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Phytochemical Constituents And Comparative Antifungal Activity Of Polarity Based Solvent Extracts Of Moringa Oleifera Seeds, Alum And Chlorine On Aspergillus Fumigatus Isolate From Wastewater.

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Abstract:
Wastewater treatment technologies are uneconomical in developing countries. For this reason, a study on bio-coagulants from plants was exploited. Moringa oleifera (MO) seeds extracted using solvents from non polar hexane to highly polar water was tested for their photochemical constituents and antifungal activity on a strain of Aspergillus fumigatus isolate from wastewater. Only alkaloid in the aqueous extract suggested that the highly polar solvents are better extracting solvent when compared to non polar solvents for active antifungal fraction from Moringa oleifera seeds. An antimicrobial peptide measuring 12kDa identified as an alkaloid has been isolated from aqueous extracts of moringa seeds with no report on the antifungal activity on waterborne fungal isolates. Flavonoids detected in all the extracts while steroids only in the polar extracts. All the extracts of Moringa oleifera inhibited mycelia growth of Aspergillus fumigatus as compared to a mycelia spread of 85mm on the control plates. The aqueous extract of MO inhibited spore germination, while no inhibitory effect was observed for Aspergillus plates treated with alum and chlorine. This is the first authentication of the antifungal activity of the crude extracts of Moringa oleifera seeds in comparison to alum and chlorine on a water borne fungal isolate.

Keywords: *Moringa oleifera, Antifungal, Aspergillus, Water, Phytochemical, Chlorine.*

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I. Introduction

Approximately five million lives are lost annually due to drinking and use of contaminated water (Madore *et al.*, 1987; W.H.O, 2006 ;ritchard *et al*., 2009; Amir *et al*., 2010). Many drinking water utilities rely solely on monitoring indicator organisms such as coliforms and *Escherichia coli* to ensure quality of water (Cheesbrough,2005) However, the presence or absence of these indicator organisms may not correlate well with the presence of fungal contaminant in water (Dogget, 2000; Hageskal et al,2006 and Pereira et al,2009). Limited attention has been given to the presence of fungi in public water supply systems (Dogget, 2000; Arvanitidou et al, 2002; Haggeskal et al, 2007 and Pereira et al, 2009). There is a need not only to monitor the fungal spectrum of aquatic systems but to apply sustainable techniques to decontaminate fungi from public water systems. One of technologies is to apply plant materials such as *Moringa oleifera*,(Amanda et al., 1991), consist of a tremendous variety of secondary metabolites diverse applications in water treatment and in clinical medicine (Muyibi and Evison 1995b; Kebreab et al,2005 and Pritchard et al,2009) The interest in studying *Moringa oleifera* has increased since Jahn in 1979 reported its significant role as a coagulant in water purification and antimicrobial activity (Olsen,1987 and Raheela et al., 2008) Many bioactive compounds in *Moringa oleifera* plant have been found to have anticancer,antibacterial,antifungal and immunostimulatory properties(Fuglie,2001) Despite the uses of Moringa extracts for water treatment and pharmaceutical applications, researchers have focussed their interest solely in the isolation of coagulant active ingredient (Okuda et al., 1999 and Okuda et al., 2000) antibacterial studies with limited studies on the application of Moringa seeds to disinfect waterborne fungi.

Moringa oleifera is widely used in folk medicine, as a human health supplement and also as animal feed due to its high protein content and high concentration of essential amino acids, vitamins, minerals and fatty

acids(Fuglie,2001). The detection of primary and secondary metabolites in the leaves, roots and seeds has been employed in various pharmaceutical applications. For example, the primary and secondary metabolites of *Moringa* have the potential as an antiviral, antibacterial, anti-inflammatory, anti-tumour as well as anticancer activity (Amanda et al., 1991; Fuglie, 2001).Coagulant and antimicrobial peptides of less than 10 kDa using various extraction and purification protocols has been reported (Ndabigengesere et al., 1995; Kebreab et al, 2005; Dorries, 2005) and antimicrobial alkaloid called pterygospermin was reported by Eilert in 1981.

Independent solvent extracts of *Moringa oleifera* seeds *were* screened and reported that the water, acetone, petroleum ether and diethyl ether extracts have considerable antimicrobial activity against many microorganisms (Dorries, 2005; Kebreab et al, 2005; Raheela et al., 2008 and Amir et al., 2010) Nevertheless, it is not well understood if the phytochemical constituents responsible for the coagulant activities is the same for the disinfection activity. The importance of analysing the phytochemical constituents is due to the fact that these chemicals are the key on the biological activities. Although many studies showed the extensive use of *Moringa oleifera* is essential for the development of pharmaceuticals, its phytochemical constituents require continuous elucidation. Water borne fungi such as *Aspergillus spp* has been implicated in many immune- compromised diseases such as cancer HIV and AIDS (Monica Cheesbrough, 2005) Therefore, in this study, the preliminary phytochemical screening of *Moringa oleifera* and the in vitro inhibitory effect of polarity based extracts from this plant on *Aspergillus fumigatus* isolate from a wastewater source was investigated in comparison to Alum and Chlorine.

II. Material And Methods

Collection, Processing and Extraction procedure of Moringa oleifera seeds.

Moringa oleifera seeds were obtained from Bamenda in Cameroon where it is used in many rural areas to treat water. The seeds that were preserved in normal room temperature were dehusked and pulverized using a kitchen blender. It was sieved using a 0.01 sieve mesh. Fifty grams of the powder was added to 250ml of five solvents sequential extraction (1:5, w/v) in 250 ml beakers (Pyrex). The soxylet extraction method was first carried out for a period of 48 hours with hexane and the extracts were filtered by gravity filtration. The solvents were evaporated under a gentle stream of nitrogen gas with a warm water bath (at 50^{o}C). The extraction was repeated with the residue by adding in toluene, acetone, methanol and finally distilled water (1:5, w/v) in the same manner, except the aqueous extract was dried under sunlight. The weight of the extracts was obtained to calculate the percentage yield (Cannel, 1998).

Analyses for phytochemical constituents

All of the sequential extracts were subjected to preliminary phytochemical screening as described by Geissman (1963), Trease and Evans (1989) and Harborne (1998) The preliminary screenings were carried out to identify alkaloids, tannins, saponins, phlobatannins, cardiac glycosides, steroids, terpenoids and flavonoids. For tannins, $FeCl_3$ test was used; for alkaloids, Wagner test was used; for cardiac glycosides, Keller killiani test was used; for saponins, frothing test was used; for phlobatannins, hydrochloric acid was used for flavonoids, alkaline solution and diluted acid for steroids and terpenoids, chloroform and concentrated sulphuric acid was used (Cannel, 1998; Trease and Evans, 1989; Mackie et al, 1978; Haslam, 1996).

Isolation protocol and identification of Aspergillus fumigatus

Wastewater sample from a mining well was cultured on Potato dextrose agar using the pour plate technique (Collins et al, 1995). One millilitre of aliquots of the wastewater was aseptically incorporated on to molten agar and swirled gently to have a homogenous mixture. This was done in triplicates and plates incubated at room temperature (23^{o}c) and growth monitored daily for one week. Mycelia strands and spores were picked using an inoculating loop and a smeared using lactophenol cotton blue stain. This preparation was examined microscopically and morphological characteristics of Aspergillus such as ascospores, greenish mycelia strands were indicative of *Aspergillus fumigatus*. *Specific taxonomic* guides specified by David et al (2007) were used to confirm the identity of this organism.

In Vitro Antifungal assay

Antifungal screening of the sequential extracts was conducted using the agar diffusion method (Collins et al, 1995). Tested organism (*Aspergillus fumigatus)* was subcultured and grown on potato dextrose agar at 27^{o}C for 7 days. The sequential extracts were dissolved in its extracting solvent and made up to a concentration of 40 mg/ml except the aqueous extract which was already in liquid form. Each of the extracts, their corresponding extracting solvents as controls, alum and 0.5ml of Chlorine were carefully incorporated into molten potato dextrose agar plates in triplicate, swirled gently to have a homogenous mixture. They were then allowed to set. After inoculation, a 6mm diameter of the test fungus (mycelia block) with approximately 5000 spores per ml was aseptically placed at the centre of each plate. The set up was incubated at room temperature and growth rates

monitored daily by direct measurement for up to two weeks. The extracting solvents, such as hexane, toluene, acetone, methanol and water; were included as a positive controls. The actual growth inhibition of the test organism was examined by measuring the rate of spread of the mycelium and spore germination ability using the tape rule measurement test.

III. Results And Discussion

The appearance and the yield of the sequential extracts of Moringa seeds were observed and presented as in Table 3. The yield of the sequential extracts ranged from 5.4% to 47.2% with the aqueous extract having the highest percentage yield.

The potential antimicrobial active compounds are mostly found from the organic solvent extracts (Kebreab et al., 2005). Coagulant and antimicrobial peptides less than 10kDA has been detected from bulk water extracts of Moringa oleifera seeds (Kebreab et al.,2005 and Amir et al.,2010). This, however, has not been tested on any fungal isolate from water or wastewater. Previous studies also showed that the organic solvent extracts from *Moringa oleifera* have been found to show antimicrobial activity (Raheela et al., 2008; Mala et al, 2009; Amir et al., 2010) In order to identify a better solvent extraction for *Moringa oleifera* seeds, a sequence of different polarity solvents were used for the extraction. In this study, all the extracts displayed varying antifungal activity against *A. fumigatus* (Table 5). The extracting solvents, Alum and Chlorine had no antifungal activity against *Aspergillus fumigatus* (Figs 1 and 2) The finding in this study is in agreement with the previous report of antifungal activity of Moringa seeds (Raheela et al., 2008).However; the fungi used in previous reports were obtained from skin infection. Extracts from Moringa have not been tested on water borne fungi particularly *Aspergillus fumigatus*.

Preliminary screening of different sequential extracts for alkaloids, tannins, saponins, phlobatannis, cardiac glycosides, steroids, terpenoids and flavonoids was evaluated in Tables, 1 and 2

The phytochemical screening of the aqueous extracts of *Moringa oleifera* revealed the presence of alkaloids. Greater antifungal activity was observed with the aqueous extract than with all the other solvent extracts. The aqueous extract totally inhibited spore germination. This observation has not been reported elsewhere. This finding possibly lends credence to the reason why most researchers prefer to use water and methanol extracts of medicinal plants for the antimicrobial testing. From this results, the polarity based sequential extraction protocol may be a better extraction solvent system than the use of one single solvent for extracting active components from *Moringa oleifera* seeds.

Flavonoids were discovered in all the extracts of *Moringa oleifera* seeds .This suggest that they are soluble in both polar and non polar solvents. This phyto-constituent have been reported to have, anti-inflammatory, anti-diabetic, anti-viral, anti-fungal and anti-bacterial properties; moreover, it plays a significant role in the central nervous system activities (Argal and Pathak, 2006;) (Lacaille- et al 1996;) (Milgate and Roberts, 1995;) (Rupasinghe, et al., 2003); (Sayyah, et al 2004)). The industrial applications of oils from moringa have also increased significantly due to its important role as a drug for skin infections. Steroids were detected only in the acetone, methanol and water extracts. This implies that steroids are highly soluble in polar solvents. Steroids are found to have the similar role as saponins, which both of these bioactive compounds are responsible for central nervous system activities (Argal & Pathak, 2006). In addition, steroids showed to possess analgesic and anti-inflammatory effect (Lerner *et a*l., 2004). The anabolic-steroids, of which are the most common drugs in this class of phytochemicals, has been used by more than 1 million people in the United States (Du Rant et al, 1995). These results seem to support this finding since polar extracts from Moringa seeds possess steroid and might have the potential to show antifungal activities. Flavonoids were found in all the extracts of Moringa seeds. There are many reports suggesting that flavonoids have anti-tumour, and anti-inflammatory effect (Al-Meshal, et al 1986) (Wang, et al 1998). Flavonoids isolated from citrus fruits also demonstrated the anticancer both in vivo and in vitro activity (Silalahi, 2002).

IV. Conclusion

In conclusion, the presence of these phyto-constituents suggested that extracts from *Moringa oleifera* seeds is a good antifungal agent for disinfection of *Aspergillus spp* from contaminated water. This observation has not been reported elsewhere. In addition, the solvents used in the extraction play an important role to obtain the phyto-constituents from *Moringa oleifera* seeds. Aluminium sulphate and sodium hypochlorite did not exhibit any antifungal activity against this isolate. This suggests that alum and chlorine may not have any effect in disinfecting water borne fungi. This is a very important observation that points to the fact that fungi monitoring in water analysis should be considered as traditional faecal indicators may not correlate well with this group of organisms. Furthermore, Moringa oleifera seed extracts could be applied in disinfecting water borne fungi. The findings also suggest that Moringa seed extracts could also be applied to treat clinical infections such as systemic mycosis, Aspergillosis in immunosuppressed patients.

Table 1: Phytochemical Components/Constituents of Moringa seeds and Shells Extract

Constituents	Hexane Extract	Toluene Extract	Acetone Extract	Methanol Extract	Water Extract
Tannins	-	-	-	-	-
Phlobatannins	-	-	-	-	-
Saponins	-	-	-	-	-
Steroids	-	-	+	+	+
Flavonoids	+	+	+	+	+
Glycosides	-	-	-	-	-
Terpenoids	-	-	-	-	-
Alkaloids	-	-	-	-	+

Key - → not detected
+ → detected

Table 2: Phytochemical Components/Constituents of Moringa seeds without Shells Extract

Constituents	Hexane Extract	Toluene Extract	Acetone Extract	Methanol Extract	Water Extract
Tannins	-	-	-	-	-
Phlobatannins	-	-	-	-	-
Saponins	-	-	-	-	-
Steroids	-	-	+	+	+
Flavonoids	+	+	+	+	+
Glycosides	-	-	-	-	-
Terpenoids	-	-	-	-	-
Alkaloids	-	-	-	-	+

Key - → not detected
+ → detected

Table 3: Nature and Yield of Extracts from seeds of Moringa oleifera (Lam) per 50 grams Samples + Shells

Extract Fraction	Nature of Extract	Extract Yield	Percentage Yield (%)
Hexane	Oil, greenish yellow Butterlike, viscous	10.97	21.95
Toluene	Oil (viscous) yellow Less viscous	2.74	5.48
Acetone	Crystal like oil Pellet-like	7.41	14.82
Methanol	Brown liquid Slightly oil, creamy	5.080	10.20
Water	Creamy liquid	23.6	47.2

Table 4: Nature and Yield of Extracts from Seeds of Moringa (Lam) Seeds without Shells per 50 gram Sample

Extract Fraction	Nature of Extract	Extract Yield	Percentage Yield (%)
Hexane	Oil, light brown Butter consistency	8.81	17.6
Toluene	Oil, light yellow Butter-like Consistency	8.71	17.5
Acetone	Pellet-like oils Butter consistency	12.78	25.6
Methanol	Brownish oil/greasy liquid	5.2	10.4
Water	Gummy, sticky Resinous, semi brownish liquid	23.6	47.2

Table 5: Inhibitory Effect of Moringa Extracts, Alum, and Chlorine on Aspergillus fumigatus isolate from waste water

Extracts Control	6mm Inoculums initially which is approx. 10,000 spores per mL													
	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aspergillus Fumigatus Control Culture Plate	6 mm	15	35	42	50	58	72	85 Size of Petri Plate						
	Profuse Sporulation													
Hexane Mo	6	6	6	6	6	6			No Germination of Spore nor Vegetative _____					
Methanol Mo	6	6	6	6	6	6								
Salt Mo	6	6	6	6	6	6								
Crude Mo Extracts	6	6	6*[1]	6	6	6								

Aqueous Sequential Mo Extract	6	6	6*[1]	6	6	6			
Aluminium Sulphate	6	15	34*[2]	42	50	56	85 mm		
Sodium Hypochlorite	6	13	34*[3]	42	50	56	85 mm		

*[1] → No spore germination
*[2] → Prefuse sporulation
*[3] → _____ sporulation

Plate.1 *Aspergillus fumigatus* control plate and plate showing inhibition by crude aqueous extract of Moringa oleifera seeds.

Plate 2: Confluent growth of *Aspergillus fumigatus* on plate treated with Chlorine.

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