

Effects of different extraction methods on in-vitro antimicrobial properties of *Lagenaria breviflora* whole fruits

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Abstract: The Steeped and Soxhlet methanolic extracts of *Lagenaria breviflora* whole fruits were screened for secondary metabolites and antibacterial activities against Enterohaemorrhagic *E.coli* (EHEC), *Salmonella typhi*, *Salmonella paratyphi*, *Pseudomonas fluorescens*, *Shigella dysenteriae*, and *Shigella flexneri*. The methanolic extracts of *L.breviflora* contain some secondary metabolites such as alkaloids, tannins, anthraquinones, terpenoids, flavonoids and reducing sugars. Soxhlet methanolic extracts were the most active, showing activities against pathogenic bacterial isolates but Steeped extracts showed resistance to *Shigella dysenteriae*, *Shigella flexneri*, and *Pseudomonas fluorescens*. Extracts of *L. breviflora* was able to exhibit higher bactericidal action against EHEC, *S. typhi*, *P. fluorescens* and *Salmonella typhi* while *S. dysenteriae* was the least affected. A significant difference was established between soxhlet and steeped extracting methods of *L. breviflora* ($\chi^2 = 10.01$; $p < 0.05$). The mean MIC of *L. breviflora* against some pathogenic bacterial agent showed a significant difference ($F = 10.25$, $P < 0.05$). Further analysis by least significant difference (LSD) showed *Salmonella paratyphi*, *P. fluorescens*, EHEC and *S. typhi* to be most susceptible isolates in increasing order while the extract was less inhibitory to *Shigella dysenteriae*. These extracts exhibit antibacterial activities against some clinical pathogens involved in gastroenteritis and wound sepsis. [T.A. Banjo, L.S. Kasim, B.A. Iwalokun, W.B.Muti, W.E. Olooto, N.G. Mba, E.S. James and T.O. Shorunmu. **Effects of different extraction methods on in-vitro antimicrobial properties of *Lagenaria breviflora* whole fruit** *N Y Sci J* 2013;6(10):60-65] (ISSN: 1554-0200). <http://www.sciencepub.net/newyork>. 11

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1. Introduction

Plants such as herbs, shrubs or trees are natural sources of organic chemical on earth, valuable in part or in whole in the treatment and management of diseases and disorders dating back to the prehistoric days. A vast reservoir of medicinal plant abounds mostly in Africa where its limited resource controlled-communities are highly predisposed to disease burdens. A claim of beneficial responses and human reliance on use of herbal remedies persists amongst Africans' large population (Fasola,2000; Okwu,2006). Extracts of plants which form the basis for all traditional systems of medicine have been used for the treatment of various diseases as reported by Kalimuthu (2010). Moreover, Ekundayo (2011) established that the use of available medicinal plants in the treatment and control of diseases in any local community would help maintain and play significant roles in medical health care accomplishment in the developing countries. And the medicinal values of

these plants lie in their component photochemicals mostly alkaloids, tannins, flavonoids and phenolic compounds (Hill, 1952) which produce definite physiological actions on the human body.

Lagenaria breviflora (*wild colocynth*) is one of those numerous plants with characteristic antibacterial and antiviral herbal remedies in local communities such as Nigeria (El-Mahmood *et al*, 2008). It is one of the collections of West Tropical African's fruits, a perennial climber ascending to the forest canopy generally widespread in tropical Africa. It belongs to the family: Cucurbitaceae (United States Department of Agriculture, 2001); genus: *Lagenaria*; and specific epithet: *breviflora* (Burkill, 1985). The leaves are extremely scabrid and sandpappy while the fruits are dark green with creamy blotches, and are ovoid to 9 cm long. The plant, and more than ever, the fruit is widely used in traditional medicine in West Africa as a herbal remedy for a wide range of gastrointestinal disorders and the treatment of human measles. Also, it

possesses broad spectrum antimicrobial activity (Tomori et al., 2007). And the phytochemical analysis of *L. breviflora* showed that it contains variety of chemical compounds ranging from saponins to phenolic acids (Elujoba et al., 1991).

Sequel to the persistent evolution of bacteria resistance to currently available antibiotics, this study seek to compare the efficacies of soxhlet and steeped extraction methods for the active phytochemical contents of *Lagenaria breviflora* whole-fruit, in relation to its antibacterial properties on certain pathogenic bacterial isolates.

2.0 Materials & Methods

Matured fruits of *Lagenaria breviflora* purchased from Sagamu retail Market in Ogun state, Nigeria, were validated at the Nigerian Institute of Medical research, Yaba, Lagos. The fruits were washed under running tapwater, then, washed again using sterilized distilled water. Thereafter, they were cut into pieces, and grinded to liquid pulp using an electric binatone fruit blender. Extraction techniques used included soxhlet and steeped methods. The ground whole fruits were shared in two parts. One part was extracted in soxhlet machine at the solute solvent ratio of 1 : 10 (1gm of the fruit pulp to 10ml of 70% methanol) for 6hrs. The other half was steeped with 70% methanol, macerated in water bath for about 6hrs daily for 3-4 days after which the methanol was drained off and replaced with a fresh one. This procedure was

continued until the fruits were no longer extracting. The crude extract was filtered with muslin cloth following which it was finally sieved using whatman no. 1 filter paper. The crude extracts were kept in plastic containers and refrigerated at 20°C. The condenser inlet and outlet were placed on the soxhlet extractor with the inlet connected to the running tap water while the outlet to the run-offs cool the water. The whole setup was placed on top of the regulated heating mantle, clipped down with the aid retort stand and clamped while the heating mantle was connected to the electricity.

The phytochemical analysis of the secondary metabolites was carried out according to Harborne (1973), Trease (1989) and Sofowora (1993) using standard methods for detecting the presence of Saponins (Abate,1989), Alkaloids (Shale,1999) Tannins (Gupta, 2005), Anthraquinones (Moody, 2006), Flavonoids (Sawadogo, 2006). All the microorganisms were clinical isolates of wounds sepsis and gastroenteritis from NIMR (table 1) but *E coli* ATCC 25923 was a standard organism. Samples of each bacteria were collected on slopes and subcultured on MacConkey agar & subsequently on nutrient agar for 24hr at 37°C. Screening for antibacterial susceptibility of methanolic extract was carried out using agar diffusion by punched hole method as described by Lino (2006); and MIC & MBC were determined according to El-Mahmood (2008).

Table 1: Clinical presentations and sources of pathogenic bacterial agents

Pathogenic bacteria isolates	Specimens	Clinical presentations
<i>Salmonella typhi</i>	Stool	Gastro-enteritis
<i>Salmonella paratyphi</i>	Stool	Gastro-enteritis
Enterohaemorrhagic <i>E.coli</i> (EHEC)	Stool	Gastro-enteritis
<i>Pseudomonas fluorescens</i>	Wound	Wound sepsis
<i>Shigella dysenteriae</i>	Stool	Gastro-enteritis
<i>Shigella flexneri</i>	Stool	Gastro-enteritis

Pure colonies of these bacteria were inoculated into sterile peptone water incubated for 24h at 37°C. Muller- Hilton agar was prepared and plated after which the plates were incubated for 24h at 37°C. Four plates were prepared for each bacteria species. The labelled sterile plates were flooded with the broth culture & incubated for 1hr at 37°C. Six holes were punched on each of the plates using sterile cork borer 6.0mm after which the plates were incubated for another 1hr.

2.1 Application of extract on bacterial culture

Five concentrations of the extract 3000, 1500, 750, 375, 187.5µg/ml were used as test while standard antibiotics ciprofloxacin 5mg from oxoid was used as positive control and sterile peptone water as negative control. The five concentrations of the extract were added into the punched holes. Ciprofloxacin 5mg was used as positive control and sterile peptone water as negative control. The plates were then incubated at 37°C for 24hr. Antibacterial activity was determined by measurement of zones of inhibition produced against the test organism after incubation.

2.2 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the extracts against the test organisms was carried out using the broth dilution method (El-Mahmood, 2008). One ml of the extract at concentrations 3000µg/ml was added to 1ml of Nutrient broth to obtain extract concentrations at 3000, 1500, 750, 375, 187.5µg/ml in different test tubes. 1ml of an 18h culture adjusted to 0.5 mcfarland turbidity standard, equivalent to 1.0×10^8 CFU/ml, was added to each test tube. They were mixed properly and incubated at 37°C for 24h. The tube with the lowest concentration or highest dilution without visible growth was considered the MIC.

2.3 Determination of minimum Bactericidal Concentration (MBC)

Two loopfuls of broth culture were taken from the tubes that showed no growth for the MIC determination and were inoculated onto agar plates. The plates were incubated at 37°C for 24h and observed for possible growth. Concentrations that did not show any growth after incubation were regarded as the MBC.

2.4 Statistical analysis:

All data were analysed by SPSS version 15. A comparison between the sensitivities of soxhlet and steeped extract was carried out by Mc Nemar chi-square; mean MIC & MBC of the extract of *L.breviflora* against pathogenic bacterial isolates were compared by ANOVA while the level of significance was determined at 95%.

3. Results

Analysis of the phytochemical contents of *Lagenaria breviflora* in methanolic solvent showed enhanced reducing sugar, anthraquinone and Terpenoids. But flavonoids, tannins and alkaloids were least while saponin and cyanogenic glucoside were absent (table 2).

Table 2: Phytochemical analysis of *Lagenaria breviflora* whole fruits

Phytochemical contents	Results of analysis
Tannins	++
Flavonoids	+
Alkaloids	++
Terpenoids	+++
Anthraquinone	++++
Reducing sugar	++++
Saponin	-
Cyanogenic glucoside	-
Phlobatanins	-

The effects of soxhlet and steeped methods of methanolic extraction on *Lagenaria breviflora* and the antimicrobial susceptibility patterns are shown in tables 3 & 4. The MIC and MBC of methanolic extract on the clinical pathogenic bacterial isolates were indicated in tables 5 and 6.

Table 3: Antimicrobial susceptibility of *Lagenaria breviflora* whole fruits against pathogenic bacterial isolates

Organisms	Soxhlet extract	Steeped extract
EHEC 1	S	S
EHEC 2	S	S
EHEC 3	S	S
EHEC 4	S	S
S. typhi 1	S	S
S. Typhi 2	S	S
S. typhi 3	S	R
S. typhi 4	S	R
S. paratyphi 1	S	R
S. paratyphi 2	S	S

S. paratyphi 3	S	R
S. paratyphi 4	S	S
P. fluorescens 1	S	R
P. fluorescens 2	S	R
P. fluorescens 3	S	R
P. fluorescens 4	S	R
S. flexneri 1	R	R
S. flexneri 2	R	R
S. flexneri 3	R	R
S. flexneri 4	R	R
S. dysenteriae 1	S	R
S. dysenteriae 2	S	R
S. dysenteriae 3	S	R
S. dysenteriae 4	S	R
Key: S: Sensitive R: Resistant		

Table 4: Comparative assessment of antimicrobial efficacy of *Lagenaria breviflora* extracts obtained by soxhlet and steeped methods

	Soxhlet extract		Steeped extract	
	Sensitive	Resistant	Total	
Sensitive	8	0	8	
Resistant	12	4	16	
Total	20	4	24	

$$\chi^2_{MN} = 10.01, P < 0.05$$

$$\text{Sensitivity for soxhlet} = \frac{8}{8} \times 100 = 100.0\%$$

$$\text{Sensitivity for steeped} = \frac{8}{20} \times 100 = 40.0\%$$

Table 5: Comparative Minimum Inhibitory Concentration (MIC) (ug/ml) of *L. breviflora* among some pathogenic bacteria.

Organisms	N	Mean ± SEM	F	P- Value	Most susceptible isolates
<i>S. typhi</i>	4	562.50 ± 108.25	10.25	<0.05	<i>S. paratyphi</i>
<i>S. paratyphi</i>	4	468.75 ± 93.75			<i>P. fluorescens</i>
<i>P. fluorescens</i>	4	468.75 ± 93.75			EHEC
EHEC	4	515.63 ± 140.63			<i>S. typhi</i>
<i>S. dysenteriae</i>	4	562.50 ± 437.50			

Table 6: Comparative Minimum Bactericidal Concentration (MBC) (ug/ml) of *L. breviflora* among some pathogenic bacteria

Organisms	N	Mean ± SEM	F	P- Value	Most susceptible isolates
<i>S. typhi</i>	4	1125.00 ± 216.51	10.25	<0.05	EHEC
<i>S. paratyphi</i>	4	937.50 ± 187.50			<i>S. paratyphi</i>
<i>P. fluorescens</i>	4	937.50 ± 187.50			<i>P. fluorescens</i>
EHEC	4	843.75 ± 235.93			<i>s. typhi</i>
<i>S. dysenteriae</i>	4	4875.00 ± 1125.00			

Discussions

It has been established that the methanolic extracts of *L. breviflora* contained some secondary metabolites such as alkaloids, tannins, anthraquinones, terpenoids, flavonoids and reducing sugars as established by Cowan (1999). Soxhlets extraction method proved to be 100% sensitive (except for *S. flexneri*) when compared to the steeped technique. The presence of flavonoids and tannins in the fruit of this plant is indicative of its anti-inflammatory and analgesic effects. The Flavonoids and tannins are phenolic compounds suggestive of its primary antioxidants as documented by Adedapo (2008) and Ayoola (2008). Also, Lai (2010) established tannins and saponins as effective antioxidants, antimicrobial, and anti-carcinogenic agents while flavonoids are known to target prostaglandins which are implicated in the late phase of acute inflammation and pain sensitivity as affirmed by Rajnarayana (2001) and Chakraborty (2004). However, cynogenic glycosides were absent in the *Lagenaria breviflora* fruits studied when compared to its presence in the leaves according to the findings of Adedapo (2013).

And the extracts were found to exhibit antibacterial activities against some clinical pathogens such as EHEC, *S. typhi*, *S. paratyphi*, *P. flourescens* and *S. dysenteriae* implicated in gastroenteritis and wound sepsis, exhibiting a mean minimum inhibitory concentrations ranging from 468.75 to 562.50 µg/ml hence becoming a potential baseline for future drug development. According to the affirmation of Egwaikhide & Gimba (2007), the pharmacological actions of the plants cannot be ascertained based on the phytochemical analysis alone.

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