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Effects of *Moringa oleifera* Leaves and Seeds Extracts against Food Spoilage Fungi

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Abstract

Fungal foodborne diseases pose serious public health problems and cause significant loss of the world's food stock as a result of toxic contamination. Hence the need to find solutions to foodborne fungal contaminants. This study investigated the antifungal and phytochemical properties of *Moringa oleifera* leaves and seeds using various extraction solvents (acetone, water, ethanol and methanol). *Aspergillus flavus* and *Aspergillus niger* isolated from food samples were used as test organisms. The Agar Well Diffusion method was used to determine the antifungal activities of *Moringa oleifera* leave and seed extracts, while standard phytochemical tests were used to analyze for the phytochemicals. *Moringa oleifera* leave and seed extracts showed the presence of glycosides, flavonoids, alkaloids, tannins, saponins, phenols and hydrolysable tannins after the chemical test. At 100 mg/ml for Methanol extract, the leaves gave wider zones of inhibition (18.33 mm against *A. flavus* and 17.17 mm against *A. niger*) than the seed extract (16.50 mm against *A. flavus* and 16.33 mm against *A. niger*) for all test organism. The activity of the extracts were however lower than Sodium benzoate (33 mm at 100 mg/ml), as standard. The Minimum Inhibition Concentration of the plant extracts was most active at 25 mg/ml. *Moringa oleifera* leaves and seeds extracts may serve as natural antifungals for controlling growth of food spoilage fungi, and therefore may be used as a bio-preservative agent for prolonging the shelf-life of food products.

Keywords

Food Spoilage, *Moringa oleifera*, Minimum Inhibitory Concentration

1. Introduction

Foodborne disease is a global issue with significant impact on human health [1].

Several factors like humidity, temperature, light, oxygen, heat and spoilage bacteria make food unsuitable and unsafe for consumption. Deteriorated food produces defects like change in colour, odour, change in texture and change in appearance [2]. Groups of microorganisms responsible for food deterioration are bacteria, molds and yeasts. These microorganisms cause typical foodborne illnesses like diarrhea, fever, abdominal cramps and dysentery [3]. Other foodborne illnesses that pose serious health threats are Botulism, Perfringens food poisoning, intestinal cryptosporidiosis, Hepatitis, Listeriosis, Shigellosis [4].

Mold and yeast are terms used to describe forms of fungi. Fungi are organisms grown for food but a smaller number are agents of diseases in animals including human. Some fungi produce poisons called mycotoxins which contaminate food and feed [5]. Kidney and Liver damage in animals are acute diseases associated with mycotoxins [6]. *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomins* produce aflatoxins which are potent natural carcinogenic compounds that cause mutation [6]. Aspergillus species are most common in stored foods such as nuts, grains and spices, and occur more frequently in tropical and subtropical climates [7]. The specie *Aspergillus clavatus* is associated with barley during malting and it can grow to unacceptably high levels if malting temperatures are not elevated [8].

Saccharomyces cerevisiae also plays a vital role in wine industry but is also the devastating cause of food spoilage [9].

Fungi in general are typical food spoilage organisms for dairy products which result in significant food deterioration and economic losses [10]. The mycotoxins produced by fungi affect agricultural commodities by lowering their market value [11]. Consumers end up paying extreme prices due to an increased monitoring at all levels of handling and in extreme cases death problems due to consumption of contaminated food products [11].

Over the years, a vast range of chemical methods of preservation have been used to protect and preserve many foods from spoilage as a result of fungal and bacterial contamination. Protection of food from microbial deterioration has been an important concern to individuals and food industries. Consumers in recent years have a high preference for natural preservatives to chemical-based preservatives due to their adverse health effects [12]. This experience led to more natural preservative alternatives so as to prolong the shelf life and safety of food [13] [14] [15].

Moringa is one of the world most important trees of Asia and Africa origin. One of these most useful and therapeutic species is *Moringa oleifera*. *Moringa oleifeera* is widely known and utilized for various purposes around the [16] [17] [18]. The *M. oleifera* has several traditional uses of which almost all the parts are used (root, bark, leaves, fruits, flowers, seeds) for many ailments [19]. These parts are also used for the treatment of inflammation and infectious human diseases like cardiac circulatory tonic and antiseptic [20].

The forage is used for livestock and also as a micronutrient powder to treat different ailments [21], leaves and immature pods (drumstick) as vegetable ac-

companiments and as health foods, used to enhance breast milk production in pregnant women, used to enhace flavour, taste and extend shelf life of cow and buffalo ghee [22]. Ayirezang and associates also improved the shelf life of pito, a local beer, through the addition of moringa leave extract [23].

Although the antimicrobial characteristics of *Moringa oleifera* extracts have been confirmed by many literature, studies on the effect of specific fungi isolates from food such as *Aspergillus flavus* and *Aspergillus niger* are limited. This study explored the antifungal potency of *Moringa oleifera* leave and seed extracts against *Aspergillus flavus* and *Aspergillus niger* that cause food spoilage.

2. Materials and Methods

2.1. Collection and Identification of Plant Samples

The fresh leaves and the matured seed pods of *Moringa oleifera* were collected at Madina in Accra. The authenticity of the plant (*Moringa oleifera*) leaves and seeds were done by a weed scientist, Mr. Chakubus Amoah (Taxonomist) in the Department of Biology at the University of Ghana, Legon.

2.2. Preparation of Plant Samples

The leaves were destalked and washed again with distilled water and air-dried at room temperature for 8 days with constant turning. The seeds were also removed from the seed pods, dehusked and dried under the same condition for 15 days. The dried leaves and seeds were later milled separately into powder and stored at 4˚C in air-tight containers for further analysis.

2.3. Sample Extraction

Eight different solvent extracts (three replicates each) were made from the leave and seed samples of *Moringa oleifera* using ethanol, methanol, acetone and water; AMSE = Acetone Moringa Seed Extract, AMLE = Acetone Moringa Leave Extract, EMSE = Ethanol Moringa Seed Extract, EMLE = Ethanol Moringa Leave Extract, MMSE = Methanol Moringa Seed Extract, MMLE = Methanol Moringa Leave Extract, WMSE = Water Moringa Seed Extract and WMLE = Water Moringa Leave Extract. Approximately, 40 g of pulverized seed sample were separately soaked in 100 ml of each solvent in different 200 ml conical flasks for 24 hours while 20 g of pulverized leave sample were soaked in 50 ml of each solvent. The extracts were filtered with Whatman No.1 filter paper. The filtrates were evaporated to dryness at 50˚C temperature in an oven [24]. The dried extracts were reconstituted in sterile distilled water to the required concentrations for the bioassay analysis. The corresponding concentration was expressed in terms of mg of extract per ml of solvent (mg/ml).

2.4. Phytochemical Analysis

Study adopted phytochemical composition in extracts analysis method as used by Sofowora [25].

2.5. Test for Saponins

To reveal the presence of Saponins, 1 g of the sample was weighed into a conical flask. 10 ml of sterile distilled water was added and boiled for 5 minutes. The resulting mixture was filtered using 11 μm filter paper. 2.5 ml of the filtrate was mixed with 10 ml of sterile distilled water in a tightly capped test tube. This was then agitated vigorously for 30 seconds using a standard laboratory agitator. Agitated mixture was then allowed to stand for 30 minutes after which it was visually inspected for the presence of Honeycomb froth which is precursor for saponins.

2.6. Test for Tannins

The powdered sample (3 g) was boiled in 50 ml distilled water for 3 minutes on a hot plate.

Resulting mixture was filtered using 11 μm filter paper and a portion of the filtrate diluted with sterile distilled water in a ratio of 1:4 with 3 drops of 10% Ferric chloride solution added.

Blue or green colour indicated presence of tannins.

2.7. Test for Phenol

The extract (2 ml) was added to 2 ml of 10% ferric chloride solution ($FeCl_3$), a deep bluish green solution is formed with presence of phenols.

2.8. Test for Glycosides

Diluted sulphuric acid (25 ml) was added to 5 ml of extract in a test tube and boiled for 15 minutes, cooled and neutralized with 10% NaOH, then 5 ml of fehling solution A and B was added. A brick red precipitate of reducing sugars indicates presence of glycosides.

2.9. Test for Alkaloids

Exactly 2 ml of extract was added to few drops of picric acid solutionin a test tube. The formation of orange coloration indicated the presence of alkaloids.

2.10. Test for Volatile Oils

Briefly, solution of extract (2 ml) was shaken in a test tube with 0.1 ml dilute sodium hydroxide and a few drops of dilute HCl. Appearance of a white precipitate indicated presence of volatile oils.

2.11. Test for Hydrolysable Tannins

Briefly, solution of extract (4 ml) was shaken in a test tube, and thereafter, 4 ml of 10% ammonia solution was added. Formation of an emulsion on shaking indicated the presence of hydrolysable tannins.

2.12. Test for Flavonoids

Briefly, few drops of very dilute solution of ferric chloride were added to 1 ml of

extract. A colour change to pale green or red brown colour indicated the presence of flavonoids.

2.13. Preparation of Media

Dichloran Rose Bengal Chloramphenicol (DRBC) medium and Potato Dextrose Agar were prepared according to manufactures protocol.

2.14. Preparation of Inoculum

The organisms were subcultured on potato dextrose agar and incubated at a temperature of 25˚C for 120 hours. Inoculum suspensions were prepared from fresh, matured (5-day-old) cultures. Colonies were carefully covered with approximately 5 ml of sterile distilled water. Then, the conidia (Non-motile spores of fungus) were carefully and aseptically rubbed with a sterile cotton swab and transferred to a sterile tube containing 5 ml sterile water. The suspension was mixed (vortexed) at 2000 rpm for 15 seconds. The suspension was then filtered with a filter (11 µm pore-size) into sterile tube. This step was used to remove hyphae in order to yield a suspension composed only of conidia. The suspension was adjusted to a concentration equivalent to McFarland 0.5 by diluting the suspension 1:10 with sterile distilled water to obtain a final working inoculum of 2×10^{-5} cfu/mL.

2.15. Standardization of Inoculum

Turbidity of the inoculum was standardized to a turbidity equivalent to a 0.5 McFarland standard prepared from 1% barium chloride and 1% sulphuric acid.

2.16. Preparation of Samples of Extract

Three concentrations (100, 200 and 300 mg/ml) of each extract were prepared by reconstituting in distilled water.

2.17. Antifungal Susceptibility Test

The Agar Well Diffusion method was employed to determine the antifungal activities of *Moringa oleifera* seed and leave extracts. Briefly, each of the fungal suspensions was spread aseptically onto the surface of Dichloran Rose Bengal Chloramphenicol medium using a sterile glass spreader. The agar plates were dried and wells (10 mm in diameter) were cut from the agar with separate sterile cork borers. The wells were then filled with different concentrations of plant extracts. Negative controls were prepared using sterile distilled water. Sodium benzoate (100 mg/ml) was used as positive reference standard. The inoculated plates were incubated at 25˚C for 72 hours. Antifungal activity was evaluated by taking triplicate readings of the diameter of zone of inhibition. All the zones of inhibition were expressed in millimetres (mm).

2.18. The Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentrations of the plant extracts were determined by doubling the dilution. Briefly, extract concentrations of 50, 25, 12.5, 6.25 and

3.13 mg/ml were prepared by serial dilutions. Each concentration of the extract was inoculated with 0.1 ml of the standardized spore suspension, and thereafter, incubated at 25˚C for 72 hours. The inoculum of each organism in Potato Dextrose Broth was observed for turbidity or cloudiness. The lowest concentrations at which turbidity or cloudiness were not seen, were taken as the MIC.

2.19. Statistical Analysis

All readings were done in triplicates, and the means determined. Differences in means between groups were determined using non-parametric statistical analysis. Differences in means were considered significant for p-value < 0.05. Microsoft Excel (2013) was used for all statistical analysis.

3. Results

3.1. Percentage Yield for Different Extraction Systems

As shown in Table 1, MMLE produced the highest yield of 17.8% among the leave extracts. In contrast, the same solvent gave the least yield of 2.275% among the seed samples. Water (WMSE) on the other hand gave the highest yield of 31.325% among all the samples.

3.2. Phytochemical Constituent of Extracts

Some of the phytochemicals were absent in some treatments with the exception of hydrolysable tannins which was not present in all the treatments. Saponins were present in AMSE, EMSE, EMLE, and MMLE but absent in MMSE, WMSE and WMLE. Flavonoids appeared in almost all the extracts except AMSE and WMLE. Tannins were seen in only EMLE, MMLE and WMLE. Alkaloids were also seen in almost all the extracts except AMLE and EMLE (Table 2).

3.3. Antifungal Susceptibility of Extracts

The zone of inhibition was significantly different ($P < 0.05$) among treatments at various concentrations for the Aspergillus species (Figure 1 and Figure 2). MMLE showed the highest zone of inhibition at 27.50 mm, 23.67 mm and 18.33 mm against *Aspergillus flavus*, when applied at various concentrations, 300 mg/ml, 200 mg/ml and 100 mg/ml respectively. This same observation was made with for *Aspergillus niger* with zones of 23.83 mm, 19.83 mm and 13.33 mm for respective concentrations (highest to lowest).

Acetone extracts gave the least zones of inhibition for all the treatments, with the seed sample AMSE recording the lowest for both *Aspergillus flavus* (20.17 mm, 17.0 mm and 13.17 mm) and *niger* (18.50 mm, 16.83 mm and 13.33 mm) when applied at the concentrations 300 mg/ml, 200 mg/ml and 100 mg/ml respectively.

3.4. Minimum Inhibitory Concentrations of Extracts

MMLE showed the least minimum inhibitory concentration of 12.5 mg/ml followed by MMSE, WMSE, AMLE and WMLE at 25 mg/ml (Table 3).

Table 1. The percentage yield of the various seed and leave extracts of *Moringa oleifera*.

Extract	A) Initial Amount of *M. oleifera* used (g)	B) Amount of Extract Recovered (g)	C) Percentage Yield (% Yield)
AMSE	40.0	9.96	24.900
EMSE	40.0	4.58	11.450
MMSE	40.0	0.91	2.275
WMSE	40.0	12.53	31.325
AMLE	20.0	2.56	12.8
EMLE	20.0	2.42	12.1
MMLE	20.0	3.56	17.8
WMLE	20.0	3.44	17.2

Column A: various amount of Moringa powder; 40 g of the seed and 20 g of the leave samples were used. Column B: were the various amount of the extracts recovered after extraction and oven concentration and column C were their distinctive percentages.

Table 2. Phytochemical constituent of ethanol, acetone, methanol and water extracts of *Moringa oleifera* leaf and seed.

	AMSE	AMLE	EMSE	EMLE	MMSE	MMLE	WMSE	WMLE
Saponins	+	+	+	+	−	+	−	−
Flavonoids	−	+	+	+	+	+	+	−
Tannins		−	−	+	-	+	-	+
Alkaloids	+	−	+	−	+	+	+	+
Glycosides	−	−	−	+	−	−	−	−
Volatile Oils	+	−	−	+	−	−	−	−
Hydrolysable Tannins	−	−	−	−	−	−	−	−
Phenols	−	+	-	+	−	+	−	+

Key: (+) = present, (−) = absent. AMSE = Acetone Moringa Seed Extract, AMLE = Acetone Moringa Leave Extract, EMSE = Ethanol Moringa Seed Extract, EMLE = Ethanol Moringa Leave Extract, MMSE = Methanol Moringa Seed Extract, MMLE = Methanol Moringa Leave Extract, WMSE = Water Moringa Seed Extract and WMLE = Water Moringa Leave Extract.

4. Discussion

The bioactive analysis of *Moringa oleifera* seed and leave extracts revealed the presence of Saponins, Glycosides, Tannins, Phenols, Volatile Oils, Alkaloids, Flavonoids and Hydrolysable Tannins. Saponins were present in Acetone Moringa Seed Extract (AMSE), Acetone Moringa Leave Extract (AMLE), Ethanol Moringa Seed Extract (EMSE), Ethanol Moringa Leave Extract (EMLE)and Methanol Moringa Leave Extract (MMLE). The presence of saponins in Ethanol Moringa Seed Extract (EMSE), Ethanol Moringa Leave Extract (EMLE) collaborates with the findings of Bukar *et al.*, (2010) but the presence of saponins in Acetone Moringa Leave Extract (AMLE) in this research disagrees with the finding of Bansode and Chavan (2012). Flavonoids were active in Acetone Moringa Leave Extract (AMLE), Ethanol Moringa Seed Extract (EMSE), Ethanol

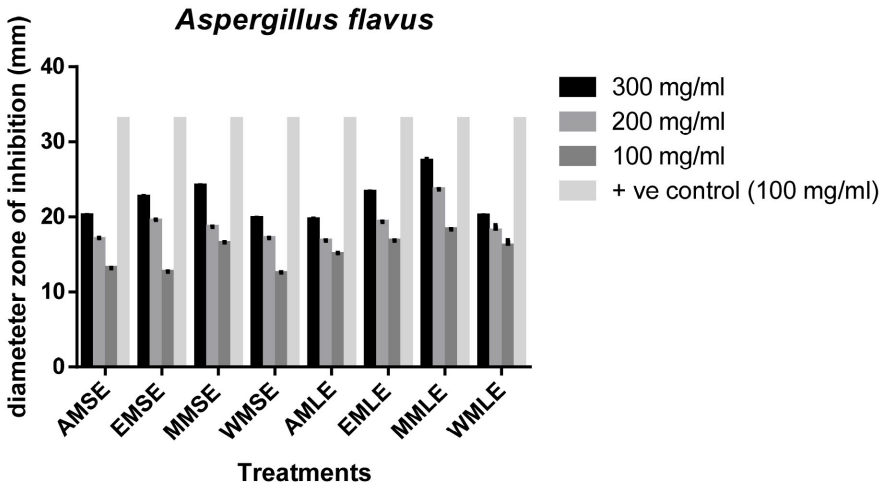

Figure 1. Mean Zone of Inhibition of *Aspergillus flavus* among Treatments. Values are means and standard deviations of diameter zone of inhibition (mm) of triplicate determinations. Values showed a significance difference among treatments (Fpr < 0.001). Key: AMSE = Acetone Moringa Seed Extract, AMLE = Acetone Moringa Leave Extract, EMSE = Ethanol Moringa Seed Extract, EMLE = Ethanol Moringa Leave Extract, MMSE = Methanol Moringa Seed Extract, MMLE = Methanol Moringa Leave Extract, WMSE = Water Moringa Seed Extract and WMLE = Water Moringa Leave Extract.

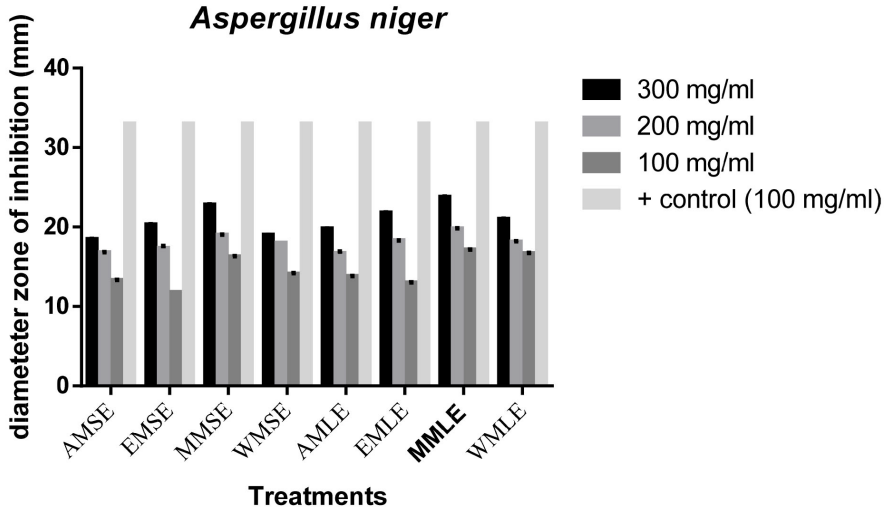

Figure 2. Mean Zone Of Inhibition Of *Aspergillus Niger* among Treatments. Values are means and standard deviations of diameter zone of inhibition (mm) of triplicate determinations. Values showed a significance difference among treatments (Fpr < 0.001). *Key:* AMSE = Acetone Moringa Seed Extract, AMLE = Acetone Moringa Leave Extract, EMSE = Ethanol Moringa Seed Extract, EMLE = Ethanol Moringa Leave Extract, MMSE = Methanol Moringa Seed Extract, MMLE = Methanol Moringa Leave Extract, WMSE = Water Moringa Seed Extract and WMLE = Water Moringa Leave Extract.

Moringa Leave Extract (EMLE), Methanol Moringa Seed Extract (MMSE), Methanol Moringa Leave Extract (MMLE) and Water Moringa Seed Extract (WMSE). Again, flavonoids in Ethanol Moringa Leave Extract (EMLE), Ethanol Moringa Seed Extract (EMSE) and Acetone Moringa Seed Extract (AMSE),

Table 3. Minimum inhibitory concentrations of the treatments.

Concentration Mg/ml	AMSE	EMSE	MMSE	WMSE	AMLE	EMLE	MMLE	WMLE
50	–	–	–	–	–	–	–	–
25	+	+	–	–	–	–	–	–
12.5	+	+	+	+	+	+	-	+
6.25	+	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+	+

+: means profuse growth; –: means no growth.

Acetone Moringa Leave Extract (AMLE) agreed with the findings of [26] [27] respectively. Alkaloids were present in Water Moringa Leave Extract (WMLE) and absent in Ethanol Moringa Leave Extract (EMLE). The presence of alkaloids in Water Moringa Leave Extract (WMLE) collaborates with the work of [27] but disagree the findings of [28] [29]. The inconsistencies in these findings could be as a result of method variations adopted by the researchers.

The percentage yield of the various seed extracts were 2.275%, 11.450%, 24.900% and 31.325% of Methanol Moringa Seed Extract (MMSE), Ethanol Moringa Seed Extract (EMSE), Acetone Moringa Seed Extract (AMSE) and Water Moringa Seed Extract (WMSE) respectively. The leave extracts Acetone Moringa Leave Extract (AMLE), Ethanol Moringa Leave Extract (EMLE), Water Moringa Leave Extract (WMLE) and Methanol Moringa Leave Extract (MMLE) yielded 11.7%, 24.2%, 34.4% and 35.6% respectively (**Table 1**). Thus, from these results in **Table 1** and **Table 2** in comparison, the leave extracts produced more extracts with Methanol Moringa Leave Extract (MMLE) being the highest than the seed extracts. This could be as a result of greater accumulation of the phytochemical compounds in the leaves than the seeds which is consistent with zones of inhibition recorded.

In this research study, the mycelia growth of the *Aspergillus flavus* and *Aspergillus niger* were found to be inhibited significantly in a dose-dependent manner by the water, acetone, ethanol and methanol extracts of seeds and leaves of *Moringa oleifera*. Although, the efficacy of the extracts were found to vary with concentration, plant parts, type of solvent used for extraction and also the pathogen, it is clear that *Moringa oleifera* has potential antifungal activity though none of the extracts had 100% mycelia growth inhibition. It was further revealed that increase in the antifungal activities of the extracts was enhanced by increase in concentration of the extract (**Figure 1** and **Figure 2**). This may be due to higher quantity of antifungal compounds in higher concentration as reported by [30].

The leaves extract of *Moringa oleifera* upon comparison gave the best result in terms of mycelia growth inhibition. This may be due to the leaves having more bioactive compounds than the seeds. This corroborates the findings of [31], where they found leaves extract of *Moringa oleifera* to have high inhibitory effect

on some pathogenic fungi than other extracts. From the result, it is evident that extracts of seeds have low inhibitory activity.

This may be due to the fact that in seeds, either there is low accumulation of these phytochemicals or they are more complex to be dissolved in the solvents used for the extraction. In this study, it was found that the plant extracts in organic solvent (methanol) provided more antifungal activities compared to those extracted in water (aqueous extract), ethanol and acetone. This may be because most of the antifungal agents may be relatively more non-polar than water.

Direct comparison of some of these results with those obtained in other studies [32] seam to contradict slightly, and this may be due to a number of factors such as variability in composition of plant extracts as a result of local climatic and environmental conditions, low number of samples tested, differences in experimental design including inoculum size, extractive procedure used, and culture medium used.

The MIC of most of the extracts (Methanol Moringa Seed Extract (MMSE), Water Moringa Seed Extract (WMSE), Acetone Moringa Leave Extract (AMLE), Ethanol Moringa Leave Extract (EMLE) and Water Moringa Leave Extract (WMLE) was 25 mg/ml. This collaborates the findings of [33] but disagrees with [34] who used micro dilution method and had MIC of 1.562 and 3.125 for *A. niger* and *A. flavus* respectively. These differences could be as a result of the reasons stated by [32] that factors such as variability in composition of plant extracts as a result of local climatic and environmental conditions, low number of samples tested, differences in experimental design including inoculum size, extractive procedure used, and culture medium used as the reason for the discrepancies.

5. Conclusion

This research work revealed that the leaves and seeds of *Moringa oleifera* possess antifungal properties. However, the efficacies of the extracts were found to vary with concentration, pathogen, plant part as well as the type of solvent used for the extraction. These results therefore show that *Moringa oleifera* leave extracts are a potential source of natural antifungal agents that can be used in food industries as a natural food preservative.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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