitosterol is by far the major sterol. The major tocopherol was γ-tocopherol.

The state of knowledge on seed composition could help us in the development by innovative fractionation process and eco-

compatible twin-screw extruder for the production and promotion of its azadirachtin, its oil and its cake according a bio refining approach.

Acknowledgements

We thank the Department for Cooperation and Cultural Action (SCAC) of the Embassy of France at Dakar (Senegal) for their financial support.

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