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Evaluation of antibacterial activity of different Moringa oleifera leaf extract on Streptococcus mutans: An in-vitro antimicrobial study

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***Abstract*---**Dental caries, which has a complex etiology brought on by several facultative anaerobes, is a well-known serious oral health issue in the majority of developing countries. The primary pathogen linked to this illness is *Streptococcus mutans*. *S. mutans* species that are resistant to numerous commercial antibiotics have been found in dental caries patients. Multi-drug resistance (MDR) is a natural occurrence that poses a significant global hazard to human health. While there are several therapeutic options for treating or preventing dental decay, the worldwide burden of MDR-related diseases is continually growing. In order to evaluate the effectiveness of different *Moringa oleifera* leaf extract as a solvent to control *S. mutans* infection the current study was created. *S. mutans* was isolated and identified, then used the disc diffusion method to assess the isolates' susceptibility to antibiotics as well as different extracts. The results of a one-way ANOVA exposed significant (P< 0.001) differences between the various extract solutions and *S. mutans*. The highest level of efficacy among the three extract was ethanol extract on another hands the cold distal water extract was the lowest efficacy against *S. mutans*. *M. oleifera* solution with ethanol extract is more effective than the other extract types.

***Keywords*---**dental caries, Moringa oleifera, Streptococcus mutans.

Introduction

One of the most common chronic oral disorders affecting people globally is dental caries (Joury *et al.*, 2022). It may be uncomfortable and distracting, which negatively impacts the patient's quality of life. The World Health Organization estimates that dental caries affects roughly 75 percent of the world's population. Dental caries affects between 60% and 90% of schoolchildren and almost 100% of adults (World Health Organization [WHO] 2013). It is a serious health issue, especially for the less fortunate populations in both industrialized and developing nations who lack access to medical care. Bacteria, the type of food consumed, and the host's immunological response are the primary causes of dental caries (Harrison *et al.*, 2020; Gokkaya and Kargul, 2022). Microorganisms are crucial to its beginning and development. Several oral streptococci strains have been related to the development of dental plaque biofilms and the occurrence of cavities (Khere *et al.*, 2019). Bacterial biofilm can be survived on all the oral cavity surfaces, overall the teeth, mucosa and restorative materials. Cariogenic bacteria colonized and resulted in recurrent caries at tooth restorative interface (Fúcio *et al.*, 2016). *Streptococcus mutans* (*S. mutans*) is one of the cariogenic bacteria moreover it is initial primary reason of dental caries (Chavez de Paz, 2007). Different prevention and treatment strategies for dental caries have been developed as a result of extensive study in the field of modern dentistry (Ismail *et al.*, 2001; Llena and Forner 2008). The best treatment options are those that stop bacterial surface colonization and proliferation. There are effective plant extracts had antibacterial agents can do that, one of them, which had powerful antibacterial effect and prevent bacterial surface colonization and proliferation, is *Moringa oleifera* (*M. oleifera*) extracts (Al-Ghanayem *et al.*, 2022). According to several studies, *M. oleifera* Lam on both Gram-negative and Gram-positive bacteria works as an antibacterial agent (Abdalla *et al.*, 2016; Ervianingsih *et al.*, 2019 and Fouad *et al.*, 2019).

A significant plant that is utilised in several parts of the world for food and medicinal is *Moringa oleifera*, which is a member of the Moringaceae family. Due to its pharmacological effects and therapeutic qualities, such as its antioxidant, antibacterial, antidiabetic, and anticancer characteristics, to mention a few, it is known as a "wonder tree" or "Tree of Life" (Karthiga *et al.*, 2022). Although the plant's entire body is active, the leaves are thought to be the most active part because they contain a high concentration of bioactive substances like flavonoids and phenolic acid, which have a variety of pharmacological effects like antibacterial, antioxidant, antifungal, and anti-inflammatory properties (Ma *et al.*, 2020).

The Moringaceae family contains 13 species, with *M. oleifera* being one of the important perennial trees in tropical and subtropical areas (Sreeja *et al.*, 2021). More than 92 effective components, including alkaloids, tannins, flavonoids, saponins, triterpenoids, vitamins, beta carotin, amino acids, and ascorbic acid, are said to be present in *M. oleifera* (Deepa *et al.*, 2021; Yaseen and Takacs-Hajos, 2022). Effective chemicals are present throughout the entire plant (Biswas and Sinha, 2021) such as flowers (Javed *et al.*, 2021), leaves (Rashid *et al.*, 2021), fruits (Kumari *et al.*, 2021), immature pods (Boopathi and Raveendran, 2021), seeds (Kachangoon *et al.*, 2022), roots (Danbature *et al.*, 2021), and bark

(Kumbhare *et al.*, 2021) has a high potential for natural medicine to humans. Africans and Indians have utilised *M. oleifera* as a traditional herbal remedy; it can treat more than 300 ailments (Balusamy *et al.*, 2019). Every component of the *M. oleifera* plant was used for numerous medical conditions, such as anaemia (Suzana *et al.*, 2017), night blindness (Dubey *et al.*, 2013), malnutrition (Dhakar *et al.*, 2011), anti-cancer (Khor *et al.*, 2018), antioxidant (Verma *et al.*, 2009), breastfeeding in nursing mothers (Kiranawati and Nurjanah, 2014), and control of the urinary system (Maurya and Singh, 2014). Natural and affordable sources of calcium, iron, vitamins A and C, beta-carotene, phenolics, and riboflavin can be found in *M. oleifera* (Nambiar, 2006). Particularly, the leaves of *M. oleifera* contain vital phytochemicals (terpenes, flavonoids, glucosinolates, steroids, saponins, and alkaloids) that have been linked to bacterial and cancer prevention as well as anti-diabetic and anti-cancer action (Szlachetka *et al.*, 2020). For both human and animal consumption, *M. oleifera* has a significant safety margin (Stohs and Hartman, 2015). This investigation aimed to assess the antibacterial activity of three different *M. oleifera* extracts with various concentrations on *Streptococcus mutans*.

Materials and Methods

Ethical approval

Ethical approval was not needful for this investigation, because no intrusive procedure was employed.

Sample Collection

Caries sample were collected from ten different children from the outpatient clinic of Pediatric dentistry and Dental Public Health Department, Faculty of Dentistry, Cairo University during their treatment. Using sterile spoon excavator and were immediately placed in a ten different tubes containing 2 ml of sterile Brain Heart Infusion (BHI) broth medium. Samples were stored in cool place and transported to Microbiology and Immunology Department, Veterinary Institute, National Research Centre (NRC), Egypt which processed within 1-2 hour after the collection.

Isolation of *S. mutans* bacteria

The collected samples were placed in ice boxes, inoculated in BHI broth medium, and then transported to the lab. The samples were streaked onto the Mitis-salivarius (MS) agar (contain 0.2 units/ml bacitracin and 20% sucrose) and sheep blood agar (5%) mediums. With maximum inhibition of the balance of the streptococcal flora typically seen on this media, the selective agents allowed the undiminished recovery of *Streptococcus mutans* at these concentrations (Gold *et al.*, 1973 and Al-Mudallal *et al.*, 2008). Cultures were incubated anaerobically at 37 ^{o}C for 48 hours and aerobically the following night.

Identification of *S. mutans*

Purified isolates were then aerobically subcultured on tryptose soya agar slant and maintained at 4 ^{0}C for later identification in accordance with their Gram-staining, microscopic examination, catalase test, biochemical, and fermentative identification tests as indicated by Friedrich's identification scheme (Gehring, 1981; Al-Mudallal *et al.*, 2008). According to manufacturer's instructions, the pure colonies were identified using the API ZYM system (API STREPT IDENT) (BioMerieux, Marcy l'Etoile, France) and the VITEK-2 compact system (BioMERieux, Craponne, France).

***M. oleifera* leaves Extraction**

The Moringa Unit, NRC, provided the moringa leaves powder. It was gathered from an NRC farm in Al-Nubaria, which is located along the desert road connecting Cairo and Alexandria. The study used plant material that was gathered and presented.

***M. oleifera* leaves - Cold aqueous extracts**

A conical flask was filled with 400 ml of cold, distilled water and 100 grams of *M. oleifera* leaf powder. The mixture was then weighed out and stirred sporadically (10 times per day) for 7 days. The mixture was separated using Whatman No. 1 sterile filter paper and placed in a clean conical flask for water bath evaporation, which caused the aqueous solvent to evaporate at its boiling point of 100°C. The obtained standard extracts were then kept in a refrigerator at 4 °C for bactericidal purposes. activity test (Rahman *et al.*, 2009; Fouad *et al.*, 2019 and Fontana *et al.*, 2022).

***M. oleifera* leaves - Hot aqueous extracts**

The plant material was boiled for 30 minutes while following the same procedure as for cold water treatment.

***M. oleifera* leaves - Ethanol (70%) extracts**

In this case, ethanol was used instead of water and the same process as for treating water with cold was applied.

Antibacterial assay of *M. oleifera*

The single pure *S. mutans* bacteria were picked up from blood agar plate and suspending it into Brain Heart Infusion (BHI) broth for 24 hrs at 37 ºC, Then, the bacteria were diluted to reach 0.5 McFarland or the equivalent of a bacterial density of 1.5×10^8 CFU/ml. The *S. mutans* bacteria were used as the test subject for the antibacterial activity of the three separate samples, which included (1) a cold water extract (CWE) of leaves, (2) a hot water extract (HWE), and (3) ethanol extracts (EEs) of leaves. However, there were 3 various extracts of leaves; different concentrations were prepared to be tested as following: one hundred; seventy five; fifty and twenty five mg/ml.

Well diffusion method

In vitro antibacterial test was carried out by well diffusion method (Barry, 1980) using 25 µl of the standardized bacterial suspension of the *S. mutans* (0.5 McFarland = 10^8 CFU/ml) spread on blood agar plates. The 10 µl of different extracts in various concentrations were poured on each well in blood agar plates. The same solvents (the plant extracts were dissolved) were used to create the negative controls. As a positive control, tetracycline (TE) (30 g/disc) was utilized to determine the sensitivity of the *S. mutans* (Incubation at 37°C for 24 h). The zones of inhibition against the *S. mutans* bacterium were measured in order to assess the antibacterial activity. The outermost point of the inhibition halo generated by the substance was where measurements were made because it was the greatest distance between two spots.

Bacterial growth turbidity method

In vitro antibacterial test was depended on the growth turbidity which measured by spectrophotometer (Barry, 1980) using twenty five µl of the standardized bacterial suspension of the *S. mutans* (0.5 McFarland = 10^8 CFU/ml) suspended into test tube contained five mileliter of Brain Heart Infusion (BHI) broth as well as different extracts in various concentrations incubation for 24 hrs at 37 ºC, Then, spectrophotometer was measured the turbidity in each test tubes according to the bacterial growth. Twenty five µl of the same solvents (the plant extracts were dissolved) to create the negative controls. As a positive control, tetracycline (TE) (30 g/disc) was utilized. By evaluating the turbidity of the *S. mutans* bacterium and using the plate count method, the antibacterial activity was assessed.

Plate count method

Tenfold serial dilution was carried out by mixing 10 µL of each concentration with *S. mutans* in the Eppendorf tube with 990 µL of PBS, and then transferring it to the vortex. A total of 50 µL of mixture was aspirated from the Eppendorf tube and examined on a Mitis-salivarius (MS) agar petri dish. A triangle glasses spreader was used to spread and then count the number of bacterial colonies that formed manually after 24 h incubation at 37°C.

Minimum inhibitory concentration (MIC)

The two-fold serial dilution approach was used to determine the MIC of several M. oleifera sample samples. To generate concentrations of 50, 25, 12.50, 6.25, 3.12, 1.56, 0.78 mg/ml and 390, 195, 97 g/ml that were used for MIC measurement, the other samples were serially diluted by 100 mg/ml individually. Briefly, 9 ml of the standardised suspension of the tested bacteria (108 CFU/ml) was put into the test tubes together with 100 l of samples at varied concentrations. For 24 hours, the test tubes were incubated at 37°C. With the test organisms, controls were employed instead of the plant extract, using distilled water. The MIC was determined by taking the sample with the lowest concentration and no discernible increase (Fontana *et al.*, 2022).

Statistical analysis

Before performing the data analysis, it was ensured that the variance in each group was homogeneous. Each experimental group's mean and standard deviation were calculated. Using analysis of variance, the mean inhibition zones of the materials against the bacterial strain were compared for the various groups (ANOVA), using the software SPSS version 16.

Results

Antimicrobial testing and statistical analysis in the current investigation showed that all *M. oleifera* extracts solution had an antibacterial effect against *S. mutans* at various concentrations. Three experimental extracts materials' mean and standard deviation for the diameter of the *S. mutans* inhibitory zone as well as the optic density (OD) of bacterial growth were calculated. A comparison using ANOVA exposed a highly statistically significant difference ($p < 0.001$) in the mean diameters of the zone of inhibition for *S. mutans* among the three experimental extracts (Table 1-2). *M. oleifera* extract solution at concentrations of 75-100 mg/ml is considered bactericidal. However, the lowest concentration is considered bacteriostatic. Moreover, the greatest antibacterial effect of *M. oleifera* extract is that obtained by ethanol and the lowest effect is that obtained by the hot water. The difference in the mean diameter of the zone of inhibition and the optic density of growth for *S. mutans* between three experimental extracts solution was found to be statistically significant (Table 3-4).

Table (1): Mean diameter of inhibition zone for *S. mutans* of three *M. oleifera* extract

Extract / Conc.(mg)	HE (mm)	CE (mm)	EE (mm)
100	13±0.577	17± 0.333	22
75	11±0.577	15± 0.333	19± 0.333
50	9±0.577	13±0.577	15± 0.333
25	6±0.577	10± 0.333	12±0.577
TE 30	11± 0.333	11±0.00	11±0.577
Control	0±0.00	0±0.00	0±0.00
F	83.938	311.867	192.547
P	0.000	0.000	0.000

There are significant differences at the same column $P < 0.01$

Table (2): Comparing inhibition zone for *S. mutans* of three extract groups using ANOVA test

		Mean (mm)	F	Sig (P)
Hot Water Extract	Between Groups	60.622	83.938	0.000
	Within Groups	0.722		
Cold Water Extract	Between Groups	103.956	311.867	0.000

	Within Groups	0.333		
Ethanol Extract	Between Groups	160.456	192.547	0.000
	Within Groups	0.833		

Table (3): Mean optical density of growth for *S. mutans* against three *M. oleifera* extract

Extract / Conc.(mg)	HE (OD)	CE (OD)	EE (OD)
100	0.317±0.004	0.340±0.001	0.204±0.002
75	0.512±0.001	0.414±0.002	0.314±0.001
50	0.619±0.004	0.490±0.002	0.412±0.001
25	0.627±0.003	0.547±0.001	0.485±0.002
TE 30	0.514±0.002	0.532±0.002	0.517±0.002
Control	0.990±0.05	0.990±0.005	0.990±0.006
F	5.437	1.212	1.342
P	0.000	0.000	0.000

Table (4): Comparing optical density of growth for *S. mutans* against three *M. oleifera* extract using ANOVA test

		Mean (mm)	F	Sig (P)
Hot Water Extract	Between Groups	0.156	5.437	0.000
	Within Groups	0.000		
Cold Water Extract	Between Groups	0.158	1.212	0.000
	Within Groups	0.000		
Ethanol Extract	Between Groups	0.224	1.342	0.000
	Within Groups	0.000		

Discussion

The main cariogenic bacterium responsible for the start of dental caries is *Streptococcus mutans* (Bowen 2016). It generates acid, which reduces the local pH below teeth's solubility limit and damages the tooth. Controlling the acid that the bacteria create might prevent cavities (Yost and VanDemark 1978). As a result, this study looked at natural substances as antibacterial agents that were both efficient against *S. mutans* and compatible with tooth tissue and its surrounds. Numerous research have demonstrated the antibacterial and biocompatible effects on tissues of *M. oleifera*, also known as Moringa leaves (Wang *et al.*, 2016). The zone of inhibition around the experimental samples in the culture plates was observed in the current investigation to determine the antibacterial efficacy of various *M. oleifera* extract solutions. This was done using an agar diffusion microbiological assay process and growth turbidity assay method. Disc diffusion method was used for the evaluation of the antibacterial activity of Moringa

extracts, and the significant difference of inhibition zones appeared (El-Kholy *et al.*, 2018).

In the current work, a patient caries sample was used to isolate *S. mutans* on an MS agar and blood agar plates. The primary methods used to identify the *S. mutans* strains were Gram staining, microscopic examination, catalase testing, API system, and VITEK-2 compact system. By applying the test solution of 10% mannitol and 4% TTC to the agar plate, which causes the colour to change to dark pink, the presence of *S. mutans* was verified. This is caused by *S. mutans'* special capacity to reduce TTC and hydrolyze mannitol into acid using the enzyme mannitol-1-phosphate dehydrogenase. The agar plate's colony shape and form were also used to differentiate the two groups of bacteria since *S. mutans* bacteria have a distinctive colony morphology that makes it simple to separate them from other bacteria (Al-Mudallal *et al.*, 2008).

The effect of *M. oleifera,* in caries control, may be due to inhibiting growth of *S. mutans* directly. It caused inhibition of growth rate of *S. mutans*. Because it includes saponins, flavonoids, tannins, alkaloids, phenolics, and triterpenoids, *M. oleifera* leaf extract possesses antibacterial properties (Wang *et al.*, 2016). Saponins' antibacterial action interferes with the bacterial cell wall's permeability (Podolak *et al.*, 2010 and Arabski *et al.*, 2012). Proteins and nucleic acids can become damaged or denaturized when flavonoid chemicals create complex compounds with proteins through hydrogen bonds, disrupting the protein's tertiary structure and impairing its ability to function. This protein denaturation coagulation and interferes with the metabolism and physiological functions of bacteria (Xie *et al.*, 2015). Also all bacterial cells had cumulative effects and inhibited the formation of cell membranes due to Flavonoids (Cushnie and Lamb 2005). Moreover, *M. oleifera* leaf extract had tannin compounds which can shrink cell walls and inhibit protein synthesis for cell wall formation, thereby disrupting cell permeability and leading to death (Length, 2006 and Akiyama *et al.*, 2001). Alkaloids interact with the peptidoglycan's constituent parts in bacterial cells to prevent the formation of intact cell wall layers, leading to cell death as part of their antibacterial, inhibitory function (Cushnie *et al.*, 2014). Terpenoid compounds can interact with porin on the bacterial cell wall's outer membrane to form strong polymeric bonds that damage the pile. As a result, compounds that reduce the permeability of the bacterial cell wall can enter, starving the bacterial cell of nutrients and preventing it from growing or killing the bacteria (Cowan, 1999).

In the current investigation, ethanol solvent was used to prepare the *M. oleifera* leaves extraction. Compared to extractions utilizing various solvent, plant extraction with ethanol solvent performed the best. This outcome is in line with study by Luginda (2018), who found that using ethanol as a solvent extract for plants that contain flavonoids produces the highest quantities of total flavonoids. This is crucial since higher flavonoid concentrations are associated with greater antibacterial efficacy (Manik *et* al., 2014). The hydroxyl groups in ethanol solvents can bind polar substances like flavonoids and alkaloids (Nuraini *et al.*, 2015). According to research by Piexoto *et al.*, 2011; *M. oleifera* extract in ethanol solvent is more effective than distilled water at preventing the growth of bacteria. Compounds in the *M. oleifera* extract solution have the ability to stop both Gram-

positive and Gram-negative bacteria from growing. Higher concentrations of the *M. oleifera* extract solution inhibited *S. mutans* growth in the current investigation. These findings are consistent with a study in which Shailemo *et al.*, 2016 investigated the antibacterial properties of *M. oleifera* extract at various doses. In contrast to distilled water, which appears more turbid, *M. oleifera* antibacterial test findings at concentrations of 10%, 20%, and 35% indicated just a small turbidity in the diluted tube (Shailemo *et al.*, 2016). According to a study by Arevaro *et al.*, 2018; *M. oleifera* (75%) is the lowest amount of *M. oleifera* extract necessary to eradicate *E. faecalis*. Therefore, *M. oleifera* extract was used to treat samples in the current investigation at doses of 25%, 50%, 75%, and 100%. The current investigation also demonstrated that *S. mutans* could be killed by solutions containing 75% and 100% *M. oleifera* extract.

Conclusion

Based on the obtained results of the present investigation, it can be concluded that *M. oleifera* extract solution has an antibacterial effect against *S. mutans* at concentrations of 50%, 75%, and 100%. *M. oleifera* extract solution at concentrations of 75% and 100% is considered bactericidal. However, the lowest concentration is considered bacteriostatic. Moreover, the greatest antibacterial effect of *M. oleifera* extract is that obtained by ethanol and the lowest effect is that obtained by the hot water.

Conflicts of interest

The authors declare no conflict of interest.

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