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Antifungi effects of essential oil from african pear seed against *Aspergillus niger* isolated from cereal grains in Delta State

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Abstract

Essential oils exhibit biostatic and biocidal activities against microorganism due to antimicrobial properties present in them. The seeds oil of African pear (*Dacrodes edulis*) was studied against fungi isolated from stored grains. The oil was extracted with soxhlet extractor and antifungal activity was determined using standard method Molecular identification of the fungus showed similar homology with previous isolate. Oil showed static effect as the level of activity decreased as the number of days increased (25.33±0.88,2/3days; 18.00±0.57, 4/5days and 0.00 7th day). Physiochemical and phytochemical parameters showed that D. *edulis* seed oil has high quality as perioxide value (12.40±0.16mEq/kg) and acid value (1.99±1.08(mgKOH/g)) were within the recommended values of oils. Saponification values were high (190-206mgKOH/g) though iodine value was low. Phytochemical result of D. *edulis* seed powder included presence of saponins, terpenoids, cardiac glycosides, alkaloids and other compounds. *Dacrydes edulis* seed oils could be used as biopreservative on stored grains since it was potent against fungi that spoil stored grains. Further phytochemical analysis could reveal the compounds within oil that had the antifungal property.

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Introduction

Essential oils are aromatic, volatile liquids, usually, extracted from leaves, stem barks, buds, seeds, peels, flower and other parts of plants (Tongnunanchan and Benjakul, 2014). Essential oils are used for food flavouring due to their odours. Essential oils contain antimicrobial, antioxidant and insecticidal activities (Pandey *et al*., 2017) making them suitable as natural additives in foods and food products.

They are also exploited in food industries for shelf life extention (Mahmud and Khan, 2018) where they are applied in making materials for packaging food and so on. Biopreservative involves the use of antimicrobial products derived naturally to preserve food and beverages to extend their shelf lives. Several studies have examined and reported promising natural antimicrobial agents including natamycin, niasin, pediocin, ruterin, bacteriocins, lactofferins, lysozyme and essential oils (Saeed *et al*., 2019). Works on applications of essential oils in cereal baked products (bread) have been reported (Mahmud and Khan, 2018). Also antibacterial effects of essential oils have been mentioned, however, few works exist on application of essential oils against stored grains especially on fungi that spoil stored grains.

African pear seed oil is obtained from the African pear plant (*Dacryodes edulis*) which is an indigenous fruit tree in the Gulf of Guinea and Central African countries (Troupin, 1950) but widely cultivated in Sierra Leone, Angola, Uganda and Nigeria (Anonymous, 2018). The plant belongs to Burseraceae family, members are characterized as ovary of 2 to 5 cells prominent as ducts in the intrastamnal disk, bark and wood (Chunduff, 1984, Ajibesan, 2011). The genus *Dacryodes* consists of 40 species (Verheij, 2002). However, other reports indicates 80 species to cover subspecies, varieties, forms and cultivars. *Dacryodes* var *edulis* and *Dacryodes* var. *parvicarpa* are two varieties recognized. *Dacryodes* var *edulis* have verticillate or subverticilllate branching while the branching are slender and opposite or bifurcate in var. parvicarps (Okafor, 1983, National Research Council, 1996).

Dacryodes edulis is a dioecious shade loving species of non-flooded forests in the humid zone. The tree reaches a height of 18-40m in the forest but 12m in plantation (Hintchanton and Dalziel, 1958, Burkil, 1985, Verheij, 2002). The medium sized evergreen tree has bole 50-170cm in diameter, short, shallow fluted and more or less sinuous. The bulk is rough with lenticels being yellowish- grey to pale-gray colour. It often exudes white aromatic resin. The bactericidal effects of plant part (leaves, stem bark, root) have been studied (Okwu and Ighodaro, 2009; Ogbomi *et al*., 2015; Hassan-Olajokum *et al*., 2020; Nna *et al*., 2017; Mordi *et al*., 2019) however the seed oil have not been studied. Seeds are used as feeds for ruminants and ground seeds mixed with palm oil are used for treating mumps (Omonhinin 2001; Ajibesan *et al*., 2008' Burkill, 1985). The seed oil contains essential oils which could be used as biopreservatives for cereal grains and their products. Many study have reported the use of essential oils for the shelf life extension of functional products of cereals (Gavahian *et al*., 2018) like bread, and other baked products from cereals but the antifungi of essential oils from pear seed against fungi that spoil grains as a means of preservation are not reported. The focus of this research therefore was to determine the antifungal effect of *Dacryodis edulis* seed oil on fungi that spoil stored grains and physiochemical parameters of the oil.

Cereals are grains which are types of fruits called Caryosis composed of the endosperm, pericarp (bran) and embryo (germ). Cereals are animal crops belonging to Gramineae family and are characterized by the possession of long, thin stalks. They include maize, millet, rice, rye, wheat, barley and sorghum. These grains are starchy food used all over the world. When not processed, they constitute rich sources of carbohydrates, oils, vitamins, fats, proteins and minerals (Goldberg, 2003, Mckevith, 2004). However, when bran and germ are removed, the remaining endosperm contains mainly carbohydrates and lack other nutrients. Cereals are important to human and livestock as they are source of stable food to humans. Most animal feeds are made from cereals and grains, baked products, milled grain products,

beverages and whole grain product and others are sources of food and energy for man all derived from grains (Sarwar *et al.*, 2013, Sarwar and Sattar, 2007). Microorganisms have been identified as one of the agents that destroy stored grains due to degradation of starch by microbial enzymes (Mckevith, 2004).

There is therefore concern for the microbial safety of these grains. The grains can harbour microorganism and dust. These microorganisms are mainly from the environment, the cereals are grown and processed (Ray, 2004, Bullerman and Bianchim, 2011).

Microorganisms render the grains unfit for consumption. The organoleptic and nutritional properties are affected. The effect of the microbial activity on contaminated cereal grain include texture loss, off-odours and flavour, visible slime or colonies, gas and pigment production due to metabolism (Adams and Moss, 2008).

Though other microorganisms may be important in cereal spoilage, the major organisms associated with cereal spoilage are moulds and are classified into field and storage fungi respectively (Harris, *et al.*, 2012) Field fungi that contaminate grains include *Alternaria, Fusarium, Helminthosporium and Cladosporium* (Magan and Aldred 2006; Harris *et al.*, 2012). These moulds infect the grains before harvest while the moulds that infect grains after harvest are storage fungi.

These include *Aspergillus, Mucor, Rhizopus* and *Penicillium* (Magan and Aldred, 2006). These grow when the grains are not properly dried (Harris *et al.*, 2012). Mycotoxin can be produced by these moulds in favourable growth conditions.

Mycotoxin are difficult to eradicate from grain during processing, this is the reason for prevention, to avoid foodborne intoxication. Also presence of these moulds are undesirable to farmers because of loss arising from the activities of these fungi during growth where spontaneous heating occur due to activities of the moulds, thereby leading to grain damage.

Materials and methods

Isolation and identification of fungi contaminants

Contaminated grains (millets, maize and rice) were bought from different sellers in Abraka market, Delta State into a sterile cellophane and taken to Microbiology laboratory. The grains were surface sterilized in sodium hypochlorite solution in two minutes and rinsed in sterile distilled water (Hocking, *et al.*, 2006; Agwande *et al.*, 2016). Each of the grains were mashed in sterile mortar and pestle.

One gram of each was diluted in 9mls of sterile distilled water to the fifth power (10^1-10^5). The diluents were inoculated into potato dextrose agar using pour plate technique and incubated for 7 days at room temperature (Onyeze, *et al.*, 2012). The growth and pigmentation pattern, colony size were cultural characteristics which aided identification. Pure cultures were stained in lactophenol cotton blue and the phenotypic properties were compared with charts. Further identification was carried out using molecular characterization.

Molecular Identification of Fungal Isolates

The DNA of pure isolate was extracted following the protocol of Zymo Bacterial/Fungal DNA mini prep extraction kit (made in USA). Polymerase chain reaction (PCR) was done by amplifying the internal transcriber region using forward ITS1 (5'TCCGTAGGTGAACCTGCG G3') and reverse ITS4 (5'TCCTCCGCTTATTGATATGC3') primers.

The PCR amplification mixture used was consist of : 0.5µL each of forward and reverse primer (10ng), with 2.0µL DNA template, 1.0µL each of PCR buffer, $MgCl_2$, and DMSO, 0.8µL deoxyribonucleoside triphosphates (Soils Biodyne), 0.1µL Taq polymerase (Soils Biodyne) and 3.1µL water. PCR conditions was; denaturation, 94°C at 30s for denaturation, annealing temperature 58 °C for 40s and extension (72 °C for 60seconds) in 45 cycles.

The final extension was 72°C for 10 minutes and cooled at 4°C. The PCR products were purified and separated on a 1.5% agarose gel using electrophoresis at 80 V for 1 hr.

Sanger sequencing was done using 3500 ABI genetic analyser at Inqaba, South Africa. The sequences generated by the sequencer was visualized using Chromaslite by base pairing and subsequently edited using Basic local Alignment Search Tool for nucleotide (BLASTN) from NCBI (national Center for Biotech Information) database was and similar sequences were aligned using Cluster. MEGA 6 software was employed for phylogenetic tree.

Solvent extraction was the method used for extracting oil

The method of Ibanga *et al.* (2014) was adopted with little modification. Ripe fruits were purchased from Effurun market, July, 2019, in Delta State and transported to the laboratory. Part of the samples were sent for identification and a voucher specimen number UBH-D373 was deposited at the Department of the University. The fruits were washed and the pulp removed with hot water. Seeds were air dried for 14days and then powdered. The oil was extracted from seeds using soxhlet extractor at 50^{o}C The weight of oil was noted.

Antifungal Activities

The method of Schroder *et al.* (2017) was adopted. Spore suspensions from fungi culture of 5-7 days were harvested into sterile water and 100µl of spore was spread on potato dextrose agar. The already inoculated plates were allowed to dry. The oil was pipetted into 10mm sterilized filter paper disc and placed at the centre of the plate where the quadrant meet after sharing the plates into four equal parts. Two plates were used for each test against isolated specimens. Each plate was incubated at 25^{o}C for 7 days. Fluconazole (250mg) and DMSO and were also tested. Clear zones around the disc was measured. The inhibition zone was measured within 2-3 days and 4-5 day. The area around the disc showing no growth was taken as inhibition zone.

Physicochemical Properties of oils

The physicochemical characteristics of *Dacryodes edulis* seed oils determined were, refractive index measured at room temperature using a digital refractometer (model RBH.32(ATC). Specific gravity, acid values, iodine value and saponification value. There were determined using method described in AOAC (1995) and AOCS, (1992).

Phytochemical Test

Phytochemical test of D edulis were performed according to methods described by Adomi (2017) and Ogboru *et al.*, 2015).

Results and discussion

Fungal isolated from stored grains included *Aspergillus* species (*A. flavus. A. terreus and A. niger), Fusarium* and *Tricoderma*. The organisms isolated from grains *Aspergillus terreus* and *A. niger* were isolated from millet and maize, A. flavus from rice alone. These organism had been formerly identified by previous researchers as spoilers of stored grains. Losses of grain due to storage was attributed to Pest (16%) of which microbes are part of it (Bhardwaj and Sharma 2020).

The study showed that all the grains (millet, rice and maize) obtained from Abraka market, Delta State were infested with *Aspergillus, Fusarium* and *Trichoderma* species. *Aspergillus* species were more prevalent including *A. niger, A. flavus, and A. terrus with A. niger* being in the three grains studied. Similar study reported the prevalence of *Aspergillus* species in grains (Ekpakpale *et al.*, 2021). Maize yielded more fungal isolates than other grains studied A. niger (27.78%), A. terreus (38.89%) and *Fusarium* sp (27.78%) rice yielded growth of the following *A. niger* (13.33), *A. flavus* (33.33%) and *Fusarium* spp (40.0%). Studies earlier carried in grains showed infestation of maize with *Fusarium* species which aligns with this study. However, Agwande *et al.* (2016) reported that *Fusarium* species was not found in their research.

The most prevalent fungus *A. niger* was subjected to molecular identification. The Phylogenetic tree (fig. 1) showed that the organism was *A. niger*. The dendogram showed that this fungus was similar to *Aspergillus niger* strain JNU-DDG08 (Gen Bank Assession Number

KX398342.1:12-405) based on the phylogenetic analysis and nucleotide homology. Also the organism was similar to *Aspergillus* sp isolate Z-Y-47(NCBI Asscession Number, MK367515.1.13-406) and about 92% homology with *Aspergillus tubingensis* strain 1210 with asscession number KF435032 1: 10-373. the internal transcribed space are often used for identification and detection of fungi (Omaime *et al.*, 2018)

Table 1. Fungi isolates and frequencies of occurrence in stored grains.

Fungi	Millet	Percentage (%)	Rice	Percentage (%)	Corn	Percentage (%)
Aspergillus niger	6	37.50	2	13.33	5	27.80
Aspergillus flavus	0	0	5	33.33	0	0
Aspergillus terreus	5	31.25	0	0	7	38.89
Trycoderma sp.	5	31.25	0	0	0	0
Fusarium sp.	0	0	8	40.00	6	27.78
	16	100	15	100	18	100

Effect of Dacryodes edulis on fungi isolated from stored grain

The result of the D *edulis* oil is shown in Table 2. *Aspergillus niger* was very sensitive to the oil though more sensitive to fluconazole. *Aspergillus flavus* and *Trichoderma* spp. were also sensitive however, the zone of inhibition produced was highest (20.00mm) for A niger than the other isolates. *Fusarium* sp. was not susceptible to the oil. The oil was used without further dilution. Very few literature exist on the antifungl effect of D. *edulis*, however the antibacterial activities of seeds oil of *D. edulis* was investigated by Mordi *et al.* (2019) and their report showed that oil was active against gram positive and gram negative bacteria.

Table 2. Nucleic acid sequence of *Aspergillus niger* isolated.

Fungi	Sequence
Aspergillus niger	GCGGGGCTTTGGGCCAACCTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCG TTTGTCAGCGGCGGGGGAGCGCCTCTGCCCGGRCCGGGCGGCGGGGACCAATAGAAACTTCTG AAGGGGAGGGAGTGATTGAAGAATGTTATATTTAAAAGGATCCTTGGTTCCGGCCAGAAAACC CAGGGGAAGGGAAAAACTAATGAAATTGAAAATTCAGGGAATCTTGGATTTTTTAAACGAATTG GCCCCCCCGGGTATTCGGGGGGGATGGCTTTCCAAAGGCATTTGTGCCCTTAACCCGGTTGGTT TTGGCCCCCCCCCCCTTCCCGGGGGAAGGGCCAAAGGGGGGGGMCCGCAACCCGGGGAAAGG GGCTTTTCACACCCCAATCCCCCCCCCATTTTTCAAAAATTTTTTCGGTTCCCCAAAGGGGGAAA CCCGAATTTAAGGGAAAAGGAGGAAA

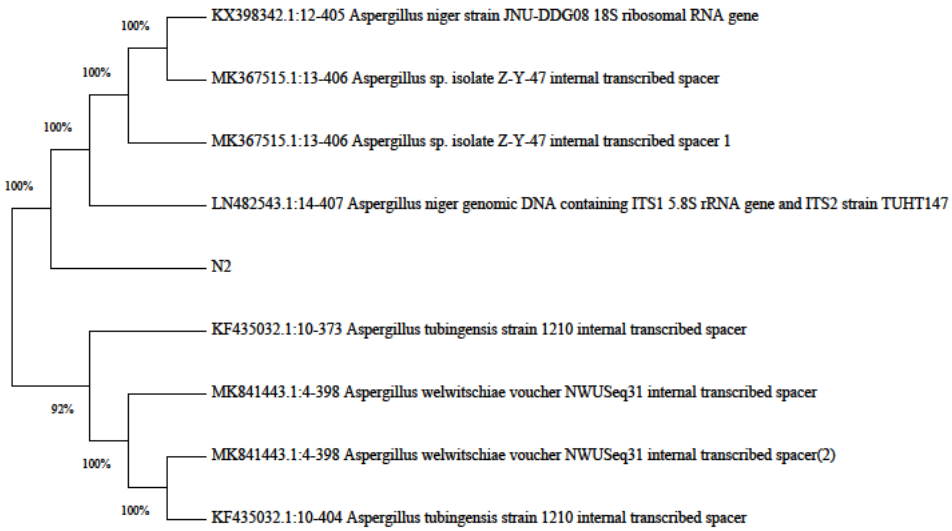

Fig. 1. Phylogenetic tree for N_2.

The physiochemical parameters of oil is presented in table 3. The percentage yield of African seed oil was 13.33% which was lower than the yields obtained by Eze, (2012) for seeds (20.92%) while Akpan *et al.* (2020) for African pear pulp (47%) but was lower than soybean and cottonseed (Rashid *et al.*, 2009). Saponification value was 190-206mgKOH/g. Results obtained from other reports were 120.8, for seed oil and 199.16± 6.09; 224.00 ±1.41mgKOH/g (Akpan *et al.*, 2020, Onwuzuike *et al.*, 2020) for African pear pulp. Saponification number determines the average molecular weight of fatty acids in the oil sample, also used to check adultration, presence of impurities and stability of oils (Onwuzuruike *et al.*, 2020) *D. edulis* in this study saponification value was high compared to soya bean oil. Higher saponification vaule was suggested to indicate more shorter chain fatty acids which favours stability (Akintayo and Bayer, 2002; Onwuzuruike, 2020) As observed in previous study, S V was higher than 189.0 to198.9mg KOH/g set by Codex Alimentarius commission for oil seeds Acid value was 1.99±1.08 and low.

Compared to previous study 44.88 by Eze (2012). Acid value determines the oxidative deterioration of oils by chemical or enzymatic oxidation (Emodah *et al.*, 2017) low value indicates possibility of oil being stable over a long period of time and potent against rancidity and peroxidation which could due to presence of antioxidants and and phytochemicals in oils (Aremu *et al.*, 2015). *Dacryodes edulis* seeds contain antioxidants such as vitamin A, B, C, ans E (Ebana *et al.*, 2017; Ogunka-Nnoka *et al.*, 2017) Iodine value (IV) obtained was 99.00±0.00 was lower than previous studies and that of soya bean oil 110.427 and 129-139. The degree of unsaturation is an identity characteristic of seeds oil and determined by iodine value. Also, used to quantify the amount of double bond present in the oil. In addition it indicates susceptibility to oxidation.

Low iodine value portrays less number of unsaturated bonds and as such low susceptibility to oxidative rancidity (Aremu, 2015). The perioxide value in this study for seeds was 12.40±0.16mEq/kg while the report of pear pulp was 11.80± 0.29mEq/kg (Akpan *et al.*, 2020). Peroixde value measures extent an oil sample undergoes primary oxidation peroide value below 20 are acceptable (Manuranjan *et al.*, 2019) High values of perioxide value are indicative of high levels of oxidative rancidity of oils and also suggest absence or low levels of antioxidant.

Table 3. Effect of pear seed oil on Fungi isolates from stored grains.

Organisms	Diameter (mm)	Fluconazole	DMSO
Aspergillus niger	20.00	35.00	-
Aspergillus flavus	10.00	24.00	-
Tricoderma sp.	18.00	28.00	10
Fusarium sp.	0.00	30.00	-

Refractive index was 1.4709±0.00 this is closer to 1.42-1.46 reported by Onwuzuruike *et al.*, 2020) of African pear oil obtained by different extraction method for pulp

Table 4. physicochemical characteristics of *Dacryodes edulis* essential oils from seeds.

Parameters	African pear seed oil in this study	Soya bean oil
Refractive index	1.4721±0.05	1.466-1.470
Acid value (mgKOH/g)	1.99±1.08	
Iodine value (mgKOH/g)	99.00±0.00	128-139
Specific gravity	0.9067±0.16	ND
Saponification value	190-206	189-195
Perioxide value (mgKOH/g)	12.40±0.16	
Relative density	1.4709	ND

Phytochemical Results of Dacryodes eduli seeds

Phytochemical parameters	Alkaloids	tannin	Steroid	Reducing sugar	Flavonoid	Cardiac glycosides	Saponin	Terpenoids
Qualitative	+++	+++	+++	+++	+++	+++	++	++
Quantitative	4.3	3.4	-	-	3.5	-	3.2	-

Specific gravity obtained from *D. edulis* seed oil in this study was 0.9067±0.16g/cm3 which concurs with other values obtained (0.90to 0.93g/cm3) (Onwuzuruike *et al.*, 2020). This oil can be exploited as biodiesel and as biopreservative which is the focus of this research. The pH is 8.18-8.20 slightly alkaline. The pH shows the corrosiveness level of oil (Emodah *et al.*, 2017). The phytochemical results of *D edulis* showed the

presence of alkaloids, tannins, steroids, reducing sugar, cardiac glycosides, saponins and terpenoids with varying quantities. This resul tallied with previous studies where alkaloids, glycosides, saponins, tannins, flavonoids, reducing compounds were present in their study (Amise *et al*., 2016)

Conclusion

Dacryodes edulis seed oil was active against fungal isolated from stored grains. Fungi isolated from grains included *Aspergllus niger, A flvus, A. terreus, Trichoderma* spp. and *Fusarium* spp. Molecular characterization of A niger showed homology with African seed oil was potent on *A. niger*. African pear oil could be used as a biopreservative against isolates from stored grains

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