

Original Article

Antifungal Activities of *Ziziphus mauritiana* against *Candida albicans*: In Vitro Study

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Abstract

Medicinal plants have been discovered and used in traditional medicine and pharmaceutical industries since centuries. In the current study, *Ziziphus mauritiana* leaves was used as it is rich with many biological active compounds such as flavonoids, polyphenols, saponins and tanins. Previous studies reported the antibacterial and antifungal effects of *Z. mauritiana* towards various microorganisms. However, the antifungal activities of *Z. mauritiana* methanol extracts on *Candida albicans* (Clinical and American Type Culture Collection Strains) have not been discovered extensively.

Objectives: Therefore, the aim of the current study is to investigate the antifungal activity of *Z. mauritiana* leaves methanol extracts against *C. albicans* ATCC strain and clinical isolate (from oral cancer patient).

Methods: Antifungal susceptibility test (AST) was performed using disc diffusion, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) to determine the antifungal activity of methanol leaves extract of *Ziziphus mauritiana* against *C. albicans* ATCC and clinical isolates.

Results: The results obtained showed that there is no zone of inhibition seen from disk diffusion test for both strains. However, the minimal inhibitory and minimal fungicidal concentration showed that *Ziziphus mauritiana* methanol extracts was able to inhibit *C. albicans* clinical isolate but not ATCC strain at 500mg/mL.

Conclusion: The finding of this study suggests that that *Ziziphus mauritiana* leaves methanol extract showed promising results against *Candida albicans*. Thus, it can be used as a source for functional ingredients for pharmaceutical drug industries in-order to reduce or inhibit oral fungal infection.

Keywords: Antifungal, *Ziziphus mauritiana*, *Candida albicans*, plant extract

Introduction

Fungal infections are common throughout much of the natural world. In humans,

fungal infections occur when fungus invades our body and the immune system unable to destroy the fungus. Fungal infections are often caused by fungi that are common in the environment. One of the most common fungus is known as *Candida albicans* which normally lives as normal flora in human body, cause no harm and has symbiotic relationship with the host. According to Singh et al. (1), about 30% to

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50% human population carries *C. albicans* as their normal flora. However, under certain conditions, it becomes an opportunistic pathogen and can quickly transform from a harmless inhabitant of mucocutaneous tissues to a highly pathogenic organism capable of killing its host under the appropriate conditions. It can be harmful in some cases, such as imbalance of immune defence system, due to trauma or post-surgery. It may cause mucosal infection in healthy individual or produce systemic infection. In the oral cavity it is commonly associated with denture-related stomatitis and oral candidiasis. According to A Akpan et al. (2), oral candidiasis is an opportunistic infection of oral cavity. It appears as whitish to yellowish patches in the oral mucosa and usually predispose to elderly who wears denture, immunocompromised individual, poorly controlled diabetes mellitus, patients on corticosteroids or psychotropic therapy and those with impaired salivary gland. The risks also increase if patient does not take good care of the oral and denture hygiene. As claimed by A Akpan et al. (2), the incidence of *C. albicans* isolated from the oral cavity has been reported to be 45% in neonates, 45%–65% of healthy children, 30%–45% of healthy adults, 50%–65% of people who wear removable dentures, 65%–88% in those residing in acute and long term care facilities. 90% of patients with acute leukaemia undergoing chemotherapy, and 95% of patients with HIV.

Oral candidiasis, if left untreated may cause pain and discomfort, burning sensation and altered taste sensation. In some extreme cases, if the infection invades until the bloodstream, it may lead to severe infection and can cause fatality. Oral candidiasis is usually treated by topical antifungal medication such as

Nystatin, Amphoterecin B and Miconazole together with chlorohexide 0.12% mouthwash Pina G et al. (5). Chlorohexidine has antimicrobial properties and has been extensively used in medical and dental field. In dental field, chlorohexidine is commonly prescribed to patient with periodontal disease as antiplaque agent. It also used as irrigation material in root canal treatment due to its broad spectrum antibacterial and antifungal properties. However, chlorohexidine has its disadvantages which are brownish staining of the teeth and alteration of the taste sensation. In a study done by Lee JA et al. (3), a short term of salty taste impairment was observed with the use of 0.2% aqueous chlorohexidine solution. So, the ideally usage of chlorohexidine is 0.12% (<https://www.drugbank.ca/drugs/DB00878>). Plants are considered as natural products that has being widely used to treat various systemic and local diseases since centuries. Medicinal plants have attained significant source of potentially new chemotherapeutics regimen. Jujube or Bidara is a common name for *Ziziphus mauritiana*. It belongs to the genus *Ziziphus* and *Rhamnaceae* family. It has many benefits due to the presence of biological active compound in the leaves.

The therapeutics potential of *Z. mauritiana* has being reported in numbers of previous published scientific papers. It is used to treat various form of systemic (i.e. gastrointestinal and genitourinary tract related disease, diabetes, bronchitis and anaemia) and local diseases (i.e. skin infections, diarrhoea and fever) stated by Mishra et al. (4). Nonetheless, the leaves extracts contain antimicrobial or antibacterial potential against *S. aureus*, *B. cereus*, *S. pneumoniae*, *C. albicans*, *B. subtilis*, *P. vulgaris* and *E. coli* [Abdallah et al. (6); Ghasham A A et al. (7)], antioxidant effect [Abdallah et al. (6); Ghasham A A

et al., (7)], and treatment for ulcer (Siddharth P et al., (8)). However, to the best of our knowledge and extensive literature review search, the antifungal efficacy of methanol extract of *Z. mauritiana* on *C. albicans* have not yet been investigated extensively. Therefore, in this current study, we investigated the antifungal efficacy of methanol extract of *Z. mauritiana* on *C. albicans* strains. This study aims to reduce the usage of synthetically derived antifungal by determining the antifungal activities of the methanol leave extract of *Z. mauritiana*.

Material and Methods

Plant extract

Ziziphus mauritiana leaves powder was purchased from local supplier.

Preparation of plant extract

First, 100g of the *Ziziphus mauritiana* leaves powder were weighted using electronic weighing machine. Then it was soaked in 500mL of methanol in a screw cap bottle covered with aluminum foil at room temperature on a shaker for 3 days. The solutions were then centrifuged at 9000rpm for 10 min using high speed centrifuge machine in-order to separate the sediment and the supernatant. The supernatant was filtered using Whatman filters paper No. 1 to get a clear filtrate and placed it into 50mL Falcon tube. The solvent was removed from the extract by using rotary evaporator. After that, it was stored in -80°C freezer for overnight prior freeze drying process. Eventually, the filtrate was freeze-dried up to 3 days until it became crystalized and finally the freeze-dried sample was stored at 4°C for further analysis.

Fungal strains

The fungal used in this study was *C. albicans* from American Type Culture collection (ATCC) strain. The clinical strain was isolated from oral cancer patient, donated by Dr Mohd Hafiz Arzmi, from Kulliyyah of Dentistry, IIUM Kuantan. Both strains were kept in glycerol (25%) at -80°C freezer.

Preparation of media

a) Sabouraud dextrose agar (SDA)

65g of SDA powder (Oxoid) was suspended in 1 litre of mili-Q water. The suspension was mixed together using magnetic stirrer and boiled using hot plate until it dissolved completely. Then, it was autoclaved at 121°C for 15 minutes. The sterilized agar was then poured into sterilized petri dishes and let it solidified inside biological safety cabinet (BSC). The plates were allowed to sterilize under Ultraviolet (UV) for 30 minutes before placed it inside sterile plastic bag and stored at 4°C chiller for further use.

b) Sabouroud dextrose broth (SDB)

65g of SDB powder was suspended in 500mL of mili-Q water. The suspension was mixed together and boiled using hot plate and a magnetic stirrer until it dissolved completely. Then, it was sterilized by autoclaving at 121°C for 15 minutes. The broth then dispensed into sterilized universal bottles. Finally, the broth was stored at 4°C chiller for further use.

Preparation of fungal suspension

C. albicans ATCC and clinical isolate obtained from glycerol stock (-80 °C) were subcultured on Sabouraud dextrose agar (SDA) and incubated at 37°C for 18-24 hours. On the next day, colony morphology of the fungal tested were observed. Single colony from both cultures were taken for

Gram staining in-order to observe the cell morphology and also to ensure that the working cultures are not contaminated with other microorganism. Single colonies were also taken from both strains and inoculated in Sabouraud dextrose broth (SDB) for the preparation of fungal suspension.

Both fungal suspensions were incubated at 37°C for 18-24 hours. On the next day, the optical density (OD) reading at 625nm wavelength were adjusted for both cultures prior antimicrobial assay. The OD reading for both cultures were standardized between 0.14-0.2 which are equivalent to 1.5×10^6 - 1.5×10^8 colony forming units (CFU)/mL for disc diffusion assay and minimal inhibitory concentration (MIC) respectively.

Antimicrobial assay

The antimicrobial assay of the methanol extracts of *Ziziphus mauritiana* leaves towards *C. albicans* were evaluated by antimicrobial susceptibility test (AST), minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC).

Antifungal sensitivity test (AST)

Antifungal assay of methanol extract of *Ziziphus mauritiana* leaves were tested using disc diffusion method for both *C. albicans* ATCC strain and clinical isolate. SDA media was used for AST test throughout the study. 1gm/mL and 500mg/mL of *Ziziphus mauritiana* leaves methanol extract were used for AST test. SDA plates were divided into 4 quadrants and labeled with positive control, negative control, 500mg/mL and 1g/mL for each quadrant respectively. Fungal suspension as prepared in the previous paragraph (ATCC strain and clinical isolate; 1.5×10^6 CFU/mL) were spread on SDA media using sterilized metal loop. Once the fungal spread dried, four wells were prepared

using cork borer size 6 mm in diameter to deposit the test samples. Freeze-dried methanol extract was used for the preparation of the stock. 1gm/mL of the freeze-dried extract was prepared in 1mL of 10% DMSO. The respective concentration of 500mg/mL was prepared by diluting the stock solution in two fold. Filtered chlorhexidine digluconate (0.12%) and 10% DMSO were loaded into the plate as a positive and negative control respectively. DMSO percentage used for this study was determined after the minimal inhibitory concentration test was conducted using a serial dilution of DMSO (starting from 100%) against both fungal strains tested. The plates were incubated at 37 °C for 18-24 hours. The diameter inhibition zone was measured in mm unit to evaluate the antifungal activity and the test were conducted in triplicate for each isolate. Aseptic technique was applied throughout the procedures.

Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) assay is used to identify the lowest concentration of the test sample to inhibit the visible growth of microorganism after 18-24 hours of incubation. In the current study, MIC assay was conducted using sterile 96 well microplates. The wells were labelled from well number 1 until well number 12. Firstly, 100µL of 1gm/mL methanol extract prepared in 10% DMSO was pipetted into the first well of each row as a starting concentration for MIC. 100µL of Sabouroud dextrose broth was pipetted into well number 2 up to well number 12 (from row A,B,C,E,F,G,H). After that, a two-fold serial dilution was performed by pipetting 100µL of the methanol plant extract from well number 1 into well number 2. The dilution was mixed well by up and down technique and this process repeated until well number 10. The

remaining 100µL of the extract dilution was discarded. Wells at row D left as a blank. Well number 11 pipetted with DMSO 10% (negative control) and well number 12 with 0.12% chlorhexidine (positive control). Then, 5µL of the standardized fungal suspension (as described in previous paragraph) was pipetted into well number 2 until well number 12. The same procedures were repeated for the blank but with the absence of fungal suspension. 100µL of Sabouroud dextrose broth was pipetted in well number 2 up to well number 10. 100µL of 1gm/mL methanol extract was pipetted into the first well for row A, B and C. Then, two-fold serial dilution was repeated same as previous microplates. These extract dilutions act as a control to be subtracted with the fungal-containing microplates to get the OD reading of the fungal itself by using microplates absorbance reader. All the test plates then incubated at 37°C for 18-24 hours. The growth of the tested fungal were observed on the next day. The well with the lowest dilution with no obvious growth of fungal by the naked eyes were considered as MIC value.

Minimal fungicidal concentration (MFC)

MFC is defined as the lowest concentration of antifungal that prevents the growth of fungal after subcultured on to antifungal-free media. Samples from MIC assay were used for MFC assay. Sample from each well that showed complete inhibition (optically clear well) same as positive control well were inoculated on sterilized SDA media. The SDA plates were divided into four quadrants and labelled. The aseptic technique was applied throughout the procedures. The SDA plates were incubated at 37°C for 18-24 hours. The quadrant with lowest concentration of the extract showed no fungal growth was considered as MFC.

Results

Antifungal Susceptibility Test (AST) of Methanol Extract of *Z. mauritiana* Leaves Against *C. albicans*.

Antifungal assay was performed using disc diffusion method for *C. albicans* ATCC and clinical isolate strains respectively. Chlorhexidine (0.12%) and DMSO (10%) were used as positive control and negative control respectively. No clear zone of inhibition were observed in the presence of 500 mg/mL and 1 g/mL of methanol extract of *Z. mauritiana* leaves for both strains (Figure 1 and Figure 2). Table 1 and Table 2 shows the measurement of the zone for all tested samples. No clear zone were observed for negative control, 500mg/mL and 1gm/mL for both strain tested except for chlorhexidine (0.12%), which is the positive control with 26.667mm ± 0.653 diameter of inhibition zone.

Minimum Inhibitory Concentration (MIC) of Methanol Extract of *Ziziphus mauritiana* Leaves Against *C. albicans*.

MIC test was performed in-order to obtain the bacteriostatic concentration of the methanol extract of *Z. mauritiana* leaves against both *C. albicans* strains using 96 well microplates. The results are shown in Figure 3 and summarized in Table 3. Based on the observation for ATCC strain (row A, B and C), all well starting from well number 2 until well number 10 shows turbidity which indicate the presence of fungal. This observation comparable to well number 11 (control negative). While, for clinical strain, well number 2 for row E, F and G did not show fungal sedimentation at the bottom of the plate or turbid broth which is similar to control positive well (well number 12). However, turbidity were observed for well number 3 until well number 10 which similar to well number 11 (control negative).

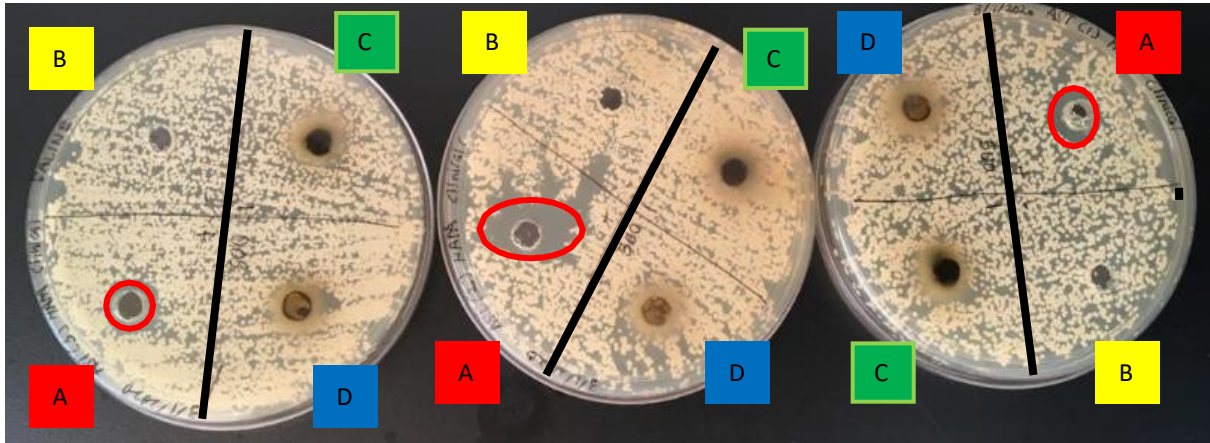


Figure 1: Antifungal susceptibility test of the methanol extract of *Z. mauritiana* leaves against *C. albicans* clinical strains. The Image shows the experiment done in triplicate for different concentration with the presence of positive and negative control.

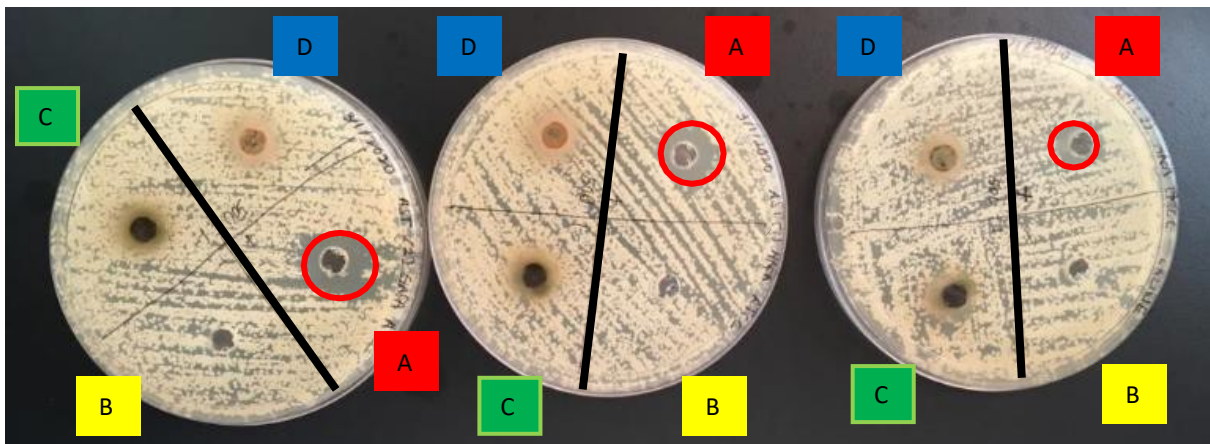


Figure 2: Antifungal susceptibility test of the methanol extract of *Z. mauritiana* leaves against *C. albicans* ATCC strains. The image shows the experiment done in triplicate for different concentration with the presence of positive and negative control

- Indicator:
- A = 0.12% Chlorhexidine as positive control
 - B = 10% DMSO as negative control
 - C = 1g/mL methanol extract of *Z. mauritiana* leaves
 - D = 500mg/mL methanol extract of *Z. mauritiana* leaves

Organisms	Well		Zone of Inhibition (mm)			
			Plate 1	Plate 2	Plate 3	Mean (SD)
Clinical <i>C. albicans</i>	Positive Control	CHX	26	27	27	26.7
	Negative Control	10% DMSO	0	0	0	0
	<i>Z. mauritiana</i>	500 mg/ml	0	0	0	0
		1000 mg/ml	0	0	0	0

Table 1: Antifungal susceptibility test analysis showing zones of inhibition (mm) around the extracts at varying concentrations towards clinical strains of *C. albicans*.

Organisms	Well		Zone of Inhibition (mm)			
			Plate 1	Plate 2	Plate 3	Mean (SD)
ATCC <i>C. albicans</i>	Positive Control	CHX	26	27	27	26.7
	Negative Control	10% DMSO	0	0	0	0
	<i>Z. mauritiana</i>	500 mg/ml	0	0	0	0
		1000 mg/ml	0	0	0	0

Table 2: Antifungal susceptibility test analysis showing zones of inhibition (mm) around the extracts at varying concentrations towards ATCC strains of *C. albicans*.

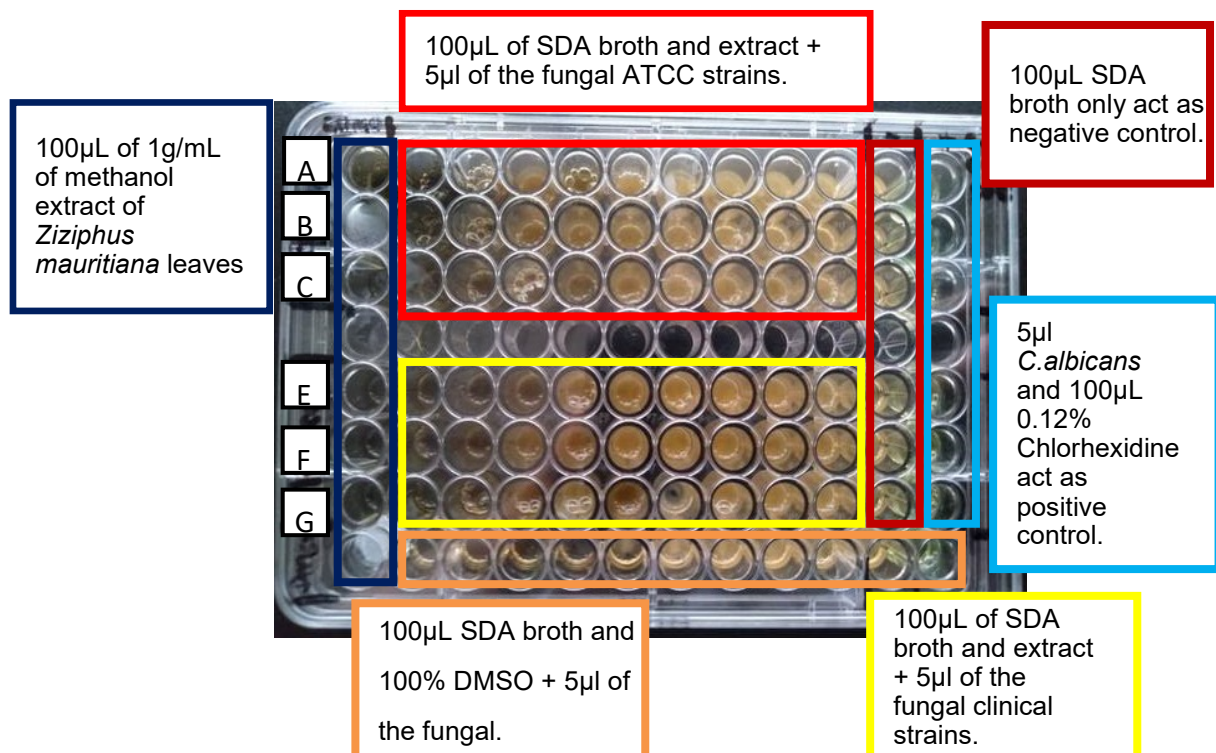


Figure 3: MIC results of *C. albicans* observed through naked eyes from 96 well microplate

Plant extracts	Organism	Concentration of methanol extract <i>Z. mauritiana</i> (mg/ml)								
		500	250	125	62.5	31.25	15.53	7.81	3.91	1.96
<i>Z. mauritiana</i>	Clinical <i>C. albicans</i>	C	T	T	T	T	T	T	T	T
		C	T	T	T	T	T	T	T	T
		C	T	T	T	T	T	T	T	T
	ATCC <i>C. albicans</i>	T	T	T	T	T	T	T	T	T
		T	T	T	T	T	T	T	T	T
		T	T	T	T	T	T	T	T	T

Key: C = Clear

T = Turbid

Table 3: Minimum inhibitory concentration (MIC) result of methanol extract of *Ziziphus mauritiana* leaves against *C. albicans*.

Minimum Fungicidal Concentration (MFC) of Methanol Extract of *Z. mauritiana* Leaves Against *C. albicans*

Minimum fungicidal concentration was performed in-order to obtain bactericidal concentration of the methanol extract of *Z. mauritiana* against both *C. albicans* strains. MFC test was performed right after MIC test was completed. Samples from well 2 until well 12 were inoculated on a fresh media to detect any growth of the fungal. After incubated 18-24 hours, the results were observed on the media. In the current study, MFC test was performed not only for the well that is clear until the first well that showed turbidity but for all wells (well number 2 until well number 12) in-order to reconfirm the inhibition of fungal growth. The extract against *C. albicans* clinical strains showed inhibition of fungal growth only from the sample inoculated from well 2 for row F and G except for well 3 from row E (Figure 4). No inhibition were observed for *C. albicans* ATCC strains from row A, B and C (Figure 5). Based on both tests, MBC and MIC values are comparable at the concentration of 500 mg/mL against clinical strain of *C. albicans* and no inhibitory effect of *Z. mauritiana* was observed against *C. albicans* ATCC strains as interpreted in the Table 4.

Discussions

Medicinal plants have been used widely around the world since ancient time for the treatment of various diseases. World Health Organization reported that about 80% of the total population in the developing country depend on medicinal plant for primary health care needs (Oyebode O et al, (9)). Its popularity increases due to their availability, low cost and less side effect compared to the synthetically derived drugs. Medicinal plants also known to have varieties of active ingredients such as phenol, flavonoids, tannins and many more that shown to confer protection against various ailments. Thus, it plays a vital role in drug development. Therefore, the search for exploitation of natural products especially of plant origin, has greatly increased in recent years. In the current study *Z. mauritiana* or commonly known as 'Bidara' in Malaysia, was chosen due to its potential and phytochemical ingredient that benefit in treating numerous diseases. Besides that, *Z. mauritiana* are grown locally and we can easily purchase from local company in Malaysia. Previous scientific reports showed *Z. mauritiana* has antioxidant effect, antibacterial effect (Abdallah et al. (6); Ghasham A A et al. (7))

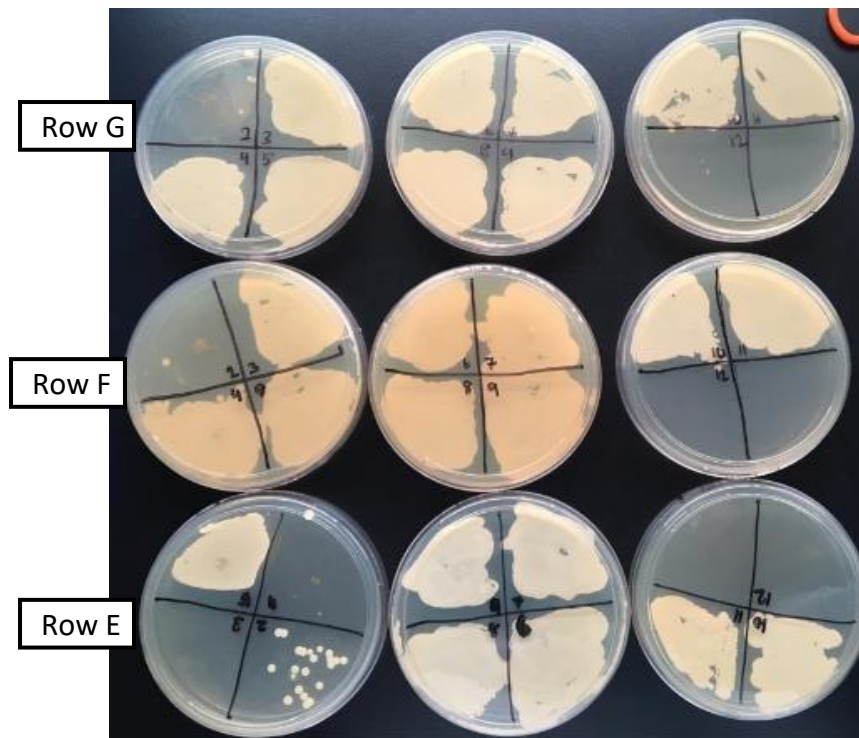


Figure 4: MFC of the methanol extract of *Z. mauritiana* leaves against *C. albicans* clinical strains.

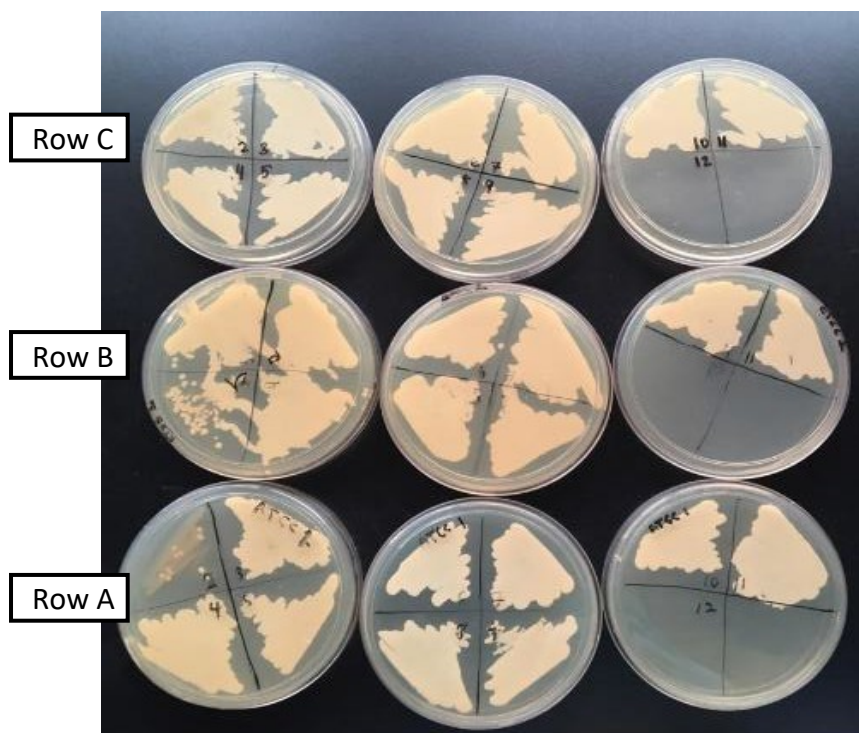


Figure 5: MFC of the methanol extract of *Z. mauritiana* leaves against *C. albicans* ATCC strains

Plant extracts	Organism	Concentration of methanol extract <i>Z. mauritiana</i> (mg/mL)								
		500	250	125	62.5	31.25	15.53	7.81	3.91	1.96
<i>Z. mauritiana</i>	Clinical <i>C. albicans</i>	+	+	+	+	+	+	+	+	+
		-	+	+	+	+	+	+	+	+
		-	+	+	+	+	+	+	+	+
	ATCC <i>C. albicans</i>	+	+	+	+	+	+	+	+	+
		+	+	+	+	+	+	+	+	+
		+	+	+	+	+	+	+	+	+

Key: - = No growth + = Growth

Table 4: Minimum bactericidal concentration (MBC) of methanol extract of *Ziziphus mauritiana* leaves against *C. albicans*.

and antiulcer effect (Siddharth P et al., (8)). However the antifungal activities of the leaves extracts have not been extensively investigated in Malaysia.

Therefore, the current study was conducted using the methanol extract of *Z. mauritiana* leaves which is mainly grown in Malaysia and investigation on the antifungal activities of the methanol leaves extracted towards the *C. albicans* ATCC and clinical strains was performed. The antifungal study was performed using disc diffusion test for both *C. albicans* strains (ATCC and clinical strains). Based on the result from the figure 9 and figure 10, the inhibition zone cannot be seen surrounding the well loaded with 500mg/mL and 1g/mL of methanolic extract of *Z. mauritiana* for both *C. albicans* strains used in the study. The inhibition zone only can be seen around the positive control which is chlorohexidine 0.12% solution. The current study is comparable with a study done previously by Abalaka et al. (10). They found that antimicrobial test

conducted analysis showed no inhibition zone seen when tested with *Z. mauritiana* leaves extract. However, the different between their study and the current study is we used methanol as solvent whereas they used ethanol in the preparation of the extracts at various concentrations. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) was conducted to determine the lowest concentration that inhibits and kills the fungal respectively. In the current study, MIC and MFC was done on both selected strains (*C. albicans* ATCC and clinical strains) using 96 well microplates. Based on the result summarized in table 3, the MIC and MFC of the methanol extracts against the *C. albicans* clinical strain was at concentration of 500mg/mL but showed no inhibition against *C. albicans* ATCC strains. It shows that methanol extracts of the *Z. mauritiana* has the ability to inhibit *C. albicans* clinical strain only, but not *C. albicans* ATCC strains. Probably higher

concentration is required in-order to inhibit *C. albicans* ATCC strain. 500mg/mL may not enough to disrupt the cell wall or the growth of the ATCC strain. Abalaka et al. (10), reported that there is no bacteriostatic and bactericidal effects shown for MIC and MFC against *C. albicans* strain used for their study. However another study conducted in India reported moderate activities of the ethanolic and methanolic extracts of *Ziziphus mauritiana* leaves on *C. albicans* tested by Sivasankri et al. (11). Whereas, study conducted in Saudi on methanolic extracts of *Ziziphus mauritiana* on *C. albicans* strains by Gasham A A et al, (7) showed significant level of antifungal effect with the tested concentrations. Variation in the previous and current study may be due to the origin the *Z. mauritiana* trees. *Z. mauritiana* grown in different geographical location may exhibit different antimicrobial effects due to the presence of different active compounds. Study conducted by Abalaka et al. (10) in Nigeria on phytochemical screening of *Z. mauritiana* leaves extract shows the presence of various bioactive compounds such as cardiac glycosides, polyphenols, resins, saponins and tannin. Another study done in Malaysia by Hasnah et al, (12) on the phytochemical screening found that *Z. mauritiana* contain alkaloids, phenols, tannins, flavonoids, and saponin when extracted using methanol as solvent. Another study on the composition of *Z. mauritiana* also done in Malaysia reported the presence of various nutrients such as crude protein, fat, carbohydrates and crude fibers (Jailani FNAM et al., (13)). The presence of various active compounds in the *Z. mauritiana* leave extracts have great potential in the treatment of various human diseases. However, further studies are required in-order to gain more information on the bioactive components and the effect of *Ziziphus mauritiana* leaves extracts on

oral fungal infection as there are not many studies reported for this organism either in Malaysia or other countries. In addition, more studies regarding the mechanism and mode of action of this plant extracts are also required.

Conclusion

In conclusion, the finding from the current study suggest that methanol extract of *Z. mauritiana* leaves has antifungal activity against the etiology agent of oral fungal infection mainly *C. albicans*. Thus, the information gained from this study, may be served as a source for development of new oral health care product especially in treating oral fungal infection. In addition, the result obtained from the current study can be used as reference by other researchers.

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