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Investigation of endophytic fungi in *Moringa oleifera Lam.* and determination of the phytochemical and proximate contents of its leaves and seeds

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Moringa oleifera Lam. is embedded with huge nutritional values hence, it is being utilized in folklore medicine for various therapeutic and pharmacological applications. This study was conducted to identify the endophytic fungi associated with commercial dried and fresh leaves and seeds of *Moringa oleifera*. The phytochemical and proximate contents of the ethylacetate extracts of the samples were also determined. Surface sterilization procedures were carried out to isolate fungi while identification was by standard identification manuals. The phytochemical content analysis was by standard protocols while the proximate content analysis was done using AOAC protocols. Endophytic fungi isolated are from genus; *Aspergillus, Rhizopus, Fusarium, Rhizoctonia, Saccharomyces, Xylaria, Mucor, Nigrospora, Trichoderma, Alternaria, Penicillium* and *Macrophomina*. From the qualitative analysis, flavonoid, tannin, steroid, alkaloid, terpenoids and cardiac glycoside were observed in all samples while saponin and phlobatannins were not detected in any sample. Reducing power was only observed in the fresh leaves and seeds samples. In quantitative analysis, fresh leaves showed the highest quantity of flavonoids (55.86±0.36 mg/g) while the fresh seed had highest quantity of cardiac glucosides, tannin and terpenoids. The dried leaves showed highest quantity of steroid and alkaloids. In the proximate analysis, the dried leaves had higher carbohydrate and crude fibre of 39.06±0.27 % and 14.32±0.01 % respectively. Moisture content was highest in the fresh leaves sample (20.26±0.06 %). Dried branded seed had the highest ash, crude lipid and crude protein contents. This study has shown that the leaves and seeds of *M. oleifera* are domains of diversity of endophytic fungi.

Keywords: Endophytic fungi, Ethylacetate, *Moringa oleifera,* Phytochemicals, Proximate

1. Introduction

Endophytes are organisms inhabiting the interior of a plant's body such as the seeds, flowers, leaves, stems, fruits and roots. Dhanalakshmi *et al.* (2013) and Talukdar *et al.* (2021) have noted that almost all forms of vascular plants can host endophytic organisms like species of bacteria and fungi. Endophytic fungi are known to be producers of secondary metabolites in relation to the host plant. Dhanalakshmi *et al*. (2013) and Khalil *et al*. (2021) stated that these metabolites serve as protective agents to the host plants as they can be antimicrobial and antiviral agents. Extraction and utilization of secondary metabolites from endophytic fungi can result in a reduction in the exploitation of medicinal plants which serve as materials for drugs and in the cost of producing medicines. Zhao *et al.* (2012), Arora and Kaur, (2019) reports revealed that endophytic fungi obtained from medicinal plants are being considered as attractive sources of novel bioactive compounds. Strobel and Daisy (2003) also reported that *Taxus brevifolia* plant plays host to endophytic fungi which were observed to have the ability to produce active compounds like paclitaxelortaxol, a beneficial anti-cancer agent.

Phytochemicals are well known to be secondary metabolites that are synthesized by most plants for medicinal or nutritional benefits. The occurrence of these compounds in plants is an essential phenomenon as they assume a largely protective health benefits in food, beverage or other products derived from the plant. Phytochemicals have been established to be the agents behind changes in colour, flavour, and

odour in foods. The report of Sadat *et al.* (2017) revealed that phytochemicals also function as immune-modulators and can exhibit properties which can be antioxidant, anti-inflammatory, anticancer, antimalarial and antimicrobial. Phytochemicals can be classified based on chemical structures and functional properties.

Sha'a *et al*. (2019) reported that it is important to have the knowledge about the proximate composition of plant resources which is essential for the motivation of more cultivation and consumption of the plants that synthesize such. *Moringa* is a tropical plant which can be found growing in the tropics and belongs to the family Moringaceae. Morton (1991) reported it to be a single genus with 14 known species, widely known and enormously utilized. It is a tree with multiple benefits, because of its vital nutritional, industrial (Mishra *et al*., 2012), medicinal (Biswas *et al.,* 2020 and Ahmadua *et al.,* 2021) and livestock feed (Manju *et al*., 2018) applications. Manju *et al.* (2018) also posited that this plant can flourish both in tropical as well as subtropical climates usually under the hot, humid or wet conditions where the amount of rainfall exceeds 3000 mm/annum. Dahot, (1988) and Unuigbe *et al.* (2014) reported the leaves, flowers and pods to be useful as vital sources of vitamins A, B and C, beta-carotene, calcium, iron, riboflavin, nicotinic acid, folic acid, alpha-tocopherol and pyridoxine. *Moringa* seeds have been asserted by Abdulkarim *et al*. (2005) to possess high levels of lipids and proteins. The major saturated fatty acids present in the seeds are palmitic, stearic, arachidic and benic acids. Makkar and Becker (1999) and Francis *et al*. (2001) also reported that being a natural source of benic acid, the Moringa seed oil is a suited option as solidifying agent in margarines and other foodstuffs containing solid and semi-solid fat.

Ogunjinmi and Oladipo, (2012) and Jumare *et al.* (2022) crude phytochemical screening on the seed extract of *Moringa oleifera* further confirms the presence of alkaloids, glycoside, flavonoids and saponin in both the hexane and methanolic extracts. Vanajakshi *et al.* (2015), observed that the aqueous and ethanolic leaf extracts of *M. oleifera* possess phytochemicals such as alkaloids, anthraquinone, cardiac glycosides, flavonoids, saponins, steroids, terpenoids, anthocyanin, tannins and carotenoids while the aqueous extract showed more of the phytochemicals. It was noted that the proximate analysis of the plant revealed the existence of ash, carbohydrate, protein, fats, fibre and moisture. Muyibi and Evison (1995), Ndabigengesere *et al*. (1995) noted that the seeds possess a non-toxic natural polypeptide which is vital in the sedimentation of organics and mineral particles when purifying drinking water, cleaning vegetable oil and in the process of sedimentation of fibers in juice and beer industries. Bukar *et al*. (2010) noticed that Moringa can also be utilized to stabilize sugar levels in certain cases of diabetes while Kasolo *et al*. (2010) submitted that it can be useful in the stabilization of arterial tension. Unuigbe *et al*. (2014) observed that the leaf and seed extracts exhibit remarkable and concentration-dependent increase in radical scavenging activities with IC_{50} values ranging from 5.72-42.56 µg/mL.

M. oleifera plant can be in symbiotic relationship with microbes which naturally inhabit plant tissues without necessarily endangering the hosts. The microbes can be species of bacteria, algae or fungi and are known as endophytes. This study aimed to investigate the fungi endophytes as well as phytochemical and proximate contents of fresh and dried packaged *Moringa oleifera* seeds and leaves.

2. Materials and Methods

2.1 Collection of Plant Samples

Healthy seeds and fresh leaves of *Moringa oleifera* were collected from trees at the staff quarters of Yaba College of Technology, Lagos, Nigeria (6° 31' 2" N, 3° 22' 39" E). They were put in separate Ziploc bags and transported to the Botany Research Lab of University of Lagos, Nigeria for storage at 4 ℃ prior to use. A deposit of the specimen was done with the Lagos University Herbarium (LUH) in Nigeria with Voucher Number LUH 5712. Two brands of dried packaged leaves and seeds of *M. oleifera* were bought from stores within the Lagos metropolis with the expiration dates confirmed (07/2023 and 02/2024).

2.2 Isolation and Identification of Fungi from the Leaves and Seed of *Moringa Oleifera*

Isolation of fungi from the collected samples (fresh and dried branded) was carried out using surface sterilization described by Talukdar *et al.,* (2021) with slight modification. The leaves were dipped sequentially in 70 % ethanol for 3 min, followed by 0.5 % sodium hypochlorite (NaOCl) for 2 min, and then rinsed thoroughly with sterile distilled water for 1 min. Finally, the leaves were dried over sterile blotting paper inside the laminar airflow chamber. Surface sterilized leaves measuring about 0.5 mm in diameter were then punched out using a sterile puncture and were inoculated onto Petri plates containing potato dextrose agar (PDA) medium which was prepared in line with the manufacturer's specification (Oxoid, Basingstoke, England) and supplemented with chloramphenicol (5 mg/100

ml). The same protocol was done for the seeds. After the surface sterilization of the seed, each was divided into four halves using a sterile surgical blade and inoculated diagonally on the agar plate. The plates with the leaf or seed fragments were then incubated at 28±2 ℃ for two weeks and observed once a day for mycelia growth. Hyphal tips growing out of the inoculated fragments were sub-cultured on freshly prepared PDA plates until pure cultures were obtained. The isolates were identified based on their morpho-cultural characteristics (i.e. the shape, size and spore formation) using standard identification manuals (Domsch *et al*. (1980), Bryce (1992) and Wattanabe, (2012). The photomicrographs of the fungi were obtained via the Motic Camera 2000, 2.0 Megapixel.

2.3 Preparation of Plant Samples

Each of the fresh leaves, de-shelled fresh seeds, dried commercial branded leaves and seeds were pulverized into fine particles using an electric grinder. A measure of 200 g was obtained from each pulverized sample and extracted in ethylacetate at 1:2 w/v for 72 hrs. Each extract was concentrated to dryness using rotary evaporator at 40 ℃. The pulverized samples were used for the proximate analysis while the extract of each sample was used for the phytochemical analysis.

2.4 Proximate Analysis of the Collected Leave and Seed samples of *Moringa Oleifera*

The Association of Official Analytical Chemists (AOAC) protocol of 2012 was followed to determine the ash, crude lipid, crude fibre, crude protein and moisture contents of the samples. Thiex (2009) and Maisarah *et al.* (2014) method was used to obtain the carbohydrate content by subtracting the sum of the ash, crude lipid, crude fibre, crude protein and moisture contents in percentage from 100 %. The proximate analysis of all samples was carried out in triplicate to ascertain the consistency of results.

2.5 Preliminary Phytochemical Screening of the Leaves and Seeds of *Moringa oleifera*

The preliminary phytochemical components of the ethylacetate extracts of the samples were analyzed through standard protocols detailed by Trease and Evans (1989), Sofowora (1993), Madziga *et al*. (2010), Hossain *et al*. (2013) and Auwal *et al*. (2014). Phytochemicals analysed included tannins, phlobatannins, saponin, flavonoids, steroids, terpenoids, cardiac glycosides, alkaloid, phenols, anthraquinones and reducing compound. These was carried out in triplicate.

2.6 Statistical Analysis

The SPSS version 22 (Statistical Package for Social Science) was used for analysis of data while data were presented as mean and standard deviation.

3. Results and Discussion

3.1 Endophytic fungal Isolates

Microorganisms are cosmopolitans and as such their habitats are unlimited. In this study, endophytic fungi associated with the fresh and commercial dried leaves and seeds of *Moringa oleifera* revealed a wide diversity of fungi. Twelve genera of endophytic fungi were identified which included *Aspergillus, Rhizopus, Fusarium, Rhizoctonia, Saccharomyces, Xylaria, Mucor, Nigrospora, Trichoderma, Alternaria, Penicillium* and *Macrophomina.* Carbungco *et al.* (2017) also reported the isolation and characterization of endophytic fungi belonging to some of these taxa from *Moringa oleifera* leaves in the Angeles City of Philippine. Although, they performed their isolation on malt extract agar plates while potato dextrose agar was used in this study. The isolates identified from the fresh leaves were *Aspergillus niger, A. fumigatus, A. flavus, Macrophomina phaseolina, Rhizopus stolonifer, Rhizoctonia* sp*., Nigrospora* sp*., Penicillium chrysogenum, Xylaria* sp*., Alternaria cryogenic, Alternaria solani,* and *Trichoderma citrinoviride.* while eight fungi endophytes belonging to seven genera were isolated from the fresh seeds of *M. oleifera*.

From the dried branded commercial samples, four fungal endophytes were isolated from the leaves of the first branded sample (B1) and five from the leaves of the second branded sample (B2) while four fungal endophytes were identified from the seeds of B1 and four from the seed samples of B2. Fifteen species of endophytic fungi were isolated and identified from the fresh and dried samples. The results showing the isolates is presented in Table 1. The high presence of *Aspergillus* species agrees with the submission of Rajeswari *et al*. (2014) that *M. oleifera* plant parts such as the calyx, leaves and stem are host to diverse endophytic fungi especially species of *Aspergillus*. Arora and Kaur, (2019) reported in their study that *Aspergillus fumigatus* an endophytic fungus isolated from *M. oleifera* showed antimicrobial properties while Zhao *et al.* (2012) demonstrated that *Nigrospora* sp. an endophytic fungus from *M. oleifera* root possesses secondary metabolites against pathogenic fungi. They also posited that the fungus is a common endophyte with a wide array of biologically active secondary metabolites like phytotoxic while Tanaka *et al.* (1997) noted that it possesses antibacterial

nigrosporins. Lu *et al.* (2012) reported the anticancer potential of some endophytic fungi isolated from *Actinidia macrosperma* while Khalil *et al.* (2021), also reported that endophytic fungi isolated from the medicinal plant *Ephedra pachyclada* have the potential of producing growth promoting hormones like indole acetic acid (IAA). The isolates from this present study might be potential candidates for secondary biochemicals.

Table 1: Fungal Endophytes Isolated from the Fresh and Dried Commercial Samples of *Moringa oleifera* Leave and Seeds

	Isolated endophytes	Fresh leaves	Fresh seeds	Branded Sample 1 leaves	Branded Sample 1 seeds	Branded Sample 2 Leaves	Branded Sample 2 seeds
1	*Aspergillus fumigatus*	+	+	+	-	+	-
2	*Aspergillus niger*	+	+	-	+	+	+
3	*Aspergillus flavus*	+	-	-	-	-	-
4	*Rhizopus stolonifer*	+	+	+	+	-	+
5	*Fusarium oxysporum*	-	+	-	-	-	-
6	*Rhizoctonia sp.*	+	+	-	-	-	-
7	*Saccharomyces cerevisiae*	-	-	-	+	+	+
8	*Xylaria sp.*	+	+	-	-	-	-
9	*Nigrospora sp.*	+	+	+	-	+	-
10	*Trichoderma citrinoviride*	+	-	+	-	-	-
11	*Alternaria exospore*	+	-	-	-	-	-
12	*Mucor sp.*	-	-	-	-	+	+
13	*Penicillium chrysogenum*	+	+	-	+	-	-
14	*Macrophomina phaseolina*	+	-	-	-	-	-
15	*Alternaria solani*	+	-	-	-	-	-

+ denotes present; - denotes absent

3.2 Qualitative Phytochemical Content

The qualitative phytochemical contents analysis of both the fresh and dried commercial extracts of the leaves and seed of *Moringa oleifera* confirmed the presence of alkaloids, flavonoids, steroids, terpenoids, cardiac glycosides, tannins in the plant (Table 2). This corresponds with the reports by Kasolo *et al*. (2010) and Unuigbe, *et al*. (2014). The results also show the absence of saponin and phlobatannins in all samples assayed while reducing power was detected only in the fresh leaf and seed samples. However, Bamishaiye *et al*. (2011) reported the absence of terpenoids and cardiac glycoside while Oluduro (2012), submitted that steroids, terpenoids and cardiac glycoside were not observed both in the aqueous and methanolic leaves extracts of *M. oleifera.* Also, Unuigbe *et al*. (2014) reported the absence of alkaloid, reducing sugars and tannins in the leaves while reducing sugars were observed only in the fresh seed of *Moringa oleifera*. Leaves of *M. oleifera* are known to be rich source of tannins, a reason for its efficacy in diarrhea and urinary tract infection treatment and in wound healing as well as control of dysentery (Fahey, 2005 and Akaneme, 2008). Fahey (2005) studies all revealed that the presence of terpenoids in *M. oleifera* is one of the reasons for utilizing the plant in the treatment of diabetes. Ullah *et al.* (2020), reported that flavonoids possess a number of medicinal benefits which include anticancer, antioxidant, anti-inflammatory, and antiviral properties. That they have neuroprotective well as cardio-protective effects. Although, these biological activities are noted to depend upon the type of flavonoid, its (possible) mode of action, and its bioavailability. This further lay credence to the utilization of *M. oleifera* for therapeutic purposes.

Table 2: Qualitative Analysis of the Collected Fresh and Dried Commercial Leaves and Seeds of *Moringa oleifera*

Phytochemicals	Commercial leaves	Fresh leaves	Commercial seeds	Fresh seeds
Saponin	-	-	-	-
Reducing power	-	+	-	+
Flavonoids	+	+	+	+
Tannin	+	+	+	+
Steroids	+	+	+	+
Terpenoids	+	+	+	+
Alkaloids	+	+	+	+
Phlobatannins	-	-	-	-
Cardiac glycoside	+	+	+	+

+ denotes present; - denotes absent

3.3 Quantitative Phytochemical Content

The quantitative analysis of phytochemical constituents revealed that the commercial leaves and seed extract had lesser amounts of phytochemicals than the fresh seeds and leaves extract. The commercial had higher alkaloid of 72.53±0.13 mg/100g in the leaf sample and 71.70±0.32 mg/100g in the seed sample. The fresh seeds and leaves had higher contents of cardiac glycoside, reducing sugar, terpenoids, flavonoid, tannins and cardiac glycosides. This result is displayed in Table 3. The quantitative analysis of the phytochemical constituents of the fresh and dried commercial leaves and seeds of *Moringa oleifera* revealed that tannin, flavonoids, cardiac glycoside, terpenoid and reducing power of the fresh leaves and seeds of *Moringa oleifera* were more in quantity than its level found in the dried commercial leaves and seeds, this could be as a result of biochemical changes that occur during the drying process. Ademiluyi *et al.* (2018) also observed that drying alters the phytochemical contents of *M. oleifera.* However, alkaloids and steroids were observed to be high in the commercial leaves. Meanwhile, phlobatannins and saponin were absent in all sampled parts which corresponds with the phytochemical analysis carried out by Ojiako (2014), where phlobatannins was not detected in the ethylacetate extract of *M. oleifera* leaves but in the n-hexane extracts.

Table 3: Quantitative Analysis of the Collected Fresh and Dried Commercial Leaves and Seeds Samples of *Moringa oleifera*

Samples	Reducing power	Cardiac glycoside	Flavonoid	Tannin	Steroid	Terpenoids	Alkaloid
				mg/100g			
Fresh leaf	31.57±0.52	58.15±0.51	55.86±0.36	41.70±1.07	26.44±0.94	20.67±0.37	63.83±0.18
Commercial leaf		53.41±0.52	47.57±0.72	38.91±0.30	33.17±0.74	19.21±0.25	72.53±0.13
Fresh seed	46.67±0.35	60.39±0.62	54.83±0.54	43.69±0.20	31.65±0.37	22.15±0.10	61.17±0.64
Commercial seed		55.44±0.52	51.69±0.32	40.39±0.35	29.54±1.30	17.51±0.21	71.70±0.32

Result presented as Mean±SD

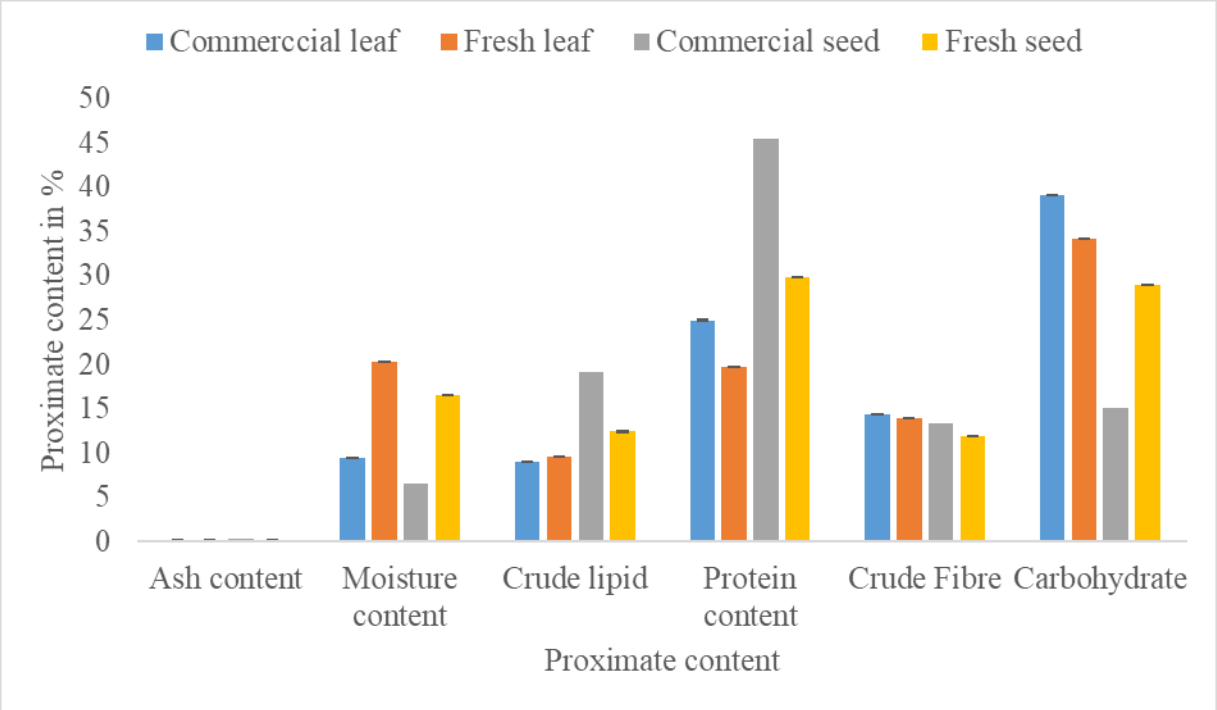
3.4 Proximate Content

The proximate analysis of the leaves and seeds of *Moringa oleifera* revealed a difference in the level of moisture content in fresh seeds and leaves which seem to be higher than that in the commercial seeds and leaves samples. The ash content of both dried commercial and fresh seeds and leaves are of significant differences. The protein content, carbohydrate and crude fibre level was observed to be high in the commercial leaves and seeds but quite reduced

in the fresh leaves and seeds. The results from the proximate analysis are in accordance with similar studies such as Chatepa and Mbewe (2018) who observed the values of 5.37, 7.90, 28.5, 23.27 and 34.91 in percentage for the ash, crude fibre, crude protein, carbohydrate and crude fat respectively for the seed of *M. oleifera* in their study from Central Malawi. Sultana (2020), study of the leaves of *M. oleifera* showed similar comparable levels of 8.05 – 10.38 % ash, 6.0 – 9.6 % fibre, 22.99 – 29.36 % crude protein, 47.25 – 56.25 % carbohydrate, 4.03 – 9.51 %, crude fat, 7.55 – 8.65 % moisture, and 81.33 – 83.73 % organic matter. The moisture contents observed in the dried commercial leaves and seeds could have served as an enabling environment for the endophytic fungi isolated. As species of *Saccharomyces, Aspergillus, Mucor, Rhizopus* and *Nigrospora* were found in these parts.

Nweze and Nwafor (2014), study on *M. oleifera* root powder revealed proximate content values of 7.95 %-ash, 9.31 %,-crude fibre, 18.92 %-protein, 2.74 %-crude fat, 4.09 %-moisture and 57.01 %-carbohydrate. However, this comparison has not been reported but it can be inferred that the variation of proximate composition could be credited to the region where *M. oleifera* is planted and the growing conditions of the plant as submitted by Olagbemide and Alikwe (2014).

The results of the proximate analysis of *M. oleifera* leaves agrees with the submissions of Krishnaiah *et al*. (2009) and Oluduro (2012) and further gives credence to the duo's reports on the consumption of the leaves as supplement for food essential in infants healthcare and nursing mothers. Preliminary proximate analysis of the various crude nutrients noted in the leaves and seed of *M. oleifera* disclosed that these plant parts contain a sufficient amount of nutrients and can be added to diets for the purpose of supplementing the daily nutritional needs of human. Furthermore, the present study of the phytochemical contents of *M. oleifera* has authenticated its usefulness by traditional herbal practitioners in ethnomedicine.



Result presented as Mean±SD

Figure 1: Proximate Content of the Collected Fresh and Dried Commercial Leaves and Seeds Samples of *Moringa oleifera*

4. Conclusion

This study has shown that the leaves and seeds of *M. oleifera* house a wide diversity of endophytic fungi. Cautions should however be directed in harnessing the potentials of the endophytic fungi in drug formulation and development. The leaves and seeds of *M. oleifera* contain high levels of terpenoids and alkaloids which may be key determinant of the antioxidant activity. These findings together with previous studies demonstrate that *Moringa oleifera* is an excellent plant candidate to be explored in improving the health and nutrients of

communities around the globe. The study can also assist in policy making for packaging of *M. oleifera* products to ensure standard practice.

Conflict of Interest

The author declares that there is no conflict of interest.

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