Time-Dependent Evaluation Of The Antibacterial And Phytochemical Properties Of Vernonia Amygdalina And Gongronema Latifolium

K Enyi-Idoh, S Utsalo, J Epoke, G Arikpo, M Eja

Abstract

Background: Many plants in Africa are medicinal including domesticated edible vegetables. The time-dependent antibacterial efficacy of two popular Nigerian vegetables (Vernonia amygdalina and Gongronema latifolium) on clinical isolates of Staphylococcus aureus and Escherichia coli was evaluated. Phytochemical compositions were also evaluated using direct chemical estimation and thin layer chromatography respectively.

Methods: Leaves were extracted with aqueous and alcoholic solvents at 2 hours and 72 hours at various concentrations and tested against bacteria using the agar well diffusion method. Minimum inhibitory/bactericidal concentrations were also determined. Results: Ethanol extracts of fresh leaves were more active than methanol extracts when were decocted in 98% alcoholic concentrations. The highest zone size obtained was 18mm at 125mg/ml concentration of ethanol extract against S. aureus. Activity was most prominent at 2 hours of decoction. Both plants had inhibition values of 2500 μg/ml each on E. coli as MIC and MBC while G. latifolium showed 5000 μg/ml each as MIC and MBC and V. amygdalina showed 625μg/ml each as MIC and MBC values respectively on S. aureus. The plants were shown to have ample quantities of saponins, flavonoids and alkaloids.

Conclusions: This result has shown the relative potency of edible vegetables against bacteria. The level of phytochemical compounds bitter vegetable plants contain points to the usefulness of G. latifolium and Vernonia amygdalina as credible supplements for antibiotics when used fresh or used as fresh solvent decoctions.

INTRODUCTION

The clinical success of medicinal extracts from plants and plant parts have rekindled the interest in medicinal plants as potential sources of novel drugs. The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into the African continent. Herbal medicine has been widely used and forms an integral part of primary health care in China, Ethiopia and Argentina. A significant proportion of pharmaceutical products in current use are designed from plants. Vernonia amygdalina Del., (African bitter leaf) and Gongronema latifolium are primarily indigenous to Nigeria/Africa and used extensively in the management and treatment of a number of ailments like diabetes mellitus.

Gongronema latifolium, of the family Asclepiadaceae, also known as ‘Utazi’ and ‘Arokeke’ in south eastern Nigeria and South-western Nigeria, is an edible tropical rainforest tree climber with woody hollow glabrous stems characterized by greenish yellow flowers. It is widely used as a staple vegetable and spice in traditional medicine in West Africa. The plant is supposedly native to South-Eastern Nigeria but also found in deciduous forests from Guinea-Bissau to Western Cameroons. Antimicrobial activity of G. latifolium and Vernonia amygdalina has been reported on the aqueous extracts and their blends on some beer spoilage organisms.

V. amygdalina is a small tree (1-3 m high) that grows throughout tropical Africa and has been domesticated in some parts of West Africa and. Nigeria, where it is locally known as bitter leaf vegetable in soups. Bitter leaf is reported to contain an alkaloid, vernomine, which is capable of reducing headaches associated with hypertension. The therapeutic relevance of all parts of bitter leaf as a useful folkmedicine and a popular vegetable adjunct in the
delicious onubu soup has been reported by some researchers 15, 16, 17, and 18. The divergent and discordant experimental methods identified with plant research, lacking any semblance of standardized procedures for plant extract preparation, plant/solvent dilution ratios, solvents used for extraction and susceptibility test methods are some factors that informed the need for this research. The purpose of the present study was to comparatively evaluate the antibacterial efficacy, minimum inhibitory and bactericidal concentrations (MIC, MBC) of freshly harvested and dried leaf samples of G. latifolium and V. amygdalina by direct decoction and Soxhlet extraction methods using aqueous and alcoholic solvents of different concentrations and solvent/plant dilution ratios against antibiotic sensitive clinical strains of Escherichia coli and Staphylococcus aureus and thus to determine the significance of plant/solvent dilution ratios and the effect of plant/solvent contact time on the antibacterial activity of the plant samples. MATERIALS AND METHODS COLLECTION OF MATERIALS The leaves of Vernonia amygdalina (Bitter leaves) were harvested from cottage gardens in Calabar, while fresh healthy looking Gongronema latifolium were bought from vegetable sellers at the Calabar Watt market. The leaves with no signs of external damage were used for ethanol, methanol and aqueous extractions by direct decoction and soxhlet extraction methods using different solvents of different concentrations and solvent/plant dilution ratios against antibiotic sensitive clinical strains of Escherichia coli and Staphylococcus aureus and thus to determine the significance of plant/solvent dilution ratios and the effect of plant/solvent contact time on the antibacterial activity of the plant samples.

SOURCE OF ORGANISM Known isolates of Staphylococcus aureus and Escherichia coli were obtained from the bacteriology laboratories of the University of Calabar Teaching Hospital, Calabar, Nigeria. They were known to be sensitive to a wide spectrum of regular prescription antibiotics.

EXTRACTION OF PLANT MATERIALS Fresh, sun dried and shade dried plant leaves were used for the research. Fresh plant leaves were washed under running tap water, air dried and chopped to fine piece that could be weighed. Leaf samples were also sun dried for several days until easily breakable and ground to a fine powder. Shade dried leave samples were dried in flat plastic trays in an airy place at ambient temperature away from direct sunlight. After the leaves were dry enough to break, they were ground to fine powder in a blender and stored in clean stopper bottles until used for soxhlet extraction. Extraction was done by a method previously described9 with a variation in the time used for extraction which in this case was 2 hours and 72 hours, with gentle agitation before refluxing. Soxhlet alcohol extracts were prepared by extracting 50g, 20g, 12.5g, 10g and 5g of dried powdered leaves of both plants with appropriate volumes of 70% and 98% ethanol and methanol for 3h.

ANTIBACTERIAL SENSITIVITY TESTING Agar well diffusion technique11 was used to determine the antibacterial activity of the extracts. The seeded plates were allowed to set and a standard cork borer of 6 mm diameter was used to cut out wells in the agar. A drop of molten agar, previously held in a water bath, was aseptically added to the wells to seal up the bottom spaces between the agar and Petri dish to avoid seep out of extracts. The wells were then filled with 0.3 ml of each extracts. Absolute ethanol and Tetracycline were used as negative and positive controls which were also put in separate wells. All the plates were incubated at 37°C for 24h right side up. Zones of clearance round each well means inhibition and the diameter of such zones were measured.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) Minimum inhibitory concentrations of the extracts were determined by the macro broth dilution technique19. PHYTOCHEMICAL ANALYSIS Phytochemical quantitative analyses were conducted on the alcoholic solvent extracts of each plant using standard methods20,21. The plant were screened for alkaloids, saponins, tannins, flavonoids, and terpenoids and qualitatively by specific solvent system thin layer chromatography using pre-coated silica gel in Shandon Unikit TLC Chromatank as follows: Alkaloids: Acetone – water - ammonia in the ratio 90:7:3 v/v/v; Flavonoids: Aluminium chloride 1% in methanol (methanol 10mls – water 90mls), Saponines: Sulphuric anisaldehyde, incubation at 105-110°C with evaluation under day light using the solvent chloroform-methanol-water in the ratio 64 :50 :10 v/v/v; Terpenoids and Tannins: Ethyl acetate – acetic acid - formic acid - water in the ratio of 100:11:12:27 v/v/v/v with
saturation. After elution, the developed plates were observed under visible as well as UV light (254 nm and 356 nm) and 
and Rf values obtained by measuring distance travelled by the 
solute/distance travelled by the solvent, noted and recorded.

RESULTS
Antibacterial activities of fresh decocted and dried soxhlet 
extracted V. amygdalina and G. latifolium are presented in 
Tables 1 to 3. The raw activity of both plants against the test 
bacteria was 14mm and 18mm respectively. The test 
organisms were very sensitive to Tetracycline antibiotic 
control used with up to 25mm clearance. G. latifolium has 
the highest activity of 14mm against E. coli at 500mg/ml 
concentration in 98% ethanolic and aqueous extracts after 2 
hours of decoction as shown in Table 1. The highest activity 
of fresh plant extracts was observed at 125mg/ml 
concentration against S. aureus (18.4±0.2mm) in 98% 
ethanol followed by 16.1±0.40mm (Table 2) against E. coli 
at the same solvent but at 500mg/ml and 100mg/ml 
concentration.

MIC and MBC values and the retention factors (Rf) values 
and qualitative values for plant phytochemicals for the test 
plants are shown in Table 4 and Table 5 below. G. latifolium and 
V. amygdalina had MIC and MBC values of 2500 μg/ml on 
E. coli and 5000 μg/ml and 625μg/ml each on S. aureus 
respectively. In Table 6, the quantitative values by direct 
chemical estimation of phytochemical compounds are 
presented. The level of saponins (750mg/g) observed in G. 
latifolium agrees with the Rf value (0.94) seen in the 
qualitative estimation by thin layer chromatography.

V. amygdalina activity was significantly high against S. 
aureus for 500mg to 100mg concentrations of 98% ethanolic 
extracts while G. latifolium activity was significant for E. 
coli at 98% ethanolic concentration extraction with higher 
zones of inhibition observed in extracts left for 72 hours.

Figure 1
Table 1: Antibacterial activity of fresh plant extracts by 
direct decoction against E.coli at 2 and 72 hours decoction

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Raw extract</th>
<th>Assay</th>
<th>Methanol (98%)</th>
<th>Ethanol (98%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. amygdalina</td>
<td>2hrs</td>
<td>14.0±0.04</td>
<td>14.55</td>
<td>14.07±0.04</td>
</tr>
<tr>
<td>G. latifolium</td>
<td>72hrs</td>
<td>15.50±0.003</td>
<td>14.07±0.04</td>
<td>14.07±0.04</td>
</tr>
</tbody>
</table>

After 72h of decoction, 15mm was the highest activity 
observed, at 200mg/ml against E.coli by G. latifolium. 
Initially no activity was observed after 2hours decoction 
against E. coli with G. latifolium but at 72hours activity 
developed (15.50±0.003 and 14.04±0.02) at 200mg/ml and 
100mg/ml concentrations respectively as shown in Table 1. 
V. amygdalina antibacterial activity concentrated around the 
100mg/ml concentration and that of G. latifolium around the 
500mg/ml and 200mg/ml concentration.

Figure 2
Table 2: Antibacterial activity of fresh plant extracts by 
direct decoction on Staph aureus after 2 and 72hours

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Raw extract</th>
<th>Assay</th>
<th>Methanol (98%)</th>
<th>Ethanol (98%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. amygdalina</td>
<td>2hrs</td>
<td>15.20±0.02</td>
<td>15.20±0.02</td>
<td>15.20±0.02</td>
</tr>
<tr>
<td>G. latifolium</td>
<td>72hrs</td>
<td>15.20±0.02</td>
<td>15.20±0.02</td>
<td>15.20±0.02</td>
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<tr>
<td>V. amygdalina</td>
<td>2hrs</td>
<td>14.00±0.002</td>
<td>14.00±0.002</td>
<td>14.00±0.002</td>
</tr>
<tr>
<td>G. latifolium</td>
<td>72hrs</td>
<td>14.00±0.002</td>
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<td>14.00±0.002</td>
</tr>
</tbody>
</table>
DISCUSSION

Fresh extracts of V. amygdalina decocted with ethanol showed greater levels of antibacterial activity against S. aureus (16/14mm) when assayed after 2 hours than when left to stand in the decocting solvent for 72 hours. This activity could not be explained as a longer time in extraction would have been thought to allow more of the active components to be extracted. This activity is limited to the ethanol extracts of the plant samples tested. G. latifolium and V. amygdalina showed significant antibacterial activity at 100mg/ml concentration.

Results obtained in this work complement previous report on the usefulness of G. latifolium as has been documented\textsuperscript{22, 23}. The highest activity of 17mm was seen at the 200mg/ml concentration of G. latifolium on E. coli in the 98% ethanol extract of shade dried plant samples. V. amygdalina in the same solvent had up to 16mm activity against S. aureus at 500mg/ml and 200mg/ml concentrations. Aqueous G. latifolium extracts showed insignificant activity of 8cm and 6cm against E. coli and S. aureus respectively, a relatively poor activity in comparison to the 10mm and 9mm obtained with the ethanol extracts against the same organisms.

A concentration dependent antibacterial activity of aqueous and ethanol extracts of V. amygdalina against E. coli and S. aureus, occurring in increasing proportions has been previously reported\textsuperscript{24}, with minimal MICs at 12.5mg/ml only for both E. coli and S. aureus.

Very significant activity of G. latifolium against E. coli and S. aureus with zones of inhibition of 17mm and 16mm respectively has also been previously reported\textsuperscript{9, 12}. The presence of phytochemical compounds in the test plants as detected by their R\textsubscript{f} values when determined qualitatively and quantitatively agrees with earlier reports of the phytochemical composition of G. latifolium which has been shown to consist of about 0.5% flavonoids, 2% tannins, 0.66% saponins, 0.33% polyphenols, 1.97% alkaloids and 13.2% hydrogen cyanide. Appropriately surmised therefore it can be said that the presence of phytochemical compounds in these plants as has been reported\textsuperscript{8} could be responsible for the antibacterial activity of G. latifolium and V. amygdalina. However, the time dependent activity which formed the focus of this research has not been adequately documented. The significance of this fact is related to the common practice by rural and urban herbal medicine sellers in Nigeria to steep plant parts in alcohol solvents and kept for very long periods. If these mixtures remain active and potent against their therapeutic targets after keeping for so long.
cannot be ascertained empirically. This research has however thrown some light on the disparity of time dependent antibacterial activity of these highly prolific and frequently used Nigerian vegetables.

There is a general higher level of activity of the ethanol extracts over the methanol extracts. It was also observed that an increase of activity occurred over time with ethanol extracted principles. The aqueous extracts of the plant samples were less active on the test organisms compared to the raw and alcohol extracts. This agrees with previous reports\textsuperscript{12,25} that aqueous extracts of \textit{G. latifolium} showed no activity against \textit{S. aureus} and \textit{E. coli} even at high concentrations. It was observed that in both forms of extraction i.e. by direct decoction in solvents and by soxhlet extraction, solvent at higher concentration of 98\% (absolute) was able to extract more of the active compounds from the plant samples. Generally, the activity of 70\% solvent concentration by direct decoction and soxhlet extractions was poor. It was also observed that activity of plant extracts showed greater activity against \textit{E. coli} than \textit{S. aureus}. Activity was also found to decrease as the solvent dilution increased, giving lesser zones of inhibition. Incidentally, activity was highest at the 200mg/ml concentration for all plant extracts and test organism accordingly.

The reason for the increased antibacterial activity of shade dried samples over fresh samples could be found in the fact that the drying process concentrated the quantity of the active principles in the leaves as the dried weight of the leaves usually contain more leaf material when dried. The drying process allows more leaf material to be powdered and when weighed the dried leaves have comparably more quantity of leaf material than when fresh saturated leave samples were weighed and used.

Shade drying also preserved the active principles as radiation and direct sunlight could have been responsible for the lack of activity of the first set of sun dried leaves used.

\textbf{CONCLUSION}

The results obtained from this work has revealed that plant/solvent contact time has relative significance with no particular pattern observed even though plant decocted at 2 hours seem to have more antibacterial activity. Ethanol solvent decocted extracts at 98\% concentration had better antibacterial activity against test bacteria than the methanol and aqueous extracts. It was also observed that activity concentrated around the 200m/ml and 100mg/ml plants extract concentrations. The presence of phytochemical secondary metabolites in the plants used attested to the plants antibacterial activity observed, however it may be possible that some of these phytochemical compounds are heat labile and affected by exposure to specific solvent systems and may exist at better states and concentrations in fresh plant materials like leaves, barks and stem and roots.

In Nigeria, it is common practice to see mobile herbal medicine sellers and shops display bottles containing solvent-soaked plant parts. Such bottles may have been prepared several weeks earlier but still sold to clients for medicinal purposes. The instability of phytochemical compounds over time in plant leaves as seen in this study casts doubt to the viability of such herbal concoctions and may pose a health hazard considering the level of clientele.

Further research to expound on the phytochemical and secondary metabolite picture of African edible vegetables may be necessary to build a data base for medicinal plant products that would be of benefit in the public health care delivery system of Africa and other floral endowed regions of the world.

\textbf{References}

10. Morebise O, Fafunso MA, Makinde JM, Olajide OA &
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