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Short Communication

Production and Characterisation of Microencapsulated Particles of *Harugana madagascariensis*: a Natural Fungicide with an Improved Delivery Potential

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ABSTRACT

Invasive fungal infections (IFI) are caused by non-pathogenic fungi in the immunocompromised host. However, ethnobotanical information suggests that *Harugana madagascariensis* (HM-E) is one of the medicinal plants used to treat IFI in Southern Nigeria. This study aimed at evaluating the effect of HM-E and microencapsulated extract of *H. madagascariensis* (HM-M) on *Aspergillus niger* and *Rhizopus* spp as well as the influence of microencapsulation on the biological activity of HM-E. The microencapsulation was done using a modified coacervation process, antifungal screening was carried out using an agar disc model, characterization of the microparticles was done using Fourier-transform infrared (FTIR), Scanning electron microscopy (SEM), and Energy Dispersive X-ray (EDX). The encapsulation efficiency of HM-M was observed to be 72.45 with a solubility value of 97.95%. SEM showed that HM-M particles exhibited relatively similar sizes, an irregular surface structure, and deep depressions in the walls. EDX analysis of HM-E at spot 1 revealed the presence of oxygen and carbon. Also, *A. niger* was resistant to HM- E and HM-M at 0.2 and 0.4 mg/mL, while no growth of *Rhizopus* spp was observed with HM-E and HM-M at 0.2 and 0.4 mg/mL in a concentration-dependent manner. In conclusion, *Rhizopus* sp was observed to be sensitive to HM-E and HM-M at both 0.2 and 0.4 mg/mL, in a concentration-dependent manner. The findings support the usage of HM and encourage microencapsulation of HM to improve drug delivery and acceptability in the treatment of *Rhizopus* sp-induced IFI.

Keywords: Antifungal, *Aspergillus niger*, *Harugana madagascariensis*, Invasive fungal infections, *Rhizopus* spp.

Introduction

A notable rise in invasive fungal infections (IFI) has been described to be associated with immunocompromised individuals.[1,2] Approximately 7% (611,000 species) of all eukaryotic species on earth are fungi, however only about 600 of these species cause diseases in humans and animals[2–4]. Fungi are extensively found in soil, plant residue, and other organic substrates.[5] Infections caused by fungi can spread through the following routes: ingestion of contaminated food or drink, penetration of the mucosa by commensal organisms like *Candida albicans*, inhalation of spores (aspergillosis, cryptococcosis, histoplasmosis), percutaneous inoculation in cutaneous and subcutaneous infections (dermatophytosis, Madura's foot).[6–9] Opportunist invasive fungi infections such as dermatophytosis and tinea versicolor are minor but can lead to serious systemic illnesses that can be fatal, such as candidiasis, aspergillosis, and mucormycosis.[10]

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The *Aspergillus* infections of the central nervous system (CNS) have the greatest fatality rate, It is also known to be associated with extended neutropenia, haematological cancers, and immunosuppression after solid organ transplantation.[11,12] However, pulmonary and central nervous system Rhizopus infections are less frequent[12] The most well-known type of this infection includes; rhino cerebral mucormycosis. This infection is linked to diabetic ketoacidosis, It also has an extremely high death rate and is regarded as the most aggressive mold infection of the central nervous system.[13,14]

The management of IFI is complex and sometimes requires surgery. Early suspicion is crucial for effective treatment, as there are limited antifungal medicines available (such as Amphotericin B deoxycholate, Voriconazole, etc), with most of which have severe adverse effects (such as nephrotoxicity, hallucinations, hypoglycemia, electrolyte disturbance, and pneumonitis, etc) and certain organisms have also evolved resistance over time.[9,15] The limitations in the conventional model of IFI treatment have called for an alternative therapy, to complement the therapeutic strategies in the management of IFI. This alternative treatment mode involves medicinal plants with ethnomedicinal usage in the treatment of fungal-related diseases. Examples of such plants with reported efficacy and low toxicity include; *Xylotheca kraussiana*, *Olinia ventosa*, *Harpephyllum caffrum*, *Bucida buceras* (black olive tree), *Breonadia salicina*, *Vangueria infausta*, *Harungana madagascariensis* (HM-E), etc.[3,16]

These antifungal medicinal herbs have been linked to several drawbacks, such as the drug's rapid release, disagreeable taste, and odour, poor absorption, decreased bioavailability at the active site owing to degradation, etc.[17–19] These difficulties have resulted in

insufficient infection clearance, to address these, microencapsulation technique is utilised.[20,21] Microcapsules are commonly utilized in the preservation of significant bioactive chemicals, the addition of flavours to pharmaceuticals, and the regulation of medication release.[21,22] Different methods are used in the microencapsulation of drugs and foods, among which include; spray drying (a technique in which a feed solution, which is a mixture of the core material and the wall material is atomized and formed into a mist inside a chamber, where hot air is applied to convert the mist into powder), spray cooling (method of encapsulation is very similar to spray drying in operation, the major difference being the use of cold air in it), coacervation (a technique which involves the formation of a homogeneous layer of the polymeric wall material around the core material), fluidised bed coating (is an encapsulation method in which coating material is sprayed onto the fluidised core material), extrusion, emulsification, cyclodextrin inclusion.[23] The Coacervation method was utilised in this research because it is the most suitable encapsulation method for natural products. It also confers no changes in the chemical integrity of the active principles and materials are readily accessible. The agar disc antimicrobial sensitivity method was used in this research because the method is simple, reproducible, consistent, and reliable. Moreover, multiple antimicrobial agents can be tested on the same agar plate simultaneously. This research aims to investigate the fungicidal effect of HM on two main non-dermatophytic fungi (*Aspergillus niger* and *Rhizopus* spp), including the production and characterisation of microencapsulated particles of HM as well as comparative evaluation of the antifungal potential of HM-E and HM-M.

Materials and Methods

Plant collection and botanical authentication

The stems of *Harungana madagascariensis* (HM-E) were harvested from the wild (June 2022) around the Forestry Research Institute of Nigeria in Ibadan, Oyo State, Nigeria (GPS Coordinates: 7.3911, 3.8582. The plant was identified at the Forestry Research Institute of Nigeria (FRIN), Ibadan, where a voucher number (UBH-R613) was assigned and the specimen deposited.

Chemicals and equipment

Acacia gum (Nexira, France), Sodium sulphate pentahydrate (Henan Tianfu, Hong Kong), Analytical grade Ethanol (Pharmatrend, Nigeria), *Aspergillus niger* and *Rhizopus* species were isolated from the human hair purchased at Okada market (Edo State, Nigeria), Fluconazole (Omega Laboratories, Canada), Potassium bromide (Kishida, Japan), Sabourauds dextrose agar (SDA) (OXOID Hampshire, England), Scanning electron microscope (EOL JSM-65, PRIOR, UK), X-ray detector (EDAX DX-4 eDXi, Thermo Fisher, Portugal), Hot plate magnetic stirrer (TK23, Kartell, Italy), FTIR spectrometer (FT/IR-6300, Jasco, Thailand), Laboratory milling machine (SM-450C, Isreal), Rotary evaporator (Buchi R-300 Evaporator, Italy).

Plant extraction

The plant sample was allowed to air dry for two weeks at room temperature (25℃) in the laboratory. The dried plant material was ground to a powder using an electric mill and kept in an airtight container until needed. The plant was macerated in ethanol with intermittent agitation for 2 days, filtered using Whatman No. 1 filter paper, and the filtrate concentrated to dryness using a rotary evaporator under vacuum at 40℃. The crude extract obtained was weighed and placed in a desiccator to ensure the complete removal of moisture.[25]

Preparation and characterization of microcapsules

About 40 g of Acacia gum was gradually dissolved with 400 mL of hot water and stirred for two minutes on a hot plate magnetic stirrer (Model TK23, Kartell, Italy). The dispersion was allowed to stand and swell for an hour. The plant sample (40 g) and 40 mL of an anti-aggregating agent solution of Sodium sulphate were added to the swelling dispersion and agitated for two minutes on a hot plate. 80 mL of 100% ethanol (for coacervation) was administered drop-wise at a rate of 1 mL/min. The resulting solution was freeze-dried and ground to powder.[17,24]

Encapsulation yield

The entire weight of the microencapsulated product and the total weight of the microencapsulated material were determined using Equation (1) to get the percentage yield:

$$\% \text{ R (yield)} = \frac{Mw\ (g)}{Tw\ (g)}\ x\ 100 \quad (1)$$

Where:
Mw = Microencapsulated weight (g)
Tw = Total weight of microencapsulated material (g).[24]

Water solubility

Purified water (20 mL) was used to dissolve 1 g of microencapsulant, which was then filtered through weighted filter paper and heated to 105℃ for 30 minutes. Following the completion of the filtration process, the filter paper and the leftover components were dried in an oven for an additional hour at 105℃. After 15 minutes of cooling in a desiccator, they were weighed again and the water solubility index was calculated.[24]

Fourier transform infrared spectroscopy of samples

The plant extracts secondary metabolites and microencapsulated samples were examined for functional groups using Fourier-transform infrared (T/IR-6300, Jasco, Thailand) analysis. The FT-IR of the extract and microcapsules was determined using the KBr method. In this method, 5 mg of the sample was mixed with solid KBr, compressed into a disc and subjected to FT-IR analysis. The transmittance was measured between 1000 and 3500 cm^{-1}.[26]

Morphology analysis

Scanning electron microscopy (SEM) of samples

Scanning electron microscopy (SEM) analysis was done using a JEOL JSM-65 10LV type scanning electron microscope. The morphology of the plant metabolite components and control microspheres were analyzed. The process involved the application of platinum to the microcapsules. The resolution was set at 4.0 nm (30 kV) for the low vacuum (LV) mode, 3 .0 nm (30 kV) for the high vacuum (HV) mode, and 0.5 kV to 30 kV for the accelerating voltage range and images of the microcapsules were taken and analyzed.[27]

Energy Dispersive X-ray (EDX) of samples

The identical SEM apparatus (JEOL JSM-65 10LV type) that came with the EDAX DX-4 eDXi System, version 2.11 was used for the energy-dispersive X-ray micro-analytical investigations of the microspheres. An X-ray detector with an exceptionally narrow window was used to acquire EDAX spectra between 0 and 20 keV. A period of 120 seconds was used to gather the spectra.[27]

Antifungi assay

Culture of Fungal strain

Strains of *Aspergillus niger* and *Rhizopus* Spp were obtained from the human hair purchased from Okada market, Edo State, Nigeria, in June 2022. The fungi were grown at 25 °C on Sabourauds dextrose agar (SDA) (OXOID Hampshire, England). A cell suspension of the organisms (*Aspergillus niger* and *Rhizopus* species) equivalent to 0.05% Mcfarland standard was employed and the agar diffusion method was used for the analysis. Colonies of fungi were selected from a week-old agar plate culture. The top of each colony was touched with a sterile loop, and the growth was transferred into a tube containing 4 mL of normal saline and compared with 0.05% McFarland standard.[28]

Antimicrobial assay of non-dermatophytic moulds

The *in vitro* antifungal assay on the non-dermatophytic moulds was conducted using the agar diffusion method on a solid agar surface. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, 0.1 mL of the suspension was dispensed on the dried surface of the SDA plates. The dispensed inoculums on the dried surface of the SDA plates were evenly spread on its surface using

a hockey stick and holes of about 4 mm were bore on the solidified agar surface. Different concentrations of HM-E, HM-M, and standard antibiotics were filled in the holes on the agar plate, allowed to diffuse for 10 minutes, and then incubated. After 72 hours of incubation, each plate was examined for the zone of clearance, which was measured in millimetres (mm) with a transparent meter rule. Subsequently, fluconazole at 10 and 20 µg/mL was used as the positive control agent and incubated for 3-5 days. The experiment was carried out for each sample in triplicates[28,29]

Statistical analysis

Results were expressed as mean ± SEM and analyzed using GraphPad Prism software version 6 (United States of America). Comparisons were performed by a one-way analysis of variance (ANOVA) followed by the means multiple comparison method of Bonferroni–Dunnett. Differences were considered significant if $p < 0.05$.

Results and Discussion

With the widespread use of antibiotic drugs worldwide and the global increase in the number of immunodeficient patients, fungal infections have become a serious threat to global public health security.[30] Moreover, the evolution of fungal resistance to existing antifungal drugs is rising.[28] To address these issues, the development of new antifungal drugs or fungal inhibitors needs to be targeted urgently from non-toxic natural sources.[16,28] Plant secondary metabolites are characterized by various chemical structures, low price, high availability, high antimicrobial activity, and few side effects.[31] Therefore, plant secondary metabolites may be important resources for the identification and development of novel antifungal drugs.[16,28] This research addresses the potential use of medicinal plants, namely HM-E as possible sources of antifungal agents and probably a clue of increasing the acceptability and improving the delivery of HM-E *vis a vis* management of opportunist infection arising from fungi. One of the quantity metrics used to gauge a microencapsulation process' efficiency and effectiveness is yield. The percentage yield value of microcapsules from this study was 92.28%. The microencapsulated particle yield is influenced by the water content of the plant extract. The lower the water content, the lower the weight of water contained in the material. If water is removed, the material will be more compact and lighter, thus affecting the yield of the final product. In addition, the amount of extract that is successfully coated also greatly affects the yield value of microencapsulated products. The greater the amount of extract that is successfully coated, the greater the percentage value of the product (microparticles) yield. Based on these findings, the 92.28% yield suggests the presence of a low amount of moisture in the plant extract before the formulation procedure, and much of the HM-E extract was encapsulated with Acacia gum (Table 1). HM-E has biological properties with mainly antibacterial, antifungal, and antiviral effects.[32] Although little or nothing has been recorded about the microencapsulation of HM-E, poly (D, L-lactide-co-glycolide) nanoparticles (PLG-NP) of HM have been shown to potentiate the oral bacterial strains largely implicated in dental care and gingivitis infections.[33,34] Physical observation of the plant extract HM-E showed a deep brown colour, which turns light brown upon encapsulation. The reduction in the colour intensity was due to the presence of whitish Acacia gum that was added (Table 1).[34,35-40] Furthermore, the solubility impacts explain the water solubility potential of microencapsulated particles and their ability to release core in an aqueous medium.[34] However, good microencapsulated particles are expected to have a high aqueous solubility value. HM-E coated with Acacia gum dressing material had a microencapsulate solubility percentage in water of 97.95% (Table 1).[35-40] Also, the encapsulation efficiency of HM-M was observed to be 72.45%. The greater the value of microencapsulation efficiency, the lower the loss of compounds during the storage process. The effectiveness of the microencapsulation depends on various factors, such as polymer concentration and solubility and the solvent evaporation rate during the microencapsulation process.[34] The type of dressing material greatly affects the encapsulation efficiency. Acacia gum is a dressing material because it has good emulsifying properties and can form a coating due to its protein content (Table 1).[35-40] Using SEM (Scanning Electron Microscopy), the morphological structure of the HM-M produced from Acacia gum dressing material was observed at 1000 and 2000 magnification variations. Figure 1 below displays a picture of the microencapsulate morphological structure. Using Acacia dressing materials during the microencapsulation process resulted in particles of relatively similar sizes, an irregular surface structure, and deep depressions in the walls.

Table 1: Physicochemical assessment of *Harungana madagascariensis* microcapsules

Physicochemical parameters	**HM-M**
Colour	Brownish
Water solubility	97.95%
Microencapsulation efficiency	72.45%
Microencapsulation yield	92.28%.

The observed morphology might cause a reduction in the stability and also make HM-M more sensitive to oxidation reactions.[27]However, it should be noted that good microencapsulants should have a homogeneous and smooth surface with a slightly rounded shape with minimal folds and wall cracks. Microencapsulants with rough surfaces are more sensitive to oxidation reactions than those with smooth surfaces.[35-43] The FTIR spectra of Acacia gum (AG), HM-E, and HM-M, showed similar peaks at close regions indicating that proper encapsulation of HM-E with a matrix (acacia) to produce HM-M. Some of the observable peaks include the presence of a peak at 3250 cm^{-1} for HM-M and AG indicating the presence of hydrogen-bonded O-H alcohol/phenol functional groups. Peaks at 2926 and 2929 cm^{-1} were observed for HM-M and AG indicating the presence of O-H functional groups in carboxylic acid monomers or hydrogen bonds in carboxylic acids. A similar observation was shown with a peak at 2117 cm^{-1}, this peak was observed to be present in both HM-M and AG, which indicated the presence of the C≡C alkyne functional group. These peaks validated the presence of AG in HM-M. A similar observation was shown at peaks 2325 and 2344 cm^{-1} for HM-M and HM-E, and also a peak was found at 1602 cm^{-1} for both HM-M and HM-E indicating the presence of C=C alkene functional groups. These observations showed that the compounds present in HM-M are also in HM-E, indicating proper encapsulation as shown in Figure 2.[38-43]

EDX analysis showed the elemental composition, element numbers, and the weight concentration of each element present in both HM-E and HM-M at two different spots, namely; spot 1 and spot 2 each as shown in Figure 3 below. HM-E at spot 1 showed the presence of oxygen and carbon, with atomic weights of 81.34 and 18.66, respectively. The weight concentrations of the organic elements were observed to be 85.31 (oxygen) and 14.69 (carbon) as shown in spot 1 of HM-E view (Figure 3A). Moreover, spot 2 of HM-E showed the presence of carbon solely, with atomic and weight concentrations of 100 each (Figure 3B).[36-43]

Moreover, upon encapsulation with Acacia gum (HM-M), the presence of organic elements such as oxygen and carbon was noted, and non-organic elements such as sodium and sulfur were also observed. Atomic concentrations of 70.85, 13.78, 11.91, and 3.46 were observed for oxygen, carbon, sodium, and sulfur present in the spot 1 of HM-M, accordingly. Atomic concentrations of oxygen and sulfur were noted to be the highest and the lowest, respectively, while oxygen also showed the highest weight concentration of 67.32 as shown in spot 1 of HM-M (Figure 3C), the observed non-organic elements present in HM-M might be as a result of the acacia matrix utilised in the encapsulation process.

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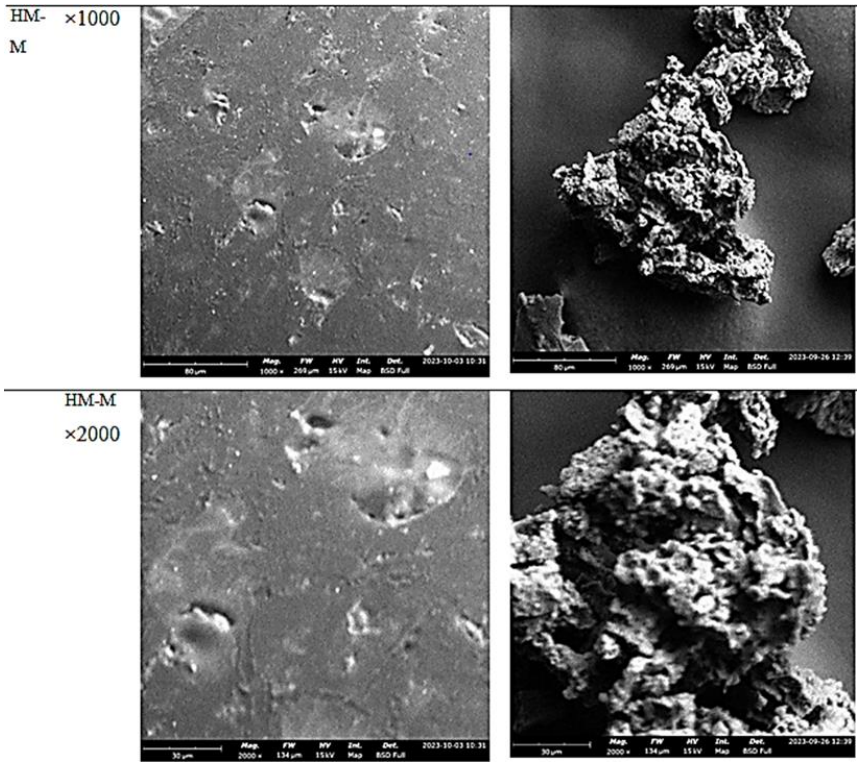


Figure 1. SEM analysis results of HM-M at ×1000 and ×2000 magnifications

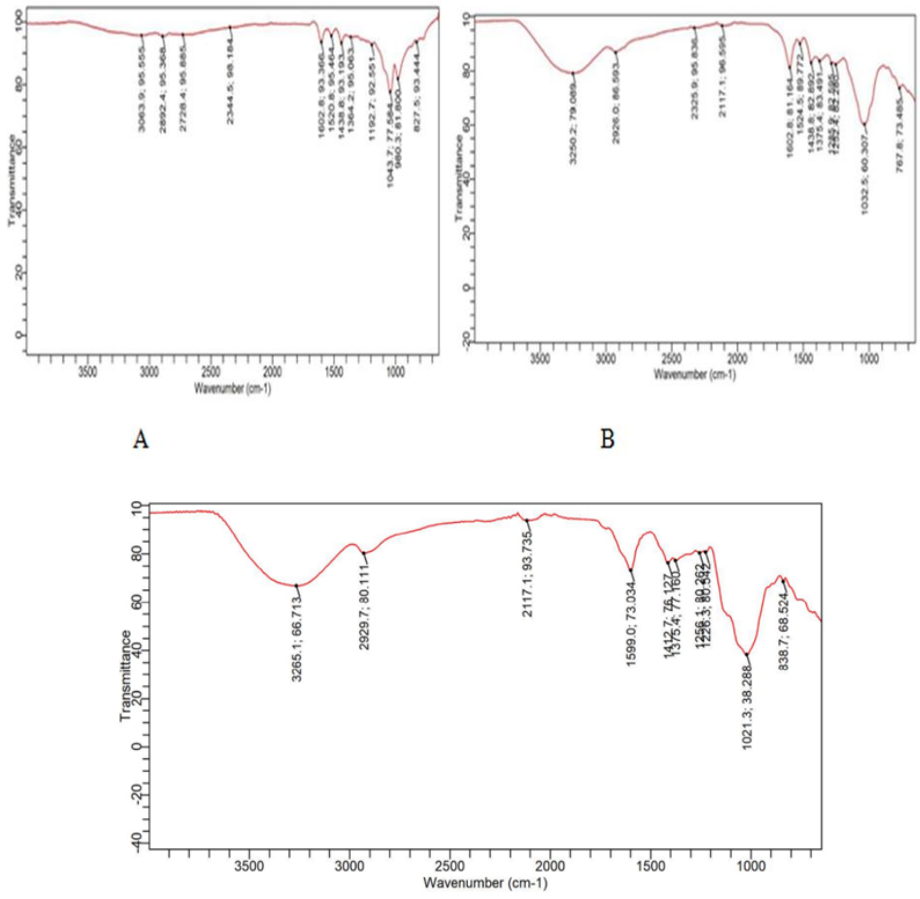
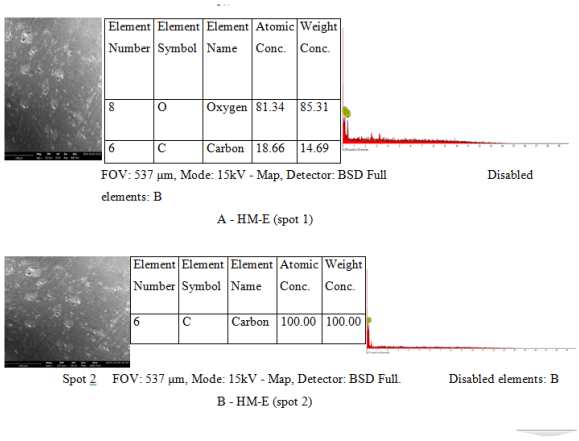


Figure 2: IR spectrum of microencapsulant HM-E (A), HM-M (B), and Acacia (C).

Moreover, in spot 2 of HM-M, oxygen showed atomic and weight concentrations of 86.03 and 89.13, respectively. The observed values were abundant in spot 2 relative to the carbon of 13.97 and 10.87 values in their atomic and weight concentrations as shown in Figure 3D.[36-43] *Aspergillus niger* and *Rhizopus* spp are pathogenic fungi that can induce disease conditions in immune-comprised humans.[13,37,38] Aspergillosis is a fungal infection caused by spores of indoor and outdoor *Aspergillus* mould species. Due to the ubiquitous nature of *A. niger*, its spores are commonly inhaled by humans from their surrounding environment. Aspergillosis infection customarily occurs in people with compromised immune systems or pre-existing lung conditions like asthma and cystic fibrosis.[11,14] Rhizopus, on the other hand, can cause localised and disseminated mucormycosis.[35] Sinusitis and pneumonia are the most common types of infection with dissemination prevalent in patients with underlying disease.[35,36]



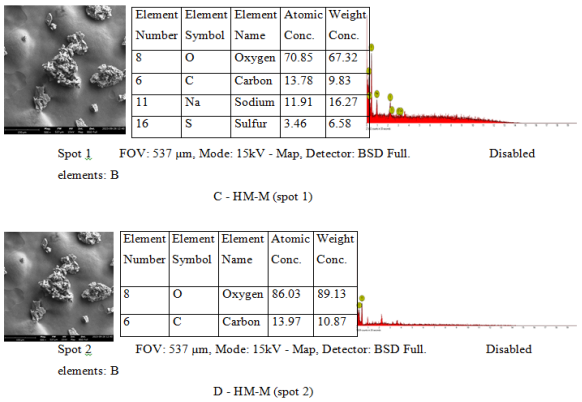


Figure 3: EDX profiling of HM-E and HM-M at two different spots (Spot 1 and Spot 2 each)

The analysis of the HM-E and the HM-M against the two non-dermatophytic moulds (Table 2) reveals that at concentrations of 0.2 and 0.4 mg/mL of both HM-E and HM-M, *Aspergillus niger* was resistant when compared (p<0.05) to the results of fluconazole with a zone of inhibition of 35 mm against *Aspergillus niger*. On the other hand, *Rhizopus* species was susceptible to the two samples (HM-E and the HM-M) both at concentrations 0.2 and 0.4 mg/mL. Furthermore, the analysis showed that after 4-5 days of the antifungal susceptibility testing, there were still no visible colonies of the *Rhizopus* species on the Sabourauds dexterous agar (SDA) plate. This could be attributed to the fact that the samples (HM-E and HM-M) have antimycotic activities or were effective against the non-dermatophytic mould, Rhizopus, as no growth of *Rhizopus* species was also seen on the agar plate when fluconazole was used as control drug against the fungi after 4-5 days of inoculation on the SDA plate. From our findings, it was noted that HM-M exhibited potent activity against *Rhizopus* species, which agrees with the findings of Ezekiel *et al*.[44] The activity of HM-E was retained upon encapsulation, and an increase in the concentration of the crude drug was observed to be linked to an increase in antifungal activity against Rhizopus species as shown in Table 2.

Table 2: Fungicidal effect of HM-E and HM-M on *Aspergillus niger* and *Rhizopus* Spp

Sample	*Aspergillus niger*		Rhizopus Spp	
	0.2 mg/mL	0.4 mg/mL	0.2 mg/mL	0.4 mg/mL
H. magadascariensis extract (HM – E)	R	R	S1	S1
H. magadascariensis microparticles (HM-P)	R	R	S1	S1
Fluconazole (Positive control)	35 mm	35 mm	S1	S1

Conclusion

The study revealed that HM-E and HM-P showed little or no effect on *A. niger* even upon encapsulation. Moreover, *Rhizopus* sp was observed to be sensitive to HM-E at 0.2 and 0.4 mg/mL. Microencapsulation of HM-E to produce HM-M was done to improve the delivery and acceptability of HM-E, and HM-M was observed to retain the integrity of the phytoactive constituents in HM-E which may have been responsible for the observed activity. The activity of both HM-E and HM-P was concentration-dependent, with the highest activity observed at 0.4 mg/mL. It can be concluded that HM-E can be used to manage opportunistic infections arising from *Rhizopus* sp in immune-compromised individuals. The delivery and acceptability of the crude drug (HM-E) can also be successfully improved by microencapsulation using commercially available acacia gum. Future research should focus on the identification of the bioactive principle(s) in HM-E.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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