Chemical characterisation of the Senegalese neem seed: Distribution of the main constituents: azadirachtins, lipids, proteins, fibers (cellulose, hemicelluloses and lignin).

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Summary: Oil and azadirachtin contents of neem (*Azadirachta Indica*) seeds depend on various origins and countries. Two batches collected in 2007 and 2008 were characterised in terms of azadirachtin, oil, fatty acids, sterols, proteins and fibbers.
Senegalese neem seed contains 32.74±0.38% of oil and 1.99±0.03 g kg^{-1} of azadirahtin in batch2008 which are located mainly in the kernel.
The fatty acid profile of the two batches showed little difference. The average values of the major fatty acid, oleic acid, were: 44.61±0.05% in batch 2007 and 43.61±0.20% in batch 2008. Distribution of total fatty acid composition showed no important difference between the whole seed and the kernel while there was some difference with the hull. Concerning the unsaponifiable fraction, total sterols were estimated at respectively 3.73±0.08 g kg^{-1} and 3.93±0.07 g kg^{-1} of oil in batch 2007 and batch 2008. For β-sitosterol, the most important one in proportion, these values were respectively 2.40±0.01 and 2.70±0.03 g kg^{-1} for batch 2007 and batch 2008 respectively.
Other important constituents in neem seed such as protein, parietal compounds were respectively 11.86±0.54% and 49.84±2.55% in batch 2007 and 11.75±0.57% and 48.47±2.32% in batch 2008. Proteins are mainly located in the kernel while parietal compounds are located in the hull.
Keywords: Neem; azadiractin; oil; fatty acid; sterol; protein; parietal compounds

Caractérisation chimique de la graine de neem Sénégalais: Distribution des composés majeurs : azadirachtine, lipides, protéines, fibres (cellulose, hémicellulose et lignine)

Résumé: Les teneurs en huile et en azadirachtine des graines de neem (*Azadirachta Indica*) dépendent de l'origine et du pays. Deux lots de graines de neem collectés en deux années différentes, 2007 et 2008, sont caractérisés en termes de teneurs en azadirachtine, huile, protéines, fibres et composition en acides gras et stérols.
Les graines de neem du lot2008 contiennent 32.74±0.38% en huile et 1.99±0.03 g kg^{-1} en azadirachtine qui sont quasiment largement localisés dans les amandes.
Les profiles en acides gras des deux lots montrent de faibles différences. Les valeurs moyennes de l'acide gras majoritaire sont : 44.61±0.05% dans le lot 2007 et 43.61±0.20% dans le lot 2008. La distribution de la composition en acide gras montre de faible différence entre la graine et l'amande cependant on note quelques différences avec la coque. Concernant la fraction insaponifiable, la teneur totale en stérol est estimée à 3.73±0.08 et 3.93±0.07 g kg^{-1} de l'huile dans le lot 2007 et le lot 2008 respectivement. Pour le β-sitosterol, composé majoritaire, ses teneurs sont respectivement 2.40±0.01 et 2.70±0.03 g kg^{-1} pour les lot2007 et lot2008.
Les autres composés important dans les graines de neem comme les protéines et les fibres sont respectivement de 11.86±0.54% et 49.84±2.55% dans le lot2007 et 11.75±0.57% et 48.47±2.32% dans le lot2008. Les protéines sont principalement localisées dans les amandes alors que les fibres sont localisées dans les coques.
Mots clés : Neem, azadirachtine, huile, acide gras, stérol, protéine, composés pariétaux

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1. Introduction

Neem (*Azadirachta Indica*) of the Meliaceae family is an evergreen tree native from the Indian subcontinent and South-East Asian countries. It has been the focus of intensive research for the past three decades since azadirachtin was isolated from its seeds as a natural insecticide.[1, 2] The neem tree has many biological activities and medicinal properties.[3] Its seed contains many triterpenoids [4, 3, 5] which show anti-feedant and growth-regulating effects on many species of insects. [6, 7] The triterpenoids can also block insects reproduction.[8]

Azadirachtin represents about 0.2 to 0.8% [9] of the kernel by weight. Nine azadirachtin isomers are reported in the literature (Azadirachtins A to K) [10, 11, 12] but azadirachtin A and B are the major active components and are used as a standard to express the neem activity. They are commercialized as technical azadirachtin and as biopesticides [13] such as Econeem Plus, Neem Azal, Fortune Aza, Neemix, Azatin EC.

Oil represents about 30 to 50% [14] of the seed kernel and is used in the formulation of pesticides, in soap and pharmaceutical industries. Fatty acid composition is one of the most important indicators of oil quality. Four important fatty acids characterise the oil: palmitic and stearic are saturated, oleic is monounsaturated and linoleic is polyunsaturated.

Many studies, carried out by Ermel et *al*.[15], Kaushik and Singh,[16] SIDHU et *al*.[17] and Nutan Kaushik et *al*.[18], showed the natural variability of azadirachtin and oil content in seeds obtained from individual trees in seasonal and geographical zone in Inde.

The objective of this study was to quantify the main components of Senegalese neem seed and their distribution in the kernel and the hull of seeds collected in two different years 2007 and 2008. In Senegal, the climate and environment conditions do not change much from a year to another; only the pluviometry and the temperature may change. These changes have some repercussions on the seed composition.

2. Materials and Methods

2.1 Plant Material

All trials were carried out with whole uncleaned neem seeds, which had been provided by SENCHIM Company (Km 13 Route de Rufisque, Dakar, Senegal) which is a firm specialising in the formulation and commercialization of pesticides. There is only one variety of neem in Senegal. The two batches used were collected in two different years 2007 and 2008 and contained each 1% of impurity. Their moisture contents were 9.04±4 and 9.10±3%, respectively. The trials were carried out on the whole seeds, kernels and hulls. Kernel itself represents nearly 52.32±0.80% of the seed weight.

Before extraction, the neem seeds (batch 2007 and batch 2008) were first crushed using a shredder MF Basic 10 with a 1 mm mesh sieve.

2.2 Solvents and reagents

All solvents and chemical reagents were analytical grades and were obtained from Sigma-Aldrich, Fluka, Prolobo and ICS (France). Water for chromatographic analyses was purified by using Milli-Q water purification system. The azadirachtin (A+B) standards were purchased from Sigma-Aldrich (Germany) with a purity of 95%.

2.3 Oil extraction and fatty acids methylation

Oil content of the whole seed, kernel and hull was determined by continuous extraction with a soxhlet (according to the French standard NF V 03-908) using cyclohexane. After six hours of extraction, the solvent was eliminated by means of

rotary evaporation at 40°C under vacuum. The weight of oil was then determined.
Fatty acids composition was determined by analyzing their corresponding Fatty Acids Methyl Esters (FAMEs) according to the norm NF of ISO 5508. Then, 20 mg of oil were solubilised in 1mL of TBME (tert-butyl-methyl ether) and 100 µL of this solution were mixed with 50 µL of TMSH (trimethyl sulphonium hydroxide 0.5 M in methanol).

2.4 Azadirachtin extraction

Azadirachtin was extracted with methanol in a mechanically stirred double layer batch reactor and purified [9, 19, 20]. A sample of 100 g of seed powder was mixed with a volume of 3×400 mL of solvent. Methanol extract was then filtered, centrifuged (8000×g, 10 min and 20°C) and concentrated at 40°C in a rotary evaporator before dilution with an equal volume of water. The aqueous methanol mixture was then extracted three times with equal volume of n-hexane. The aqueous methanol phase was extracted with dichloromethane in the same manner as n-hexane extractions. Dichloromethane extract was concentrated to dryness at 40°C under vacuum by rotary evaporation. The dark orange solid obtained was then dissolved in 25 mL of acetonitrile and analyzed by HPLC.

2.5 Gas chromatography: analysis of fatty acids and sterols

FAMEs were analyzed with a CP-Select 3900 gas chromatograph equipped with a FID (flame ionization detector) and a fused silica capillary column, CP-select (0.25 mm×50 m, 0.25 µm film thickness). The carrier gas was H_2 with a flow rate of 1.2 mL/min; split ratio was 1:100. The temperature program was: isotherm at 185°C during 40 min, increase from 185°C to 250°C at a rate of 15°C/min and isotherm at 250 during 10 min. The detector and injector temperatures were at 250°C. Fatty Acids were identified by comparison of their retention times with those of pure reference standards.
Sterols samples were analyzed by GC Varian with FID, a CP-Sil 8CB capillary column (0.25 mm×30 m and 0.25 µm film). The carrier gas was H_2 with a flow rate of 1mL/min (split-splitless injection was used). Analyses were performed under the following temperature program: isotherm at 160°C during 0.5 min, increase from 160°C to 260°C at a rate of 20°C/min, 2°C/min to 300°C and 45°C/min to 350°C. Injector and detector temperatures were maintained respectively at 340°C and 365°C.

2.6 High Performance Liquid Chromatography (HPLC): Azadirachtin analysis

Analysis was performed using HPLC ASI 100 (DIONEX SUMMIT) with C18 column and visible UV detector [3, 21]. Wavelength was set at 217 nm. The mobile phase was a mixture of two solvents: acetonitrile and water at a rate of 0.8 mL/min. The injection volume was 20 µL. The program of the gradient flow was as follows: 20% acetonitrile from 0 to 5 min, increase from 20 to 65% from 5 to 15 min and then 65% for 5 min.

2.7 Protein and parietals constituents

Protein content was determined by Kjedahl method according to the French Standard NF V18-100, consisting of mineralization of organic nitrogen content in the sample to mineral nitrogen.
Parietal constituents (hemicellulose, lignin and cellulose) were estimated by Van Soest and Wine method [22, 23, 24] consisting of two attacks. During the Neutral Detergent Fiber (NDF) attack, the neutral detergent used solubilizes all the non parietal constituents (proteins, pectins). The insoluble fraction (residue) represents the sum of the following constituents: hemicellulose, lignin and cellulose. During the first Acid Detergent Fiber (ADF) attack, the acid detergent used solubilizes all the non parietal components and

hemicellulose. A second attack ADF with permanganate solubilises the lignin and the residual fraction corresponds to the cellulose.

2.8 Statistical analyses

Data were subjected to statistical analysis using the statistical program package STATISTICA.[25] Results are means±SD of three experiments. The one-way analysis of variance (ANOVA) followed by Duncan multiple range test was employed and the differences between individual means were deemed to be significant at $P < 0.05$.

3. Results and discussion

3.1 Oil yield and fatty acids composition

The results reported in Figure 1 showed that oil contents of batch 2007 and batch 2008 were respectively 32.21±0.68 and 32.74±0.38% and that almost all of the oil is concentrated in the kernel. The oil contents in the kernel were 58.38±2.64% and 55.94±2.79% respectively in batch 2007 and batch 2008 while they were 3.55±0.76% and 3.46±1.29% in hull respectively. Sidhu et *al.*[26], studied the variability of azadirachtin and oil contents of neem seed kernel from 43 origins among seven states of India. They reported that oil content ranged from 32.9 to 51.6%. Other papers reported a range from 30 to 50% in Indian kernel [14] and 32.9 to 45%.[27] Kaura et *al.*[28] noted that neem whole seed in India presented an oil yield ranging from 20 to 32.61%. Muñoz-Valenzuela et *al.*[29] reported 18.7 to 24.5% in the Mexican fruit (whole seed and pericarp).

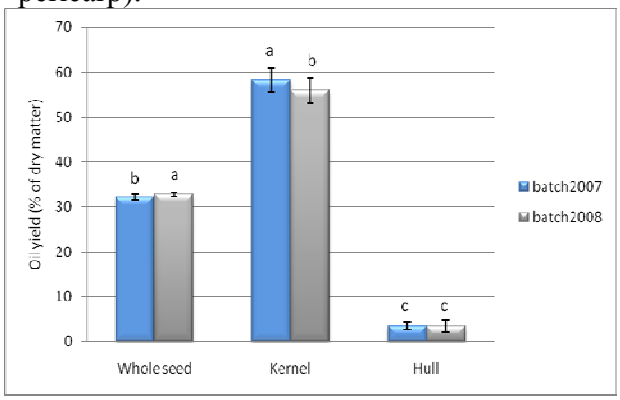

Figure 1. Oil content (% of dry matter) of different parts of neem seed (batch 2007 and batch 2008).Values were means of three replicates with different superscripts (a–c) are significantly different at $p < 0.05$ (Duncan test).

The fatty acids profile of the neem seed oil of batch 2007 and batch 2008 is shown in Table I. Fourteen fatty acids were identified. Oleic acid (C18:1n-9) was the major one; its percentage was 44.61±0.05% in batch 2007 and 43.61±0.20% in batch 2008. Oleic acid is an important element providing energy. Oils rich in this acid are also used to make cakes of soap. Neem oil can be strongly used for pesticide formulation. Researchers reported oleic acid contents varying from 25 to 58%.[14, 3, 29].

Table I: Fatty acids composition (% of total fatty acids) of neem seed in batch 2007 and batch 2008

Fatty acids	batch 2007	batch 2008
Myristic Acid (C14:0)	0.06±0.01^{b}	0.05±0.00^{b}
Pentadecanoïc Acid (C15:0)	0.23±0.03^{a}	0.07±0.01^{b}
Palmitic Acid (C16:0)	16.88±0.02^{c}	17.51±0.08^{b}
Palmitoleic Acid (C16:1)	0.12±0.00^{b}	0.13±0.00^{b}
Heptadecanoïc Acid (C17:0)	0.16±0.01^{b}	0.33±0.07^{a}
Stearic Acid (C18:0)	15.94±0.02^{b}	16.51±0.67^{a}
Elaidic Acid (C18:1)	1.35±0.01^{b}	0.20±0.18^{d}
Oleic Acid (C18:1)	44.61±0.05^{b}	43.61±0.20^{c}
Petroselenic Acid (C18:1)	0.49±0.00^{a}	0.43±0.01^{b}
Linoleic Acid (C18:2)	16.71±0.02^{c}	19.15±0.81^{a}
Arachidic Acid (C20:0)	1.27±0.00^{a}	1.24±0.08^{a}
Linolenic Acid (C18:3)	0.37±0.01^{c}	0.48±0.06^{b}
Behenic Acid (C22:0)	1.73±0.06^{a}	0.23±0.03^{c}
Erucite Acid (C22:1)	0.07±0.01^{a}	0.04±0.01^{c}
Saturated fatty acids	35.00±0.15^{a}	34.71±0.86^{a}
Unsaturated fatty acids	65.00±0.10^{a}	65.29±1.37^{a}
Saturated/unsaturated fatty acids	0.54	0.53

Data were means±S.D. of three replicates. Values with different superscripts (a–c) are significantly different at $p < 0.05$.

Other important fatty acids were identified such as linoleic acid (C18:2n-6) with 16.71±0.02% and 19.15±0.81%, stearic acid (C18:0) with 15.94±0.02% and 16.51±0.67% and palmitic acid (C16:0) with 16.88±0.02% and 17.51±0.08% in batch 2007 and batch 2008 respectively. Arachidic acid (C20:0), α-linolenic acid (C18:3n-3), behenic acid (C22:0),

palmitoleic acid (C16:1n-7) and other acids were the minor ones in both batches.

Saturated fatty acids represented 35.00±0.15% and 34.71±0.86% of total fatty acids while unsaturated fatty acids represented 65.00±0.10% and 65.29±1.37% in batch 2007 and batch 2008 respectively.

Table II shows the distribution of total fatty acids on seed, kernel and hull (batch 2008). Oleic acid (predominant fatty acid) had its lowest content in the hull with 39.58±0.13% and its largest in the kernel with 45.53±0.61%. The opposite phenomenon is observed with linoleic, palmitic, stearic, elaidic and linolenic acids. However the fatty acids composition of the whole seed was globally close to the kernel one as up to 95% of oil was concentrated in the kernel.

All parts (whole seed, kernel and hull) were characterized by high amounts of total unsaturated fatty acids compared to saturated ones. Saturated fatty acids represented respectively 33.67±1.19% and 37.93±0.38% of total fatty acids of the kernel and hull while unsaturated ones were respectively 66.33±1.27% and 62.07±1.08%.

3.2. Sterols composition of neem seed oil

Sterols constitute the major fraction of the unsaponifiable matter in many oils. Total sterols were estimated at 3.73±0.08 g kg^{-1} and 3.93±0.07 g kg^{-1} of oil in batch 2007 and batch 2008, respectively (Table III). β-sitosterol was the predominant sterols in the neem seed oil with 2.40±0.01 g kg^{-1} and 2.71±0.03 g kg^{-1} in batch 2007 and batch 2008, respectively. High β-sitosterol contents was found in the majority of vegetable oils, such as olive oil, grape oil, groundnut oil, sunflower and date seed oil, in which the mean relative contents were 84.3%, 62.2%, 62.3%, 61.9% and 80.98% of total sterols, respectively[30, 31].

Other major sterols were stigmasterol (0.52±0.01 g kg^{-1} and 0.47±0.01 g kg^{-1}), campesterol (0.34±0.01 g $kg^{-1}$and 0.35±0.01 g kg^{-1}) and the Δ^5-avenasterol (0.17±0.02 g kg^{-1} and 0.14±0.01 g kg^{-1}) in batch 2007 and batch 2008, respectively. Cholesterol, stigmastanol, cycloartenol and methylene cycloartanol were detected in lower levels.

3.3. Azadirachtin content of neem seed

Azadirachtin content of whole seed, kernel and hull is reported in Figure 2. Almost all azadirachtin was located in the kernel. Thus, its content was 3.86±0.07 g kg^{-1} in the kernel in agreement with Sidhu et *al*.[17] who showed that azadirachtin of Indian neem kernel ranges from 0.78 to 3.16 g kg^{-1}. These results confirm that Senegalese neem seeds are rich in azadirachtin.

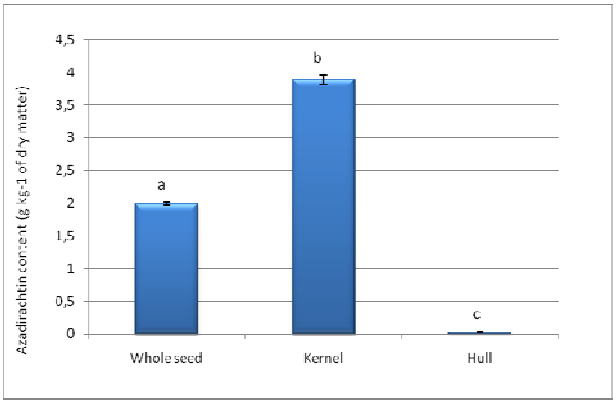

Figure 2: Azadiractin distribution in different parts of neem seed (batch2008). Values were means of three replicates with different superscripts (a–c) are significantly different at $p < 0.05$ (Duncan test).

3.4. Minerals, proteins and parietal components of the neem seed

The seed mineral contents were 4.25±0.03% and 4.44±0.23% respectively in batch 2007 and 2008 (Table IV). But no differences were noted in the different parts of the seed. The protein content was 11.86±0.54% in batch 2007 and 11.75±0.57% in batch 2008 (% of dry matter). Muñoz-Valenzuela et *al*.[29] reported that protein content of Mexican neem fruit was 14.3%. Proteins were mainly located in the kernel with 20.07±0.55% and 22.86±0.20% in the batch 2007 and batch 2008, respectively while the three parietal constituents (cellulose, hemicellulose and lignins) were located in the hulls. The total parietal constituents of batch 2007 and batch 2008

Table II: Fatty acids composition (% of total fatty acids) of oil in different parts of batch2008 seed

Fatty acids	**Seed**	**Kernel**	**Hull**
Myristic Acid	0.05±0.00^{b}	0.06±0.01^{b}	1.08±0.03^{a}
Pentadecanoïc Acid	0.07±0.01^{b}	0.23±0.03^{a}	0.22±0.02^{c}
Palmitic Acid	17.51±0.08^{b}	16.70±0.05^{c}	19.75±0.33^{a}
Palmitoleic Acid	0.13±0.00^{b}	0.12±0.00^{b}	1.64±0.42^{a}
Heptadecanoïc Acid	0.33±0.07^{a}	0.16±0.02^{b}	0.17±0.02^{c}
Stearic Acid	16.51±0.67^{a}	15.57±0.40^{b}	16.67±0.01^{a}
Elaidic Acid	0.20±0.18^{d}	0.85±0.04^{c}	2.70±0.10^{a}
Oleic Acid	43.61±0.20^{c}	45.53±0.61^{a}	39.58±0.13^{d}
Petroselenic Acid	0.43±0.01^{b}	0.48±0.01^{a}	0.48±0.02^{a}
Linoleic Acid	19.15±0.81^{a}	17.64±0.52^{b}	15.37±0.22^{d}
Arachidic Acid	1.24±0.08^{a}	1.23±0.04^{a}	1.18±0.16^{a}
Linolenic Acid	0.48±0.06^{b}	0.43±0.05^{c}	1.07±0.01^{a}
Behenic Acid	0.23±0.03^{c}	0.94±0.68^{b}	0.44±0.02^{c}
Erucite Acid	0.04±0.01^{c}	0.06±0.00^{ab}	0.06±0.01^{b}
Saturated fatty acids	34.71±0.86^{a}	33.67±1.19^{b}	37.93±0.38^{a}
Unsaturated fatty acids	65.29±1.37^{a}	66.33±1.27^{a}	62.07±1.08^{b}
Saturated/unsaturated fatty acids	0.53	0.51	0.61

Data were means±S.D. of three replicates. Values with different superscripts (a–c) are significantly different at $p < 0.05$

Table III: Sterols content of the oil from batch2007 and batch2008 (g kg^{-1})

Sterols (g kg^{-1})	**Batch2007**	**Batch2008**
20Meth, Cholesterol	0.09±0.001^{b}	0.11±0.01^{a}
Campesterol	0.34±0.01^{a}	0.35±0.01^{a}
Campestanol	0.01±0.006^{a}	0.02±0.002^{a}
Stigmasterol	0.52±0.01^{a}	0.47±0.01^{b}
β - Sitosterol	2.40±0.01^{b}	2.71±0.03^{a}
Stigmastanol	0.04±0.002^{a}	0.04±0.001^{b}
Δ^5 - Avenasterol	0.17±0.02^{a}	0.14±0.01^{b}
Cycloartenol	0.06±0.007^{a}	0.02±0.001^{b}
Methylene cycloartanol	0.08±0.001^{a}	0.02±0.008^{b}
Citrostadienol	0.03±0.005^{b}	0.05±0.004^{a}

Data were means±S.D. of three replicates. Values with different superscripts (a–c) are significantly different at $p < 0.05$

Table IV: Minerals, proteins and parietal components of the neem seed (% of dry matter)

	Whole seed		Kernel		Hull	
	Batch2007	Batch2008	Batch2007	Batch2008	Batch2007	Batch2008
Minerals (%MS)	4.25±0.03^{b}	4.44±0.23^{b}	4.45±0.08^{a}	4.78±0.02^{a}	4.27±0.07^{c}	4.15±0.76^{c}
Proteins (%MS)	11.86±0.54^{b}	11.75±0.57^{b}	20.07±0.55^{a}	22.86±0.20^{a}	1.30±0.43^{c}	2.23±0.42^{c}
Parietal components (% MS)	49.84±2.55^{b}	48.47±2.32^{b}	6.57±0.10^{c}	6.14±0.46^{c}	86.83±0.36^{a}	83.25±0.98^{a}
Cellulose (% MS)	29.52±0.42^{b}	27.86±1.65^{b}	1.85±0.35^{c}	1.72±0.53^{c}	54.03±0.57^{a}	51.33±0.63^{a}
Hemicelluloses (% MS)	14.36±2.48^{b}	14.64±1.32^{b}	2.56±0.24^{c}	2.25±0.37^{c}	24.18±0.62^{a}	28.22±2.16^{a}
Lignins (% MS)	5.96±0.50^{b}	5.64±0.87^{b}	2.15±0.20^{c}	2.18±0.36^{c}	8.61±0.41^{a}	6.69±0.61^{a}

Data were means±S.D. of three replicates. Values with different superscripts (a–c) are significantly different at $p < 0.05$

were respectively 49.84±2.25% and 49.14% in the whole seed and 86.83±0.36%and 89.80±0.98% in the hull in batch 2007 and batch 2008 respectively.

4. Conclusion

The present results show that Senegalese neem seed kernel are rich in oil and azadirachtin content compared to Indian ones. They confirm that the most important compounds of the neem seed (azadirachtin, oil, proteins) are mainly located in the kernel. The neem seed oil is characterized by a high content of β-sitosterol in unsaponifiable fraction and of oleic acid in total fatty acids. In addition to the neem seed insecticidal and medicinal properties its high content in protein can be exploited as a second added value.

However, the hull can also be use as an important source of parietal components such as cellulose, hemicelluloses and lignin.

These results on the characterization of the Senegalese neem seed allow a new ways for its fractionation for production of bio-pesticide.

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