Assessing the cytotoxic effect and antimicrobial activity of Moringa oleifera aqueous and ethanolic extract against oral pathogens extracted from periodontal and orthodontic patients – an in vitro study

Shanmugapriya Ramamurthy¹, Sheeja Varghese², Umarevathi Gopalakrishnan¹, Mahesh Kumar³, Mayma Nathasha⁴, Jeyaram Palinivel⁴

¹Sri Venkateswara Dental College & Hospital, The TamilNadu Dr MGR Medical University, Chennai, India, 600 130, Research Scholar, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai 6000077.
²Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai 6000077.
³Karpagavainavaga Institute of Dental Sciences, Chengalpattu (DT) 603 308.
⁴Consultant Orthodontist


Received: 24/08/22 Accepted: 12/10/22 Web Published: 28/11/22

Doi: 10.56501/intjorthodrehabil.v13i4.438

Abstract:
Background: Periodontitis is the result of inflammation caused due to the activity of microorganisms. The prevalence of anaerobic organisms is more when it comes to periodontal pockets and orthodontic patients. Plants with phytochemicals that could exert antimicrobial effects could aid in host modulation for management of periodontitis caused by these bacteria in periodontal and orthodontic patients.
Aim: To assess the antimicrobial effect of aqueous extract of Moringa oleifera Lam (MOL) and cytotoxic effect of aqueous and ethanol extracts of MOL.
Materials and methods: Moringa oleifera Lam. extracts were prepared by maceration. Subgingival plaque samples were collected, and microorganisms were cultured in anaerobic environment. The microorganisms were treated with the extracts and minimum inhibitory concentration and minimum bactericidal concentration was assessed. The cytotoxic effects were assessed by brine shrimp assay.
Results: Aqueous extract showed antimicrobial effect in dose and time dependent manner and both extracts exhibited cytotoxic effects in a dose and time dependent manner.
Summary and Conclusion: The antimicrobial effect of MOL could be utilized to develop a nature derived local drug delivery system for treating plaque induced periodontitis in different clinical situations.
Key words: anaerobic organism, anti-microbial, cytotoxicity, dental plaque, Moringa oleifera Lam, periodontitis.

Address for Correspondence
Dr. Shanmugapriya Ramamurthy
Sri Venkateswara Dental College & Hospital,
The Tamil Nadu Dr MGR Medical University,
Chennai, India, 600130,
Email Id : drshanpriya@gmail.com

© 2022 Published by MM Publishers.
Introduction:

WHO has reported that in developing nations approximately eighty percent of the population prefers herbal and traditional medicine for disease management.[1] This could be attributed to the fact that herbs are easy to procure, cost effective and less likely to cause side effects as most of them can be included in staple diet. Hence the research interest for exploring the medicinal properties of herbs has increased recently.


This awareness of the medicinal benefits of drumstick plant had brought about increased consumption of these leaves by many people as a part of their diet in different ways to maintain their nutritional status. In countries like Africa and India, research emphasizing on developing both therapeutic and nutritional supplement from Moringa leaves is in progress.[19] In Republic of Philippines and Niger, *Moringa oleifera* is included as therapeutic supplement in national nutrition rehabilitation program targeted for malnourished children.[20] Thus recently *M. oleifera* is being explored for the treating various chronic illness and systemic diseases. The leaves have been used for the treatment diabetes mellitus, cancer, obesity, scurvy, hysteria.[21, 22] Among the various inflammatory condition affecting the oral cavity, periodontitis is the most common chronic inflammatory disease with a microbial etiology, wherein combined effect of oxidative stress, microbial activity and inflammation leads tissue destruction.[23] The chances of this type of tissue destruction is more when there are plaque retentive factors like multiple restorations of decayed teeth, restorations replacing missing teeth and in patients wearing orthodontic appliances. In these situations, the routine mechanical plaque control is challenging. Addition of Chemical plaque control agents in routine oral care in patients with these predisposing factors will aid in prevention of plaque induced diseases. And management of periodontal disease is by a combination of surgical and nonsurgical strategies along with local drug delivery. [24] Hence a local drug delivery agent with antimicrobial properties against could be a better choice in improving the prognosis of the condition. Hence various herbs and their active constituents are being explored to aid as a therapeutic adjunct for periodontal disease management. The pharmacological benefits of *Moringa oleifera* Lam (MOL) in the treatment of systemic diseases in humans and cattle are well established.[25, 26] But literature of its actions in the management of oral diseases is scarce Few laboratory studies have explored its anti-microbial and host modulating activities in the management of oral diseases.[27]

In the microbial etiology of periodontal disease several anaerobic bacterial organisms have been attributed as a major cause.[28] Taking into consideration of this fact the present study was to assess the antimicrobial properties and cytotoxicity effects of aqueous (A) and ethanolic (Et) extracts of MOL against
anaerobic pathogens pooled from subjects with periodontitis and patients undergoing orthodontic treatment to explore its use as a chemical anti-plaque agent and local drug delivery system in future.

Methods

This invitro study design for testing the antimicrobial activity and cytotoxicity was approved by the ethics committee of Saveetha Dental College (SDC/Ph.D18/32).

EXTRACT PREPARATION

The drumstick plant leaves grown in Southern part of India were procured from the local market. The leaves were washed in running water and air dried in shade for two weeks. After weighing it was dry grounded and stored in airtight containers. For the preparation of alcoholic and ethanolic extracts to 100 gm of the powdered leaves 1 L of water and ethanol were added respectively. It was macerated for three days. Later the filtrate obtained with Whatman filter paper #1 was reduced further to obtain a solid residue. To prepare 5% aqueous and ethanolic of the extract 0.5 gm of the solid residue was dissolved in 10 ml of water or ethanol respectively.[29]

Antimicrobial Activity

Collection of plaque sample

A pooled plaque sample was collected from two periodontitis and two orthodontic patients visiting the dental college. Sampling was done only in patients with moderate periodontitis patient, with sites exhibiting 5-6 mm probing pocket depth and clinical attachment loss of 3-4mm sample according Armitage criteria 1999 was collected. And subjects who had undergone periodontal therapy or undertaken antibiotics in the past immediate six months were excluded. In orthodontic patients only subjects who is under orthodontic therapy for a minimum of 6 months was chosen for sample collection. After removal of supragingival plaque, the subgingival plaque samples were collected with sterile absorbable paper points. Samples contaminated with blood was discarded and immediately transferred to 2 ml Robertson's cooked meat medium that was preheated.

Anaerobic culture

Trypticase agar plate was used to obtain subculture from Robertson cooked meat media The Gaspak system had a transparent jar, air-tight lid, screened catalyst chamber with palladium-sized aluminium pellets. Addition of water was done to an aluminium foil packet with pellets of tartaric acid, sodium bicarbonate and sodium borohydride and placed inside the jar immediately. Carbon dioxide was produced as a result of chemical reaction to which the mounted agar plate was placed, and lid was tightly clamped. Incubation of the jar for 48 hours at 37°C was done in an incubator. Microbial growth was checked and CFU for each plate was done with a colony counter, CFUs for each plate were counted. [30]

Minimal inhibitory concentration:

To assess the minimum inhibitory concentration Brain Heart Infusion broth was prepared. And six ml of the prepared broth were subsequently taken in test tubes. To each test tube isolated bacterial colonies at a
density of 5×10^5 CFU/mL. was added. The bacterial samples were treated with various concretions (25µL, 50 µL, 100 µL) of 5% aqueous extract of *Moringa oleifera* Lam. An untreated bacterial suspension was considered as positive control and sample devoid of organisms were negative control. The test and control samples were incubated under anaerobic conditions for 1 hour, 2-hour, 3-hour, 4-hour and 5-hour. The percentage of dead cells was calculated at 600 nm at all the above time intervals. [31]

**Minimum bactericidal concentration by Agar dilution method.**

The prepared Brain Heart Infusion agar was poured in sterile petri plates. To the broth the anaerobic suspension and different concentrations of 5% aqueous extract of *Moringa oleifera* Lam. Extract were added to prepare the test samples. Finally, the test samples, positive and negative control were incubated in an anaerobic chamber for 24h following which colonies were counted.[32]

**Brine Shrimp Lethality Assay:**

**Saltwater preparation:**

2g of iodine free salt was weighed and dissolved in 200ml of distilled water to prepare the saline water for growth of Nauplii. Then 10-12 ml of this saline water was added to each of the six well plates. Followed by transfer of 10 nauplii slowly to each well. Then the wells were treated with various concentrations of 5%aqueous and ethanol extracts of (5µL,10 µL,20 µL,40 µL,80 µL) *Moringa oleifera* L. The untreated samples were treated as control. The samples were incubated for 24 hours and 48 hours. Following incubation period, the number of live nauplii’s present were calculated.[33]

**Results**

**Minimal inhibitory concentration:**

The microbial culture samples when treated with 25µL, 50 µL,100 µL of aqueous extract of Moringa demonstrated antimicrobial activity in a dose dependent and time dependent manner with increase in activity with higher concentration and increased antimicrobial activity with incubation time ranging between one hour and 5 hours. The results were comparable with control or standard. On the contrary, negative control showed increased microbial growth with an increase in incubation time. The results are depicted in Figure 1.
The plaque samples when treated with 25µL, 50 µL, 100 µL aqueous extract of *M. oleifera* demonstrated significant antimicrobial effect in the dose dependent manner as depicted by decrease in colony count with increase in concentration 25µL (450), 50 µL (304), 100 µL (206) which was comparable with positive control (57), whereas negative control had a colony count of (613). The results are shown in Figure 2.

![MBC](image)

*Figure 2- Minimum bactericidal concentration of aqueous extract of MOL.*

**Cytotoxic effects of aqueous extract, ethanolic extract of *M. oleifera***

In the first 24 hour of incubation, there was no difference in number of live nauplii at various concentrations of 5µL, 10 µL, 20 µL, 40 µL, 80 µL in both aqueous and ethanol extract of *M. oleifera*. The number of live nauplii was 10 which was the same as the control. But at 48 hours there was a decrease in live Nauplii in both the test groups. And this decrease to 5 viable Nauplii, was more pronounced in wells with highest extract concentration of 80 µL. As the concentration of extract increased, there was a dose dependent decrease in live nauplii. When aqueous (10) and ethanol(8) groups were compared, the aqueous group showed a greater number of live cells at 5 µl. But as the concentration increased both the groups were comparable (5 viable Nauplii) but more cytotoxic than control group (10 Nauplii). The results are shown in Figure 3, 4 and Table 1.
Table 1: Percentage viable Nauplii after treatment with aqueous and ethanolic extracts of MOL at 24 and 48h

<table>
<thead>
<tr>
<th>Conc in µL</th>
<th>Aqueous extract 24 h</th>
<th>Ethanolic extract 24 h</th>
<th>Aqueous extract 48 h</th>
<th>Ethanolic extract 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>5µL</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>10µL</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>20µL</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>40µL</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>80µL</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>CONTROL</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 3- Cytotoxic effect of ethanolic extract of MOL against brine shrimp
Discussion

Periodontitis an inflammatory condition of tooth supporting tissues with microbial etiology. This leads to tissue destruction as a result of inflammation associated with host-microbial interaction. This is of concern to all patients with poor plaque control and in patients with plaque retentive factors which makes them vulnerable to these damages. In periodontal patients even shallow periodontal pockets of 5 mm is inaccessible for routine periodontal care might precede to further progression.[34] Similarly in orthodontic patients daily oral hygiene practices are complex which allows for a microbial shift from aerobic to anaerobic microorganisms and thus nurturing an increased bacterial load. [35] The anaerobic organisms causes more destructive changes in the periodontal apparatus and jeopardizes both periodontal and orthodontic treatment outcome. Hence antimicrobial agents could aid in reduction of periodontal pathogens thereby preventing further tissue destruction. Although several antimicrobial agents have been used as an adjunct therapy, search for herbal alternative continue owing to diverse pharmacological effects such as antioxidant, antimicrobial and anti-inflammatory properties.

In this regard Moringa oleifera L. also known as drumstick is known for its diverse pharamcognostic effects. Although antimicrobial effects of drumstick leaves against aerobic bacteria have been reported so far, the antimicrobial effect of MOL against anaerobia bacteria from dental plaque samples have been less explored. Also prior to conduct of clinical trials, the determination of cytotoxic concentration is essential. Hence, we aimed to determine the antimicrobial and cytotoxic effects of MOL against anaerobic pathogens from subgingival dental plaque samples and cytotoxic effects.

The results of minimum inhibitory concentration assay revealed that MOL exhibited antimicrobial effects in a dose dependent manner 25µL, 50 µL, 100 µL, with decrease in activity with decrease in concentration and time dependent manner with increase in activity with increase in incubation period of one to five hours. Similarly, the results minimum bactericidal concentration revealed that MOL exhibited antimicrobial effects in a dose dependent manner with decrease in colony count with increase in concentration. The results are concurrent with the findings of Fouad et al 2019 who assessed the antimicrobial effect of aqueous and ethanol extract of leaves of drumstick against bacteria isolated from abscess of camels and reported that ethanol extract
showed better antimicrobial activity than aqueous extract.\textsuperscript{[16]} Since in our study the cytotoxicity of aqueous was comparatively less than ethanol, the antimicrobial effect was assessed against 5 % aqueous preparation.

Similarly, Lucia et al assessed the antimicrobial effect of methanol extract of \textit{M. oleifera} leaves against anaerobic bacterium \textit{Enterococcus faecalis}. They reported MIC of 75 \( \mu \text{g/ml} \) bactericidal concentration of 75 \( \mu \text{g/ml} \). Considering cytotoxic effects the IC 50 value was 70 \( \mu \text{g/ml} \).\textsuperscript{[37]} Kim et al reported the antimicrobial activity of methanol extract of drumstick leaves against dental plaque bacteria. They reported that the isolated compounds Niazinin A, \( \beta \)-Stigmasterol, Quercetin-3-O-\( \beta \)-D-Glucopyranoside and Kaempferol-3-O-\( \beta \)-D-Gluco pyranoside from Moringa exhibited antimicrobial activity against the tested bacteria.\textsuperscript{[36]} Studies have reported that quercetin exerts antimicrobial effects against \textit{Staphylococcus aureus} (at 10 \( \times \) MIC). And \textit{E. coli} (at 50 \( \times \) MIC) by causing cell wall damage.\textsuperscript{[39]} Hence the antimicrobial effect of MOL could also be via the same mechanism due to the presence of quercetin, however further molecular research warranted in this field.

In a systematic review by Nurul et al, the antimicrobial effect of MOL against oral pathogens and anti-inflammatory properties have been reported. They have also complied the results of a few studies that have reported the antimicrobial properties of MOL against drug resistant \textit{Staphylococcus aureus} and \textit{Escheria coli}.\textsuperscript{[40-42]} The antimicrobial property of MOL could also be attributed to the presence of lectins as reported by Khatun et al 2009 who demonstrated the antimicrobial effects of three lectins obtained from MOL against \textit{E. coli}, \textit{S dysenteriae} and \textit{S aureus}.\textsuperscript{[43]}

Considering cytotoxicity, but there was a dose dependent increase in cytotoxic effects as depicted by decrease in number of live nauplii in both aqueous and ethanol extract of \textit{M. oleifera} at 48 hours of incubation. the results of the present study are concurrent with the findings of Jafarain et al who reported dose dependent cytotoxic effects of MOL in HeLA cell line. They attributed the cytotoxic properties to the phenols present in the leaves.\textsuperscript{[38]} Similarly Khatun also reported the cytotoxic effect of MOL derived lectins by the brine shrimp (Artemia salina L.) lethality bioassay.\textsuperscript{[44]}

The limitation of the study is that it has an in-vitro design and further well controlled clinical trials are warranted to determine the exact therapeutic effect. The cytotoxicity assay sheds light on selection of non-toxic concentration for developing local drug delivery system and the antimicrobial properties of MOL could be further explored clinically as an adjunct to dental plaque control. This study opens up new avenues for further research especially in relation to intraoral bacteria which are capable of causing corrosion in the intra oral environment. The study by Odusote et al has mentioned about the possibility of the inhibitory effect of moringa leaf extract on the corrosion of stainless steel.\textsuperscript{[45]} This is of significance in the wake of increasing use of stainless-steel appliances intraorally.

\textbf{Conclusion:}

The 5% aqueous extract of \textit{Moringa oleifera} Lam. demonstrated a dose dependent antimicrobial activity against oral anaerobic organisms. This effect was pronounced as the exposure time of the treated sample increased. And the aqueous extract was marginally better in lesser concentration compared with ethanol extract in cytotoxicity assay which was revealed by a greater number of live Nauplii. These observations together with other published research support the pharmacological effects of \textit{M. oleifera} for the management of disease and
malnutrition in communities. This difference in higher concentration was not observed. Thus, the antimicrobial property of *M. oleifera* against anaerobic pathogens could be explored further for management of periodontitis an inflammatory condition of the tooth supporting structures. Development of a local drug delivery such as thermo-reversible gel ointments, mouthwashes containing ideal non-toxic concentration of MOL could be formulated and a clinical trial could be conducted for its effect in treatment of periodontitis similar to the current antimicrobial agents such as tetracycline, chlorhexidine, and doxycycline. Thus *M. oleifera* could be used as a nature-derived host modulatory agent for treating dental biofilm induced diseases caused by amicrobial aggregations as found in periodontal and orthodontic patients.

**Ethical approval:** The study was approved by the Institutional Ethics Committee of Saveetha Dental College ((SDC/Ph.D18/32).)

**Conflict of interest:** No conflict of interest among authors in this study.

**Sources of Funding:** Nil

**Authorship Contributions:** Conception and design of the work was presented by Shanmugapriya & Sheeja, laboratory studies, data collection was executed by Dr. Uma Revathy & Dr. Mayma, data analysis and interpretation was carried out by Dr.Mahesh, Dr.Jeyaram drafted the article and finally the article was critically reviewed by Dr.Shanmugapriya.

**Acknowledgement:** The authors thank Dr. Sabitha Sudarsan, Professor, Sri Venkateswara Dental Chennai for her guidance and support in conducting this study.

**References**


27. The antimicrobial effects of MOL on aerobic caries causing organisms to have been researched Nurul M , Muhammad Harun. Systematic Review of Moringa oleifera's Potential as Antibacterial and Anti-Inflammatory in the Oral Cavity. European Journal of Molecular & Clinical Medicine, 2020; 7(10): 144-161.


