In vitro Growth Inhibition of *Candida albicans* Caused by Antifungal Properties of Miswak (*Salvadora persica* Linn.) Ethanolic Extract and Commercial Mouthwash

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Abstract

Aim: To investigate the *in vitro* antifungal activity of ethanolic extract of Miswak wood (to) compared with three commercial mouthwashes that contain alcohol, fluoride and povidine iodine.

Methods: Miswak wood was extracted by using sterilized distilled water and ethanol 96%. Before being used for testing, each crude extract was added with 0.5% Tween 80 to serve two ready-made solutions, each containing 300 mg/mL and 200 mg/mL. Miswak wood extracts and commercial mouthwashes were prepared at an initial concentration of 10% and were then diluted serially before being tested for antifungal properties to growth.

Result: The Miswak wood extract antifungal properties were performed on isolated from gargling water of volunteers. Miswak wood extract inhibited growth compared to the control solution (significantly different at 5% level test). Antifungal properties of Miswak wood extracts were not seen on subsequent dilution concentrations.

Conclusion: The ability of Miswak wood extracts inhibiting growth was lower than the three commercial mouthwashes tested. The commercial mouthwash with povidine iodine ingredient inhibited C. albicans growth better than the other two.

Key Words: Salvadora persica Linn., Miswak antifungal activity, Commercial mouthwash.

Introduction

Candida albicans yeast is a common microflora in the oral cavity. Some factors that could cause the *C. albicans* into pathogenic microbes include optimum temperature (37 °C), the presence of serum, a high pH environment and an adequate carbon source [1]. The yeast plays an important role in oral health; thus, its presence needs serious attention because the resulting infection can lead to oral thrush (oral candidiasis). Infection can be more severe when *C. albicans* penetrates the deeper tissues, especially for immunocompromised patients. Superficial infection may change to invasive (Invasive Fungal Infections, IFI) [2]. Some herbs have different contents of bioactive secondary metabolites, such as tannins, terpenoids, saponins, alkaloids, flavonoids and other compounds that have been reported to have the ability as an antibacterial and antifungal [3,4]. One of the plants that have the antifungal properties is Miswak wood (*Salvadora persica* Linn.). Miswak wood has been used for oral care and hygiene since centuries ago, especially by the Arabs. Until now, they are still using it as a tool in addition to oral hygienic toothbrush and toothpaste. A comparative study on dental care has been done on wood users and non-users of Miswak wood. It indicates that the Miswak wood user community spent lower dental expenses than non-user community. Miswak wood has also been scientifically proven to prevent tooth decay even in the absence of other tooth cleaning action [5]. Miswak wood has similar ability to modern toothbrush and commercial mouthwash solutions containing highly-concentration of trichlosan and chlorhexidine gluconate in plaque controlling [5,6].

World Health Organization (WHO) recommended and promoted the use of Miswak wood for oral health [7]. The mouthwash solution containing Miswak wood extract has been shown to prevent gingivitis and plaque, and reduce risk of teeth carries caused by cariogenic bacteria [8,9].

Aim

This research aimed to observe *in vitro* antifungal activities of ethanol extract of Miswak wood in inhibiting *C. albicans* growth and compared them with commercial mouthwashes.

Materials and Methods

Media and reagen

The Sabauraud Dextrose Broth (SDB) and Sabauraud Dextrose Agar (SDA), duck egg albumin, sterilized distilled water and ethanol 96% were used in this research.

Miswak wood powder

Miswak wood, obtained from Saudi Arabia, was cut into small pieces and crushed to obtain Miswak wood powder [10]. Miswak wood was extracted by using sterilized distilled water and ethanol 96%. Fifty grams of Miswak wood powder was mixed with 250 ml of extracted solution in sterilized dried bottles. Bottles were stored for nine days at 25-27°C and shaken at speed of 400 rpm. The solution was replaced every 24 hours and the supernatant was stored in other bottles at 40-60°C. The solution was weighed. The volume of each extract was reduced by evaporation at 35-38°C and the remaining solvent was allowed to evaporate by drying for 2-4 days at 25-

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27°C. Lastly, the solution was weighed again and stored in a dry place at 4°C until being used in tests.

Before being used for testing, each crude extract was added with 0.5% Tween 80 to serve two ready-made solutions, each containing 300 mg/mL and 200 mg/mL. These solutions were centrifuged 15800 *g* for 20 minutes at 10°C. Supernatant was sterilized using 0.2 mm filter. Each solution was diluted by serial dilution.

Tested commercial mouthwash solution

Three commercial mouthwash solutions were used in this research. The mouthwash solution 1 contained alcohol.

The mouthwash solution 2 contained fluoride. The mouthwash solution 3 contained Povidone iodine. The mouthwash solution was diluted two-fold serially from 1:10 to dilutions of 1: 2560 (v/v) using SDB. The negative control was SDB added with *C. albicans* containing no tested materials.

Tested *C. albicans*

The yeast of *C. albicans* was isolated from gargling water of volunteers using the sterilized water prior to brushing teeth in the morning. It was cultured on SDA to isolate *C. albicans*. The isolated suspect colonies were identified according to [10].

Experimental design

The experimental design was set based on previous experiment [5,10-13] with some modifications.

The Miswak wood extract solution and commercial mouthwashes were separately diluted by transferring one milliliter of each solution to be examined in nine milliliters of SDB and another in SDA. These additions made a 1:10 dilution (v/v). Similarly, the previously mix dilution was re-diluted to obtain the dilution of 1:20. This was repeated with similar patterns until the dilution reach to 1:2560 (v/v).

Ten microlitres of *C. albicans* inoculum solution was transferred into each test tube and the plates containing SDA. After it solidified, all the media were incubated at 37°C for 72 hours.

After the incubation period was achieved, the colonies were then counted visually on solid agar media. The yeast growth in broth media, were observed by UV-VIS spectrophotometer at a wavelength of 650 nm.

Result

After the volunteers gargled water cultured on SDA and incubated at 37°C for three days, *C. albicans* colonies started being suspected. Germ tube test was performed on these isolates by culturing the suspect colonies into duck egg albumin medium which was incubated for three hours at 37°C. At the end of the incubation period, germ tube was formed as shown in *Figure 1a*. The biochemical test, namely sucrose assimilation test, was performed on germ tube positive isolates. The positive results were obtained from the test due to the presence of visible fungal colony growth (*Figure 1b*). Yeast colony isolates were confirmed as *C. albicans* colonies based on the positive results of germ tube test and assimilation test.

The results of the antifungal activity of Miswak wood extracts and some commercial mouthwash solutions in inhibiting the growth of *C. albicans* are shown in *Table 1*.

Ethanol extracted from Miswak wood showed the ability to inhibit the *C. albicans* growth at 10% concentration when compared to controls (significantly different at level test of 5%). Similar results were also obtained by other researcher, who obtained any inhibition at the test concentration of 50% and 100% [14]. Although, the dilution of the ethanol extract from Miswak wood had different concentrations the result was similar inhibition.

Discussion

Miswak wood extracts have long been known to contain active ingredients that have antibacterial and antifungal capability; thus, they play a role in maintaining oral health. Some of the active ingredients include flavonoids, salvadorina, cyanogenic glycosides, lignans, saponins, alkaloids, tannin, linoleic acid, acidic acid sterarat, salvadourea, vitamin C, silica and some salts [15-17].

Acetone-extracted Miswak wood showed the antifungal ability of Miswak wood at a concentration of 300 mg/mL [18]. Miswak wood extracted with solvent water showed the antifungal ability at a concentration of 15% [19] and inhibited the growth of *C. albicans* with Minimum Inhibitory Concentration (MIC) of 6.25 mg/mL [5]. Alcohol-extracted Miswak wood inhibited *C. albicans* growth at concentration of 750 µg [20]. The results indicate that diluting the concentration of Miswak wood extract had no effect on the inhibition of *C. albicans* growth.

Antifungal properties of extract Miswak wood on the growth inhibition of *C. albicans* were not as good as the three commercial mouthwash solutions. Similar results were also obtained by other experiments [21]. The results showed mouthwash solution 3 containing povidone iodine was more effective to inhibit *C. albicans* growth than the other three groups.

The solvent solution influenced extracted active ingredient of Miswak wood [10]. Therefore, there was a difference in chemical content between the Miswak wood using ethanol, methanol, kloform, or others as a solvent. The strength of antifungal properties of Miswak wood extract may also be influenced by the pH of the extraction results. For example, solvent extraction using ethanol had the lowest pH but the opposite result would be obtained when using water as solvent. Ethanol was used as solvent in this research. Ethanol solvent extracted the active ingredients, such as tannins, polyphenols, poliacetilen, flavonols, terpenoids, sterols, alkaloids, and propolis [22]. Miswak wood extract contains tannins, flavonols, terpenoids, sterols and alkaloids [20,23,24].

Tannin belongs to the category of the polymeric phenol

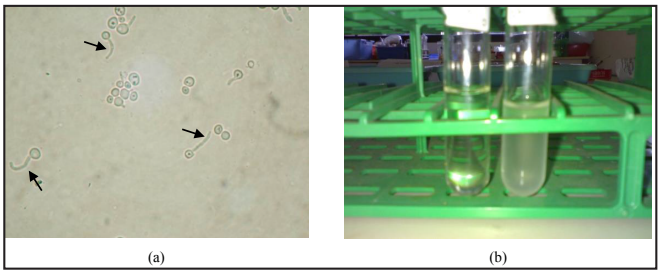

Figure 1. *The germ tubes were formed from the C. albicans cells (arrow) (a). The sucrose assimilation test was set up for C. albicans growth. The left was the control tube and the right tube was sucrose with the turbidity indicating growth of the yeast cells (b).*

Table 1. *Logarithmic growth of C. albicans in the Miswak wood extracts solutions and commercial mouthwash solutions.*

Tested material concentrations	Solutions (CFU/ml)			
	Miswak [x]	I [y]	II [w]	III [z]
0%	2.062 [a]	2.062 [a]	2.062 [a]	2.062 [a]
10%	0.690 [b]	-5.504 [h]	-2.660 [g]	-4.678 [h]
5%	0.691 [b]	-2.044 [fg]	-1.458 [ef]	-4.678 [h]
2.5%	0.693 [b]	-0.314 [d]	-0.857 [de]	-4.678 [h]
1.25%	0.697 [b]	0.551 [bc]	-0.556 [d]	-4.677 [h]
0.625%	0.705 [b]	0.984 [b]	-0.406 [d]	-4.674 [h]
0.313%	0.720 [b]	1.200 [b]	-0.331 [d]	-4.670 [h]
0.156%	0.751 [b]	1.308 [b]	-0.293 [cd]	-4.662 [h]
0.078%	0.812 [b]	1.362 [b]	-0.275 [cd]	-4.645 [h]
0.039%	0.935 [b]	1.389 [b]	-0.265 [cd]	-4.611 [h]

group. Tannins are formed from the condensation of flavan derivatives that spread to the woody tissue of plants. Tannins were extracted from Miswak wood can inhibit *C. albicans* by forming irreversible complexes with proline-rich proteins to inhibit the attachment of *C. albicans* to host mucosa [25]. Tannins will stimulate phagocytic cells and inactivate adhesins and enzymes [22]. Tannin may stimulate host tissue to excrete various proteolytic and lipolytic enzymes, such as lipases, phospholipase B and Secreted Aspartyl Proteinase (SAP) so that *C. albicans* penetration to host tissue may be inhibited [26]. Flavonols are phenolic structures containing one carbonyl group coupled with the 3-hydroxyl group. Flavonols are produced by plants with the presence of microbial infection. Therefore, flavonols showed good antimicrobial activity in *in vitro* studies. Flavonol will disrupt fungi membrane, forms a complex with the extracellular, and forms a complex with soluble protein [22]. Alkaloids are heterocyclic nitrogen compounds. Miswak wood contains alkaloids salvadorin [5]. But, it is still not clear how the salvadorin may against fungal infections. In general, groups of alkaloids have the ability to perform intercalation with DNA fungi [22].

Methanol extracted from Miswak wood contains other active ingredients, such as furancarboxaldehyde-5-2-(hydroxymethyl) furan-2-carboxylic acid-3-methyl-trimethylsilyl ester and D-erythro-2-deoxy-pentofuranose-1, 3,5-tris-O-(trimethylsilyl). The three furan derivatives contain hydroxyl groups which have high antioxidant capability with high levels of peroxidase, and catalase and low polifenoloksidase [27].

Miswak wood extracts showed different antifungal ability from Miswak leaf extracts. Miswak leaf extracts did not inhibit the *C. albicans* growth. However, the same extract could inhibit the growth of *Aspergillus niger*, *A. flavus* and *A. xylinium* [28].

Commercial mouthwash solution used in this research contained alcohol, fluoride and iodine povidin. Alcohol will control microbial biofilms production. Microbes produced acids, endotoxins and antigens, all of which were potentially damaging to the teeth and supporting tissues [29]. Alcohol can be used as a complementary therapy after mechanical removal of the biofilm by a dentist or used after brushing teeth. The structure of the bipolar configuration of alcohol played a role in dissolving the hydrophobic and hydrophilic components in fungal cells [30]. However, the use of commercial mouthwash containing alcohol should be avoided because a high concentration of alcohol has been proven to be able to cause lesions hyperkeratotic, both in humans and in laboratory animals [31]. Fluoride inhibited glycolysis and prevented the transfer of glucose into cells [32]. They denatured proteins and inactivated enzymes in the cell membrane that disrupted bacterial metabolism. Recent research on fluoride stated that fluoride had antifungal activity at concentration of 10-20 mg/mL [33].

Povidone iodine has been known to have anticandida and anti-adhesion activity to *Candida* and oral mucosa cells; thus, it could eliminate the *C. albicans* ability to penetrate into the epithelial cells of the oral cavity [34]. The research results showed mouthwash containing povidone iodine was more effective to inhibite *C. abicans* growth than others. Chlorhexidine is a popular ingredient of commercial mouthwash because they prevent dental plaque forming caused by bacteria. But, Miswak wood extract could inhibit bacteria growth more effectively than chlorhexidine. Miswak wood does not cause tooth discoloration, bad taste and burning in the oral cavity compared with chlorhexidine [35].

Conclusion

The research result concluded the ethanol extracted Miswak wood has antifungal activity against *C. albicans*. However, the activity was lower than commercial mouthwash.

Authors' Contribution

ESP contributed with the study design, acquisition, analysis and interpretation of data and took part in drafting of the manuscript. HPR contributed with the design, analysis and interpretation of data and took part in drafting of the manuscript. HSD contributed with the design, and analysis of data. All authors listed on the title page have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission.

Acknowledgements

The authors would like to thank to laboratory technicians for their assistance and kind cooperation throughout the experiment.

Conflict of Interest

None declared.

Sources of Funding

Nil.

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