In-Vitro Antibacterial Activity of Crude Defatted *Moringa Oleifera* Seed Extract: Kill time study

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Abstract: Earlier research conducted on *moringa oleifera* seed extract not only reveals its excellent coagulant property; it also being proven to possesses antibacterial activity against microorganism. An antibacterial agent 4(α-L-rhamnosyloxy) benzyl isothiocyanate, a plant synthesized derivatives of benzyl isothiocyanates has been identified as the active antibacterial agent present in the seed. In this study, the mode of antibacterial action was determined using the kill-time study on *e.coli* and *pseudosomonas aeruginosa* bacterial strains. The minimum inhibitory concentration (MIC) assay was done using macrodilution broth. MIC values were varied from 0.0125mg/ml to 0.1mg/ml. Kill-time assay was carried out based on standard procedure. The growth of the microbes was monitored for every 30 minutes by viability counting on agar plate. Log reduction of viable cells counts ranged from 0 to 3.6 log10 for *e.coli* and 1log10 to 4log10 for *p.aeruginosa*. The results of the kill-time study revealed that the extract was bactericidal against *e.coli* and *p.aeruginosa* within 30 minutes of contact resulting in about 90% elimination of the strains. Most of the microbes were killed at high MIC value of 0.1 mg/ml, which might suggest that the seed extract exhibits concentration-dependent killing that revealed that it can be used as a natural disinfectant for water treatment.

Key words: benzyl isothiocyanates; *Moringa oleifera*; kill-time; *e.coli*; *p. aeruginosa*

INTRODUCTION

*Moringa oleifera* is one of the most studied and widely cultivated species of monogeneric family Moringaceae. It is locally known as horseradish tree, drumstick tree, kelor, or Ben oil tree. It is a rapidly growing tree of about 5m to 10m tall commonly grown in semiarid, tropics and sub tropic region of the world. It has very high resistance against drought hence its presence in hot, semi arid regions. It is commonly referred to as a ‘miracle’ tree because almost all its parts have been utilized either as food, medicine or to clarify turbid water (Ali *et al*., 2004). It has been used as fertilizer, manure, machine lubrication, pulp, perfume, and hair care products. There are other several biological properties of this plant that have been reviewed over the last decade and reports have revealed that leaves of *moringa oleifera* is an abundant source of both macro and micro nutrients such as protein, calcium, potassium, Vitamin C, β-carotene, and natural oxidants (Aney *et al*., 2009). The seeds of *Moringa oleifera* have been reported by several literatures as excellent coagulant that is also reported to have anti microbial activity (Futi *et al*., 2011). The seed has been extensively used in the treatment of drinking water and wastewater. The seeds are considered as one of the best natural coagulants, which are great substitutes for the chemical coagulants (Ali *et al*., 2010). The seeds have been reported to effectively treat high turbid waters, possessing natural buffering capacity. It also exhibits softening properties as well as being a pH-correcting agent (alkalinity reduction) (Muyibi *et al*., 2003; Muyibi *et al*., 1994).
A careful examination of the phytochemical constituents present in the seed reveals vast abundant compounds rich in glucosinolates and isothiocyanates. Common phytochemicals found in the seed irrespective of the solvent used for extraction includes alkaloids, resins, tannins, flavonoids, glycosides etc (Anwar, F., and Rashid, U. 2007). It is also believed that the seed contains protein that are responsible for its coagulant property while an antibacterial agent 4 (α-L- rhamnosyloxy) benzyl isothiocyanate, a plant synthesized derivatives of benzyl isothiocyanates is believed to be responsible for its antibacterial property (Valarmathy et al., 2010).

An enormous literature has shown that the seeds have been extracted with various solvents, which has led to the presence of different phytochemicals and commonly used solvents are water ethanol, methanol, etc. Extraction of bioactive compounds from the seed is commonly done using water as the solvent and the resulting filtrate is referred to as the crude extract. The seed also contains about 30-40% w/v amount of oil that can be removed for more isolation of bioactive compounds from the seed (Walter et al., 2011; Oluduro, A. O., and Aderiye, B. I. 2007). The oil is commonly referred as ben oil, which is used in cosmetics and lubrication of some parts of machine. In this study, the seed oil was removed using n-hexane. The seed extract was prepared by taking a small quantity if the defatted seed cake and dissolving it in sterile distilled water.

The main objectives of this study is to determine the antibacterial activity of the crude extract from the defatted seed cake by determining its MIC, MBC and the kill-time study on e.coli and pseudomonas aeruginosa bacterial strains.

**MATERIALS AND METHODS**

**Collection of Seed:**

Dry seeds of *Moringa oleifera* were collected from Bayero University Nigeria, the seeds were de-husked and ground to fine powder. The ground powder was sieved through 210µm sieve (Walter et al., 2011).

**Preparation of Crude Seed Extract:**

Electro thermal soxhlet was used to extract oil from the seed powder using n-hexane as the extraction solvent. During electro thermal soxhlet extraction, 170 ml of n-hexane was used in the heating chamber, extraction of the oil from the seed was ensured within three cycles of hexane evaporation. The defatted seed cake was dried and kept at room temperature (Ali et al., 2010). To extract the active ingredients from the defatted seed cake, 10gms of the defatted *Moringa Oleifera* seed powder was measured into 500mls of sterile distilled water in a beaker mixed for 30minutes at 150rpm to extract the active ingredients. The solution was filtered through No.1 Whatman filter paper and the resulting filtrate was as the extract for the kill-time study as well as for the determination of both MIC and MBC.

**Preparation of Inoculum:**

*Escherichia coli* and *Pseudomonas aeruginosa* bacterial cells were obtained from laboratory stock solution. Colonies of the bacterial cells were picked from cultures grown on LB agar and were inoculated into 10mL of LB broth. This was incubated with shaking overnight at 37 oC. The inoculum density of the bacterial cells was verified to be 0.1 using UV spectrophotometer at 600nm.

**Minimum Inhibitory Concentration:**

This is the lowest dilution of antibacterial agent that inhibits growth of microbes, which is judged by lack of turbidity in the tube. This was done using the two-fold macro broth dilution method as described by Penna et al., (2001). Initial concentration of 50mg/mL was diluted to 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09, 0.048, 0.024 and 0.012mg/mL in LB broth. All the tubes were inoculated with about 1000 cells/mL bacterial strains and incubated for 18hrs at 37°C. After incubation, the tubes were examined for any visible trace of growth. The tube in series with no visible growth was taken as the MIC.

**Minimum Bactericidal Concentration:**

This was determined by collecting loopful of broth from the clear test tubes that were used for MIC determination. Then, it was inoculated on sterile LB agar. The plates were incubated at 37 0C for 24 hours and after the incubation, the concentration with no visible bacterial growth on the solid agar medium was noted as the minimum bactericidal concentration. 0.01ml of contents of MIC tubes will be sub-cultured by streaking on LB nutrient agar plates. The plates were incubated for 18-24 hours at 37°C. The plates producing no colony at all were reported as the minimum bactericidal concentration (MBC).

**Mode Of Action (Bactericidal or Bacteriostatic):**

The mode of action of the processed moringa seed extract was calculated using the ratio of MBC/MIC as described by Konate et al., (2012) to evaluate if the observed antibacterial effect was either bactericidal or
bacteriostatic. If the ration of MBC/MIC was less or equal to two, the effect is considered as bactericidal or otherwise bacteriostatic.

**Kill Time Study:**

The extract was added into 10 mL of LB broth and inoculum concentration of 10^5 cells/ml was used. Two controls, one LB broth without the extract which is the negative control and the other with chlorine as the positive control. MIC values ranged from 0.5 MIC to 4MIC that is from 0.0125mg/ml, 0.025mg/ml, 0.050mg/ml and 0.100mg/ml. Incubation was done with agitation at 120 rpm on an orbital shaker at 37°C. At every 30 minutes interval, 100 μl was removed and diluted in 10 fold sterile distilled water to remove the extract. After, 100 μl of the diluted solution was plated on LB agar plate, incubated at 37°C for 24 to 48 hours. After incubation, bacterial colonies were counted using total viable count and the results obtained were compared with the count of both positive and negative control. The reaction was monitored for six hours (Konate et al., 2012).

**Results:**

**Minimum Inhibitory Concentration (MIC):**

The results of MIC and MBC for the bacterial strains are shown in Table 1. The macrobroth dilution assay to determine the antibacterial activity showed that MIC of moringa seed extract was very effective against both the bacterial strains microbes. The MIC value obtained for *e.coli* was 0.025 mg/ml while that of *pseudomonas aeruginosa* was obtained at 0.049 mg/ml. The MBC values obtained for *e.coli* and *pseudomonas aeruginosa* are 0.025 mg/ml and 0.049 mg/ml respectively.

**Table 1:** MIC and MBC values obtained for *e.coli* and *p.aeruginosa*

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Concentration (CFU/ml)</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>MBC/MIC</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>e.coli</em></td>
<td>1000</td>
<td>0.025</td>
<td>0.025</td>
<td>1</td>
<td>bactericidal</td>
</tr>
<tr>
<td><em>p.aeruginosa</em></td>
<td>1000</td>
<td>0.049</td>
<td>0.098</td>
<td>2</td>
<td>bactericidal</td>
</tr>
</tbody>
</table>

**Kill-Time Study:**

The kill-time assay is an important procedure that explains the time at which 99.9% kill of bacteria is achieved. The results are presented in terms of changes in log_{10} cfu/ml of viable colonies. The bactericidal activity was defined as reduction in the viable colony count resulting to 3log_{10} cfu/ml relative to the initial cell number [13]. The results obtained for the kill-time study for both *e.coli* and *pseudomonas aeruginosa* are shown in Figure 1 and Figure 2 respectively.

**Fig. 1:** kill-time study of *e.coli*

Log reduction of viable cells counts ranged from 0 to 3.6 log_{10} for *e.coli* and 5log_{10} to 4log_{10}. From the figures, after about 30 minutes incubation with different concentrations, log reduction in the viable cell count was about 0 log_{10} cfu/ml for *e.coli* bacterial strains. For *pseudomonas aeruginosa*, 3log_{10} cfu/ml bacterial reduction was not obtained. Average viable cells were only reduced from 5 log_{10} cfu/ml to 4 log_{10} cfu/ml.

**Discussion:**

In this study, the crude defatted seed extract showed a high inhibitory as well as bactericidal effects against *e.coli* and *pseudomonas aeruginosa*. The results obtained in this study obtained were in close agreement with earlier research conducted by Suarez et al., (2003). They reported that 1 – 6mg/mL of flo were found to decrease viable cell counts in high magnitudes. Low MIC and MBC values obtained show that the seed extract exhibits adequate efficacy. The ratio MBC/MIC value obtained for *e.coli* was 1 and that of *pseudomonas*
aeruginosa was 2. Hence the mode of action of the seed extract shows that it is highly bactericidal against both the bacterial strains however, e.coli was strongly inhibited.

![Kill-time study of p.aeruginosa](image)

**Fig. 2: Kill-time study of p.aeruginosa**

Kill-time study is often used as basis for in-vitro investigation of antimicrobial agents. It provide qualitative information on the pharmacodynamics of antimicrobial agents. The results for the kill-time study as shown in figure 1 for e.coli bacterial cells revealed that for the first 30 minutes, the cells were completely destroyed for all the MIC range (0.5MIC to 4MIC). For 0.5MIC value, the cells were destroyed around 30 minutes as there were no colonies counted when plated on an agar plate but regrowth occurred about 30 minutes later. This could be as a result of insufficient extract which only killed few bacteria at 30 minutes contact but regained their consciousness 30 minutes later. For both MIC and 2X MIC, complete bactericidal action was observed for over 120 minutes while for 4X MIC, bactericidal action was observed for over 180 minutes. However, regrowth started to occur which was rapid for both 0.5MIC and MIC. For 2X MIC and 4X MIC, regrowth also occurred but remained constant for close to 4 hours. What can be deduced from the result is that the extracts exhibit strong bactericidal action against e.coli cells at the initial time contact but over the period of time, this effect become bacteriostatic. However, for chlorine, completely bactericidal action was observed as the bacterial cells were killed throughout for over the period of 6 hours. This indicates that moringa oleifera seed extracts exhibited concentration-dependent killing which means that by increasing concentrations, microbes are killed faster.

On the contrary, pseudomonas aeruginosa bacterial strains was slightly inhibited even at high concentration of 4X MIC. This was observed from Figure 2 which could be as a result of its growing resistance against the seed extract. Pseudomonas aeruginosa is notorious for its resistance against most antibiotics which is due to its permeability barrier afforded by its outer membrane. Also, its tendency to colonize surfaces in a biofilm form makes the cells impervious to concentrations of antimicrobial agents. The result obtained from MIC values also revealed that pseudomonas aeruginosa was not strongly inhibited when compared with e.coli which reflected from the kill-time study. The degree of antibacterial activity may be accounted for by the protein as well as other bioactive compounds present in the extract. Literature on the kill-time study of moringa oleifera seed extract is scare, the results obtained could not be compared.

Although in vitro tests do not necessary give accurate results, but it provides a basic understanding and gives an insight into efficacy of plant materials which leads to search into various application such as their traditional use in medicine, or application as a disinfectant. In this study, the use of moringa oleifera seed extract for the reduction of microbes showed that the seed extract has a great potential for use as a disinfectant for drinking water treatment.

**Conclusion:**

The use of moringa oleifera seed extract has been extensively studied as an excellent coagulant for the treatment of high to low turbid water. It has also been reported to drastically reduce microbes in water hence it posses antibacterial property. In this study, the use of Moringa oleifera seed extract for the reduction of microbes showed that the seed extract has a great potential for use as a disinfectant for drinking water treatment. In order to establish its disinfectant ability, extensive investigation of its mode of action, toxicological as well as the determining its optimum condition for disinfection of water is still ongoing. In this study, we conclude that Moringa oleifera seed extract exhibited a strong antibacterial activity, which can be harnessed as disinfectant for drinking water uses.
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