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Endo-1,4-D-glucanohydrolase assisted extraction of essential oil from the seed kernels of Nutmeg by using a two-step protocol

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Abstract

The cell walls of plants are made up of cellulose as the major composite and the hydrolysis of this polysaccharide has proven to be a major step in the extraction of many biomolecules. This research was on both pure and partially purified endo-1,4-D-glucanohydrolase, EC 3.2.1.4 (cellulase) assisted extraction of essential oil from the seed kernels of Nutmeg by using a two-step protocol that involved the pretreatment of the sample with cellulase followed by the distillation of the essential oil using Clevenger apparatus. The essential oil obtained after pretreatment with pure cellulase was 8.2 % while 5.8 % of essential oil was obtained when treatment prior to hydro-distillation of oil was done with partially purified cellulase. An oil yield of 3.8 % was observed when no enzyme pretreatment was carried out. The GC-MS analysis of the essential oil obtained from the two-step protocol showed the presence of 25 and 24 components from the sample pretreated with the partially purified cellulase and pure cellulase respectively. There were three major groups of components observed from the essential oils. These are the monoterpenes made up of sabinene, α-pinene, and β-pinene; the sesquiterpenes made up of safrole and alpha-copaene; and the phenylpropanoid/aromatic compounds, composed of myristicin and methyl eugenol. The presence of these principal compounds in the major groups has given nutmeg essential oil improved value due to the possibility of incorporating them as functional ingredients in several products with possible applications in the pharmaceutical, agricultural, fragrance, flavour, and cosmetic industries.

Keywords: Enzyme assisted, Extraction, Myristicaceae, cellulase, Essential oil.

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Introduction

Cellulose is a structural polysaccharide which is the main constituent of plant cell wall, and it is made-up of glucose residues linked by β-1,4-glycosidic bonds (van de Ven and Godbout, 2013; Strakowska *et al.,* 2014; Quiroz-Castaneda and Folch-Mallol 2016; Dini *et al.,* 2019). The cellulose polymers give rigidity as their primary function to plants (de Vries and Visser 2001; Byrt *et al.,* 2012). The sturdiness of the cellulose polymer and its structural prominence in the cell wall has motivated a lot of research in the hydrolysis of cellulose (Schwarz 2001; Sarkar *et al.,* 2009). Both the use of chemicals and enzymes have been explored (Barati and Sadegh, 2015) with a positive indication that enzymes from microorganisms especially fungi from the species of Aspergillus and Trichoderma have remarkable abilities to degrade cellulose (de Vries and Visser, 2001; Andrade *et al.,* 2011; Okwonkwo 2014; Strakowska *et al.,* 2014; Gupta *et al.,* 2016; Sulyman *et al.,* 2020).

Cellulase is widely used in the hydrolysis of cell wall polysaccharides, deinking of wastepaper and in enhancing the permeability of cell wall for efficient processes leading to extraction of active bio-compounds within the cell walls (Dourado *et al.,* 2004; Cheng *et al.,* 2015; Shaibu *et al.,* 2019). Due to the often very complex composition of the plant cell wall components and the minute amount of the constituents present in plants, the choice of the extraction method is of great importance (Yrjonen, 2004).

Enzyme assisted extraction has attracted the attention of researchers for the extraction of target compounds in many plant materials (Wang *et al.,* 2018). The enzymatic breakdown of the plant cell wall for extraction is based on the ability of enzymes to hydrolyze cell wall components and disrupt the structural integrity of the plant cell (Cheng *et al.,* 2015). In this research, Nutmeg (*Myristica fragrans)* seed kernel is considered a plant material of preference because of the quality of its essential oil. Generally, the techniques commonly employed in extracting essential oils from their storage structures include hydrodistillation (Ellouze and Abderrabba, 2014), steam distillation (Colecio-Juarez, 2012), and solvent extraction (Tunchaiyaphum *et al.,* 2013). These conventional techniques suffer from common drawbacks of low yield (Longo and Sanroman, 2006; Handa *et al.,* 2008) due to the incomplete extraction of essential oil components from the plant milieu.

The increase in the demand for essential oils spurred by the consumer's predilection for natural products in food against synthetics has made it needful to research in processes directed at maximizing the recovery of essential oils. Efforts have been made by many without achieving significant improvement in the essential oil yield (Chavez-Gonzalez *et al.,* 2015). The need therefore arises for alternative methods.

This study is therefore aimed at investigating the effect of pretreatment of *Myristica fragrans* seed kernels with partially purified cellulase and pure cellulase obtained from Sigma respectively and how such pretreatment would influence the yield of essential oils distilled using clevenger apparatus. The extracted essential oils were analyzed using GC-MS.

MATERIALS AND METHODS

Sample collection

Myristica fragrans seeds were obtained from Ogige Market in Nsukka LGA of Enugu State. The identity of the sample was confirmed by the Taxonomist in the Department of Pharmacology and Environmental Medicine of the University of Nigeria. The seeds were deposited at the herbarium of the Department with Voucher number PCG/UNN/0334.

Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical grade and were freshly prepared.

Methods

Pretreatment of *Myristica fragrans* seed kernels with cellulase

Pretreatment of *Myristica fragrans* seed kernels with cellulase (endo-1,4-D-glucanohydrolase, EC 3.2.1.4) was carried out by a modification of the method of Amudan *et al.,* 2011 as shown below: *Myristical fragrans* seed kernels were blended into fine powder and 25 g of the powder was soaked in 25 ml of acetate buffer pH 5.0. About 2 ml each of the partially purified cellulase (PPC) preparation was added to this and incubated at 25 ^{o}C for 6 h. The above procedure was also used to pre-treat the seed kernels using the pure cellulase obtained from Sigma.

Hydro-distillation of Powdered Nutmeg seed kernels using Clevenger Apparatus

All the pre-treated *Myristica fragrans* powdered seed kernels were then hydrodistilled for 3hr in a clevenger apparatus containing 200 ml of distilled water. Powdered *Myristical fragrans* seed kernel (25 g) that was not pretreated with enzymes was also hydrodistilled for 3hr in a clevenger apparatus containing 200 ml of distilled water to extract the essential oil. The volatile oils were then collected and analyzed according to the procedure of Al-Jumaily and Al-Amiry (2012). The experiments were performed in duplicates and the average values were used to determine the yield using the formula below:

$$\text{Yield (\%)} = \frac{Amount\ of\ essential\ oil\ recovered\ (g)}{Amount\ of\ plant\ material\ distilled\ (g)} \text{ X } 100$$

Preparation of essential oil samples for GC-MS analysis

The essential oil sample was diluted with chloroform to 7%. The inert gas (helium) from the large storage cylinder was introduced through the injection part to the column and the detector. To ensure reproducible retention time and minimize detector dirt, the flow rate of the carrier gas was adjusted. A micro-syringe was used to inject the sample through a heated injection part that vaporized and carried the sample into the column made up of a long tube closely packed with solid particles. The supporting solid was uniformly covered with a thin film of highly boiling liquid as the stationary phase. The mobile and stationary phases separated the sample into individual components which emerged from the column together with the carrier gas and passed through a detector. The components generated a signal registered electrically as detected by the device which was passed to the detector.

Gas chromatography- mass spectrometric (GC-MS) analysis of essential oil

The essential oil was analyzed by electron ionization (EI) method on GC-MS-QP2010SE SHIMADZU, JAPAN. The conditions of the MS employed during the analysis were: ionization voltage 70 eV; ion source temperature 230 ^{o}C; mass scan range: 40–440 mass units. The GC settings were as follows: the column oven temperature was 60 ^{o}C, the injection temperature was 250 ^{o}C, the injection mode was split, flow control mode was linear velocity, while pressure was 144.9 kPa, with the total Flow at 103.1 mL/min, the column flow at 3.22 mL/min, the linear velocity at 46.3 cm/sec, with the purge flow at 3.0 mL/min, and split ratio set at 30.1. The mass range was 45 m/z to 700m/z. The carrier gas used was helium and the samples (1 µL) were injected with a split ratio of 1: 30 according to the procedure of Fan *et al.,* (2018). The chemical components were identified through comparison of their retention times and mass spectra with those in the MS data library of the National Institute of Standards and Technology (NIST 11). The relative quantity of each component was determined by calculating the peak area of the TIC chromatogram.

RESULT

Enzyme assisted extraction

The essential oil obtained from pretreatment of powdered *Myristica fragrans* seed kernels using partially purified cellulase and the pure cellulase obtained from Sigma before hydro distillation resulted in a yield of 5.8 % and 8.2 % respectively (Table 1).

Table 1: Cellulase assisted extraction of essential from *Myristica fragrans* seeds

Treatment	Percentage
Distillation ^{NP}	3.8
Distillation + Cellulase ^{CPP}	5.8
Distillation + Cellulase^{CC}	8.2

NP= No enzyme pretreatment; CPP = partially purified cellulase enzyme;CC = commercially obtained enzyme from Sigma company

GC-MS analysis

The results of the GC-MS analysis were presented in the form of graphs (Chromatograms) which were used to monitor the essential oil components in the GC over time. The chromatogram of the essential oil extracted from the seed kernels of *Myristica fragrans* revealed the presence of 25 components in the oil obtained after the sample was pretreated with partially purified cellulase and 24 components in the oil obtained in the sample pretreated with the pure cellulase obtained from Sigma (commercial cellulase) as shown by the peak numbers in the chromatograms in Figures 1 and 2. The essential oil components of both the partially purified and the pure enzyme pretreatments are presented in tables 2 and 3 together with the percentage occurrences of the individual components.

Table 2: Components of the essential oil obtained after pretreatment with partially purified cellulase

Peak number	Chemical compound	Molecular weight (g/mol)	Retention time (min)	Compound(%)
1	Alpha-thujene	136	5.592	2.20
2	Alpha-pinene	136	5.708	18.71
3	Camphene	136	5.882	0.34
4	Sabinen	136	6.179	23.48
5	Beta-pinene	136	6.249	17.22
6	Beta-myrcene	136	6.372	2.07
7	3-Carene	136	6.692	0.70
8.	4-Carene	136	6.747	1.35
9	m-Cymene	134	6.783	2.00
10	m-Mentha-6,8-diene	136	6.910	6.20
11	Gamma-terpinene	136	7.276	2.01
12	Cyclohexene	136	7.668	0.81
13	Linalool	154	7.739	0.71
14	5-Caranol	154	8.050	0.21
15	Terpinen-4-ol	154	8.724	4.41
16	Alpha-Terpineol	154	8.858	0.55
17	Safrole	162	9.923	0.55
18	1-vinyladamatane	162	9.967	0.47
19	2-Azidomethyl-1,3,3-trimethyl-cyclohexene	196	10.643	0.38
20	Nerol acetate	196	10.905	0.28
21	Methyl eugenol	178	11.025	5.51
22	Alpha-copaene	204	11.135	0.53
23	Isoeugenol methyl ester	178	11.975	0.49
24	Myristicin	192	12.240	7.88
25	Elemicin	208	12.494	0.77

Table 3: Components of the essential oil obtained after pretreatment with commercial cellulase

Peak number	Chemical compound	Molecular weight (g/mol)	Retention time (min)	Compound(%)
1	Alpha-thujene	136	5.594	2.3
2	Alpha-pinene	136	5.705	16.65
3	Camphene	136	5.883	0.27
4	Sabinen	136	6.182	25.75
5	Beta-pinene	136	6.250	12.30
6	Beta-myrcene	136	6.374	1.90
7	Alpha-phellandrene	136	6.585	0.46
8	3-Carene	136	6.693	0.67
9	4-Carene	136	6.749	1.49
10	m-Cymene	134	6.787	2.33
11	m-Mentha-6,8-diene	136	6.912	6.66
12	Gamma-terpinene	136	7.277	2.35
13	Bicyclo(3.1.0)hexanol	154	7.357	0.43
14	Cyclohexene	136	7.672	0.82
15	Linalool	154	7.743	0.58
16	Terpinen-4-ol	154	8.726	4.68
17	Alpha-Terpineol	154	8.862	0.45
18	Safrole	162	9.927	0.55
19	Methyl eugenol	178	11.026	8.66
20	Alpha-copaene	204	11.136	0.30
21	Isoeugenolmethylester	178	11.977	0.43
22	Germacrene D	204	12.161	0.10
23	Myristicin	192	12.241	9.80
24	Elemicin	208	12.497	1.06

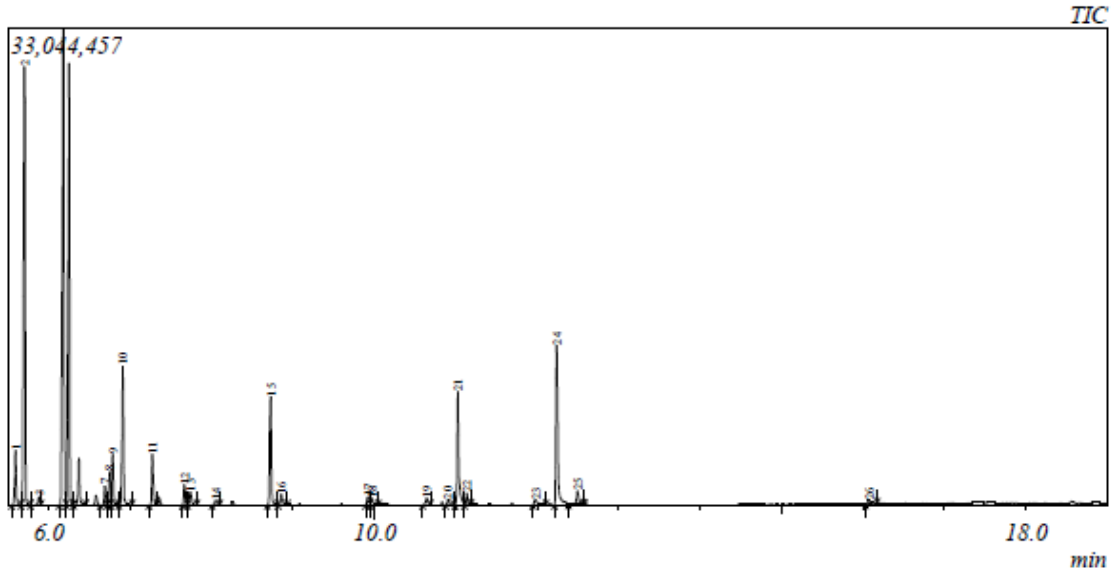

Figure 1: Chromatogram of the GC-MS analysis, the peak numbers and retention times of the components of the essential oil obtained after pretreatment pretreatment with partially purified cellulase

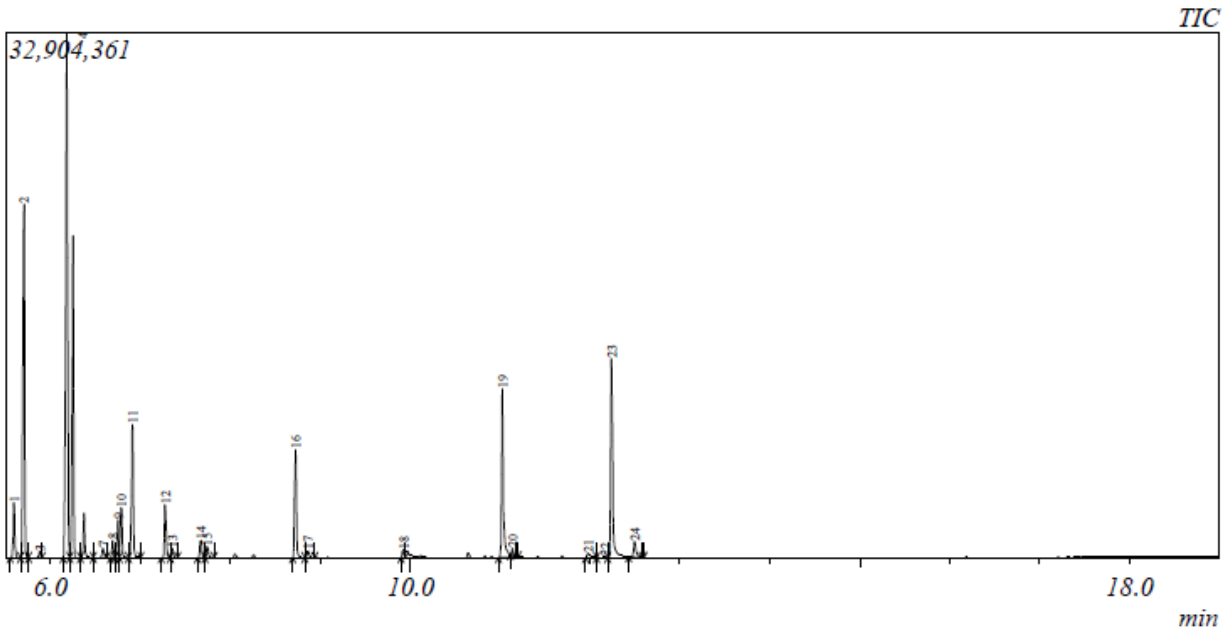

Figure 2: Chromatogram of the GC-MS analysis, the peak numbers and retention times of the components of the essential oil obtained after pretreatment pretreatment with commercial cellulase.

DISCUSSION

The essential oil yield obtained from the enzyme assisted pretreatment with the partially purified cellulase and the pure cellulase (Sigma) were higher than the yield of 3.8 % obtained from the hydrodistillation of *Myristica fragrans* without pretreatment with any enzyme. This agrees with the report by Anwar *et al.,* (2013) in which cellulase was used to assist in the extraction of essential oil from flax seed with an enhanced yield from 32.5 % to 38.0 %. Amudan *et al.,* (2011) reported a 50 % increase in essential oil obtained after pretreatment with cellulase and pectinase respectively before hydrodistillation of the extract from *Syzygium aromaticum.* In another enzyme assisted study, Jung *et al.,* (2006) reported on the effect of the pure cellulases (I Puradex HA and IndiAge Super L) on the extraction of protein from soy flakes with a reported increase of 9% and 17% respectively. Patindol *et al.,* (2007) reported an increase in the extractable oligosaccharides from 13.4 % to 39.9 % with pretreatment of rice bran with cellulase before hydro distillation. In the study of Cao (2010), the effect of using different enzymes in the extraction of geniposidic acid was determined with the pretreatment with cellulase (1.23%) giving a higher yield than that of pectinase (1.13 %) and glucanase (0.74 %). Yanday *et al.,* (2013) while working on the extraction of garlic acid from the dried fruit of *Emblica officinalis* reported a 1.23-fold increase when cellulase was used for pretreatment against the control. The pretreatment of *Myristica fragrans* seed kernels with pure cellulase obtained from Sigma yielded 8.2 % more of essential oil than the use of partially purified cellulase (5.8 %). This contrasts with reports by some researchers that commercially available (pure) cellulases do not improve the extraction yield of biomolecules when compared to the partially purified cellulase (Zhang *et al.,* 2017).

Essential oils are a mixture of multiple components which cut across different groups of chemical compounds. The quality and applications of essential oils are often defined by the presence of these components. Muchtaridi *et al.,* (2010) while analyzing the components of essential oil obtained from *Myristica fragrans* showed the result of 26 essential oil components which is like the result of the present research. 16 and 17 components of essential oils from the seed kernels of *Myristica fragrans* have been

reported in literature by Ibrahim *et al.,* (2010) and Jukic *et al.,* (2017) respectively. Also, 43, 37 and 40 components of the essential oils from the seed kernels of *Myristica fragrans* have been reported by Matulyte *et al.,* (2019), Saputro *et al.,* (2017) and Ogunwande *et al.,* (2003) respectively. The disparity in the composition of essential oils from the seed kernels of *Myristica fragrans* might be attributed to factors such as the difference in climatic and environmental conditions of growth as well as the methods used in extraction.

The essential oil from the seed kernels of *Myristica fragrans* from the partially purified cellulase contains 64 %, monoterpenes, 4 % sesquiterpenes and 32 % phenylpropanes/other aromatic compounds while the essential oil from the seed kernels of *Myristica fragrans* pretreated with pure cellulase obtained from Sigma had 70.8 % monoterpenes, 4.2 % sesquiterpenes and 25 % phenylpropanes/other aromatic compounds. The monoterpenes sabinene, α-pinene, and β-pinene constitute the major fractions of essential oils from *Myristica fragrans* seeds while safrole and alpha-copaene were the main sesquiterpenes, while myristicin and methyl eugenol were the main phenylpropanoid/aromatic compounds as observed from the oils. Saputro *et al.,* (2016) and Rodianawati *et al.,* (2015) also reported that sabinene, α-pinene, β-pinene and myristicin were the major components of essential oils from the seed kernels of *Myristica fragrans*. Muchtaridi *et al.,* (2010) found sabinene, 4-terpeneol, myristicin and α-pinene as the main components in their studies on the identification of compounds in *Myristica fragrans* seeds while Ogunwande *et al.,* (2003) reported the major fractions from *Myristica fragrans* seed kernels as sabinene, α-pinene, β-phellandene and terpinene-4-ol using GC-MS analytical methods. The major component of the essential oil from the seed kernels of *Myristica fragrans* as observed in this investigation is monoterpene. This agrees with Wojtunik-Kulesza *et al.,* (2019) that monoterpenes are the principal fraction of most essential oils.

The major essential oil components have many benefits when incorporated in products which have applications in the pharmaceutical, agricultural, fragrance, flavor, cosmetic and various other industries. Sabinene is widely used in pharmaceutical and cosmetic industries because of its radical scavenging capacity, insecticidal activity, and antimicrobial properties with strong to moderate antibacterial activity against Gram positive and pathogenic fungi (Berger, 2010; Zhou *et al.,* 2019). The essential oils α-pinene and β-pinene have diverse bioactivities with various applications as flavours, fragrances, fungicidal agents, antiviral, and antimicrobial agents (Yang *et al.,* 2016; Salehi *et al.,* 2019). Pinenes have also been used in the synthesis of polymers. The sesquiterpene safrole is commonly used as a fragrance in perfumes and soaps and a flavouring agent in drugs and in the manufacture of heliotropin, piperonyl butoxide (with insecticidal properties) (Gad and Pham, 2014). Eugenol has applications in dentistry due to its antiseptic and analgesic properties. For instance, it is used as a disinfectant in mouthwash and when mixed with zinc oxide it forms the cement for temporary filling of the teeth (Bendre *et al.,* 2016). As a fragrance and flavouring agent, eugenol is used in a variety of cosmetics and food products (Kaufman, 2015). Another flavouring agent myristicin is also known for its insecticidal activity, fungistatic activity and important psychopharmacological responses (Rahman *et al.,* 2015; Pineda *et al.,* 2018). Several other minor components have also found importance even in industries with β-myrcene serving as the primary constituent of hops and bay oils in the production of alcoholic beverages (Koziol *et al.,* 2014). Linalool, a phenolic compound has antimicrobial activities and antioxidant properties. It is often used as an important preservative for the prevention of microbial damage and lipid peroxidation (Peana and Moreti, 2008; Mughal, 2018).

CONCLUSION

In summary, the study on the use of pure and partially purified cellulase to pretreat the seeds of *Myristica fragrans* before extraction improved the essential oil yield when compared with the no enzyme treatment. The analysis of the essential oil by GC-MS showed the quality and quantity of the essential oil. The GC-MS also revealed the oil as a complex mixture of

numerous compounds, many of which are in trace amounts. However, sabinene, α-pinene, β-pinene were dominant components in all the treatments. Future studies need to be done on the use of two or more cell wall degrading enzymes to pre-treat the *Myristica fragrans* seed sample before the essential oil extraction determine the efficacy of enzyme combination on the yield of essential oil.

Authors contributions

ESOO conceived and designed the experiment, and contributed the samples, chemicals, and reagents. IW performed the experiment and wrote the article with OKO, OVE. ESOO edited the manuscript. CFC supervised the study.

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