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Effect of processing method and concentration of *Mangifera indica* leaf extract on their antibacterial activity against *Staphylococcus aureus*

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Abstract
Mangifera indica leaves play a major role in combating antibiotic resistance. They are processed using various methods to yield powder for phytochemical extraction. Present study was conducted to determine effect of processing method and concentration of leaf extract on their antibacterial activity against *S. aureus*. Four by four factorial design was employed involving two factors namely processing method (mortar and pestle, blender, laboratory mill and stone) and concentration of extract (6.25, 12.5, 25 and 50 mg/ml). Antibacterial activity was determined by disc method. Both factors influenced antibacterial activity, but there was no interaction effect ($p = 0.353$) of the factors. Highest inhibition zones were recorded for Laboratory mill (14.5mm) and 50 mg/ml concentration (16.7mm). Inhibition zones for other methods were similar ($p = 0.624$). MIC was 6.25mg/ml while MBC was 12.5mg/ml. The study has provided evidence on effectiveness of laboratory mill compared with other processing methods in Malawi.

Keywords: *Mangifera indica*, processing method, concentration, antibacterial, *Staphylococcus aureus*

1. Introduction
Staphylococcus aureus infection and other bacterial infections have been of great concern over the decades. The organisms have emerged resistance to a great deal of conventional medicine leading to increased cases of infectious diseases, prolonged stay in hospitals and death, contributing to escalating healthcare costs[1,2].Several attempts and efforts have been made to contain antibacterial resistance including use of natural products such as *Mangifera indica* (Mango) leaves [3]. In Malawi, *M. indica* continues to play a major role in primary health care and it has antibacterial activity against *S. aureus* [4, 5, 6]. *M. indica* leaves are processed using different methods to yield powder from which bioactive compounds with antibiotic activity such as alkaloids, flavonoids, tannins, steroids, gallotannin and mangiferin are extracted [7, 8]. However, there is no evidence on the effect of different processing methods on antibacterial activity of the leaf extract against disease causing clinical isolates.

In Sub-Saharan Africa, *M. indica* leaves are often processed using less costly manual methods to yield powder due to poverty and unreliable electricity. People rely on two simple traditional methods namely grinding by a stone and wooden mortar and pestle methods. In the earlier method, leaves are placed in a plastic bag and are ground manually by hammering using a metal hammer or stone. Mortar and pestle involves placing the leaves in a wooden mortar and are ground by hitting using a wooden pestle. These methods are tedious and slow as compared to modern methods. Particle size, temperature and pressure are not uniform and cannot be regulated. They also expose the powder to contamination due to broken wooden particles and plastic paper chemicals in a stone method.

Laboratory mill and home blender are modern methods designed to achieve rapid grinding rate. Proponents of these modern methods claim that they do not degrade phytochemicals as compared to traditional methods where pressure during grinding is variable. While the opponents claim that temperature in traditional methods is lower as compared to modern methods since temperature influences antibacterial activity [9, 10], thereby preserving most of the phytochemicals. It is also believed that extraction rate is higher in modern methods, although we were unable to find evidence to support this claim.

To address this gap in knowledge the current study was conducted to systematically compare performance of the four techniques with respect to *M. indica* leaves and *S. aureus* organism. The primary objective was to establish whether there is a difference in antibacterial activity of *M. indica* leaf extract against *S. aureus* processed using different techniques. Another aim was to find out the effect of the concentration of *M. indica* leaf extract against growth of *S. aureus*.

It is expected that the findings of the study will help to reduce antibacterial potency losses of the plant extracts due to poor processing methods.

2. Materials and Methods

2.1 Source of raw plant materials and organisms

Local fresh *M. indica* (*Maboloma* variety) leaves were collected from Malamulo College of Health Sciences in Malawi. The plant's identity was confirmed by a botanist. *S. aureus* organisms (BGT25N-STA) were obtained from Malawi Liverpool Welcome Trust.

2.3 Preparation of *M. indica* leaf extract

Initially, fresh leaves were sorted and cleaned from any extraneous material and contaminants using distilled water. They were put in plastic zip lock bags and taken to Malamulo College of Health Sciences Microbiology Laboratory for experimentation. The leaves were cut into pieces and were shade dried for 7 days. After drying, they were ground into fine powder using different processing methods (mortar and pestle, stone, home blender and laboratory mill). The bioactive compounds were extracted using the methods of Olasehinde *et al*. [6] with slight modification. 100g of the powder was soaked into 1000 ml of ethanol for 72 hours at 6 ºC. The extract was shaken vigorously and filtered using Whatman filter paper number 1 to obtain 100 % concentration. The extract was stored in a refrigerator at 4 ºC ready for experiment.

2.4 Determination of antibacterial activity of *M. indica* leaf extract against *S. aureus*

Antibacterial activity of differently processed leaf extracts was evaluated using agar well disc diffusion method. The extract solutions were prepared at different concentrations of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml and 50 mg/ml. Then paper discs were impregnated with extracts and placed onto the Mueller Hinton nutrient agar inoculated with *S. aureus* (0.5 McFarland's standard) using sterile swab stick [11]. They were incubated at 37 ºC for 18 hours and 25 g/l of Amoxillin was used as a control. After incubation, the zone of inhibition was measured to the nearest millimeter (mm) to assess antibacterial activity of different concentrations of leaf extracts.

2.5 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC)

Two-fold serial broth macrodilution method was used to estimate MIC of the extracts. Sequential extract concentrations of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, and 50 mg/ml were poured into test tubes containing 2 ml nutrient broth. According to NCCLS [12], standardized inocula of 0.1 ml of *S. aureus* was added to each tube and were incubated at 37°C for 24 hour. MIC was taken as the least concentration of *M. indica* leaf extract that showed no observable growth (no turbidity). Samples from tubes that showed no visible bacterial growth during MIC determination were inoculated into separate nutrient agar plates. The plates were incubated at 37 ºC for 24 hours. The least concentration of the extract that showed no colonies on the surface of the medium after incubation period was regarded as MBC [12].

2.6 Data analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS) version 22 (SPSS Inc. Chicago, IL, USA) at 0.05 significance level. All the laboratory analyses were conducted in triplicates and SPSS was used to generate the mean. ANOVA and general linear model were run.

3. Results and discussion

3.1 Antibacterial activity of differently processed *M. indica* leaves on *S. aureus*

The results of different processing methods on antibacterial activity of ethanol leaf extracts of *M. indica* on *S. aureus* are presented in Table 1. The largest zone of inhibition of 14.5 mm was recorded on laboratory mill method which was significantly higher than other processing methods. Mortar and pestle, stone and blender methods achieved similar zone of inhibitions.

Table 1: Zone of inhibition of the extract against *S. aureus* for different processing methods

Processing method	Mean zone of inhibition (mm)	P-value (<0.05)
Mortar and Pestle	13.4[a]	0.77
Grinding by Stone	12.3[a]	
Blender	13.0[a]	
Laboratory Mill	14.5[b]	

The means with similar superscripts are statistically the same.

Antibacterial activity of *M. indica* leaf extract was due to the presence of gallotannin and mangiferin bioactive compounds [7]. The leaves also contain mangiferin, Tannin, saponin, steroid, flavonoid and glycoside which are major phytochemicals that contribute to the inhibitions [8, 13, 6, 14]. These findings suggest that laboratory mill processing method could maximize retention of bioactive compounds and preserve degradation of these compounds during processing of plant leaves into powder. It is likely that higher antibacterial activity of the leaves processed by laboratory mill resulted from production of fine powder which retained more bioactive compounds compared to other processing methods [15] Data on effect of temperature on levels of phytochemicals during production of powder is limited. Thus, a laboratory mill method can be cost-effective venture, especially in developing countries for retaining the bioactive compounds activity of *M. indica* leaves. In countries where electricity is not reliable or available people can use simple methods such as stones and pestle-mortar because they can yield similar results to home blender. This research work corroborates with similar findings of Islam *et al*. [17] who reported that ethanol extract of *M. indica* leaves have antibacterial properties.

3.2 Antibacterial activity of different concentrations of *M. indica* leaf extracts against *S. aureus.*

Data on antibacterial activity of different concentrations of *M. indica* leaf extract on *S. aureus* is provided in Table 2. The mean zone of inhibition ranged from 10 mm to 16.7 mm and increased significantly with increase in extract concentration. However, there was no significant difference between concentrations of 6.25 and 12.5 mg/ml. Similarly, concentrations of 25 and 50 mg/ml were statistically the same.

Table 2: Antibacterial activity of *M. indica* leaf extract against *S. aureus*

Concentrations (% w/v)	Mean zone of inhibition (mm)	P-Value (<0.05)
6.25	10.0[a]	0.000
12.5	12.1[a]	
25	14.5[b]	
50	16.7[b]	

The means with different superscripts are significantly different at alpha 0.05.Values are mean zones of inhibition of triplicates.

These findings show that increasing concentration from 6.25 mg/ml to 12.5 mg/ml did not affect antibacterial activity of the leaf extract formulations but from 12.5 mg/ml to 25 mg/ml since there were more bioactive compounds in higher concentrations against *S. aureus.* Interestingly, activity of 25 mg/ml and 50 mg/ml concentrations were significantly the same. Application of *M. indica* leaf extract does not require increasing concentration of extract from 25 mg/ml which in any way does not significantly enhance its efficacy in the treatment of patients. This trend of zone of inhibition was not influenced by different processing methods that the leaves were subjected to before extraction of bioactive compounds (Table 3). The implication of the findings is that people from poor resource and even industrialized countries can cost effectively use 25 mg/ml concentration to treat *S. aureus* infection hence the results were similar to 50 mg/ml concentration's activity. Observed antibacterial activity of 25 mg/ml leaf extract concentration corroborates published report by Shabani and Sayadi [5].

3.3 Effect of processing method, concentration of the extract and their combined effect on antibacterial activity

Processing method and concentration of the extract had an effect (p = 0.000) on antibacterial activity of *M. indica* leaf extract against *S. aureus*. However, there was no interaction effect (p = 353) of these two factors on antibacterial activity (Table 3.0).

Table 3: Effect of processing method, concentration and their interaction on antibacterial activity

Tests of Between-Subjects Effects						
Dependent Variable: Zone of inhibition						
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig**	**Partial Eta Squared**
Corrected Model	342.979[a]	15	22.865	20.708	.000	.907
Intercept	8506.687	1	8506.687	7704.170	.000	.996
Methods	29.729	3	9.910	8.975	.000	.457
Concentration	301.729	3	100.576	91.088	.000	.895
Methods * Concentration	11.521	9	1.280	1.159	.353	.246
Error	35.333	32	1.104			
Total	8885.000	48				
Corrected Total	378.312	47				

Methods * Concentration = interaction between processing method and concentration of the extract. R Squared = .907 (Adjusted R Squared = .863).

Absence of interaction between processing method and concentration of the extract means that zone of inhibitions for the processing methods were not influenced by the concentration of the leaf extract and vice versa. In other ways, these two factors presented their effects independent of each other. As such, one would choose any processing method and any concentration in order to maximize the results since there was no significant combined effect of the two factors in influencing antibacterial activity of *M. indica* leaf extract.

3.4 MIC and MBC of *M. indica* leaf extract against *S. aureus*

The MIC was observed at 6.25 mg/ml while the MBC was 12.5 mg/ml (Figure 4).

Table 4: MIC and MBC of the extract against *S. aureus*

Leaf extract Concentration	MIC	MBC
6.25	N	P
12.5	N	N
25	N	N
50	N	N

Key: N = no observable growth; P = observable growth.

Although MIC was observed at 6.25 mg/ml, the actual MIC could be lower than this because this was the lowest concentration of the extract tested against *S. aureus*. Most importantly, MIC and MBC values against *S. aureus* were low and this means that *M. indica* leaves have the potential to treat any infection associated with *S. aureus* effectively. The MIC of *M. indica* leaf extract observed in this study differs with earlier findings where MIC ranging from 62.5 mg/ml to 75 mg/ml were recorded for *S. aureus* [6, 17]. These differences are due to variation in solvents, temperature, strains of *S. aureus*, concentration of extract and varieties of *M. indica* used in the experiments.

4. Conclusion and recommendations

The study has demonstrated that laboratory mill method is the most effective method compared to pestle and mortar, stone and home blender methods of processing *M. indica* leaves into powder as a means of retaining more bioactive compounds activity of *M. indica* leaves against *S. aureus* in Malawi. But we cannot rule out the use of traditional methods which demonstrated that they are capable of retaining bioactive compounds. Both processing method and concentration of the extract influenced antibacterial activity of *M. indica* leaves against *S. aureus* although combined effect was not observed. Additional work is encouraged to determine the levels of bioactive compounds following preparation of extracts using different processing methods.

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