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Research Article

INVESTIGATION ON INTERACTIVE ANTIBACTERIAL ACTIVITY OF *MILLETTIA ABOENSIS* AND *GONGRONEMA LATIFOLIUM* LEAF EXTRACTS AGAINST *ESCHERICHIA COLI* AND *SALMONELLA* KINTAMBO TYPED CULTURES

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Abstract

Due to bacterial resistance to synthetic antibiotics and associated problems, treatment of bacterial infections with single antibacterial agent is becoming a challenge. There are growing needs to search for combined agents from plant source that can be effective in the treatment of bacterial infections, hence, overcoming the problem of bacterial resistance. *Millettia aboensis* extract (MAE) and *Gongronema latifolium* extract (GLE) are widely combined concoctions by tradomedicine practitioners for treatment of gram-negative bacterial infections in South Eastern Nigeria. The aim of this study was to investigate on interactive antibacterial activity of *Millettia aboensis* and *Gongronema latifolium* leaf extracts against *Escherichia coli* and *Salmonella kintambo* typed cultures. This was done by extraction, determination of individual MICs of MAE and GLE using microdilution technique in a 96-well microtitre plate, subsequent determination of combined MICs using Checkerboard technique and determination of fractional inhibitory concentration index (FICI) value required for interpretation of type of interaction. From the results, individual MICs of MAE and GLE on *Escherichia coli* are 64mg/ml and 16mg/ml, while combination MICs is 32mg/ml and 16mg/ml respectively. On *Salmonella kintambo*, individual MICs of MAE and GLE are 32mg/ml and 16mg/ml, while combination MICs is 16mg/ml and 8mg/ml respectively. The FICI values on *Escherichia coli* and *Salmonella kintambo* are 1.5 and 1.0 respectively. Therefore the interactions between MAE and GLE on *Escherichia coli* and *Salmonella kintambo* are indifference and additive respectively.

Keywords: Extracts, Microdilution, Checkerboard, Fractional Inhibitory Concentration Index

INTRODUCTION

Bacterial infections have been on the increase due to human and environmental factors [1]. Therapeutic gains of synthetic antibiotics have been compromised by potential development of tolerance or resistance to antibacterials from the time they were first employed [2]. More so, the growing incidence of bacterial resistance, especially treatment of infection with single antibiotics with limited action, is a global health concern currently associated with higher mortality and morbidity rates, higher expenditure in health care and prolonged hospitalizations [3,4]. Therefore there is urgent need to search for medicinal plants that contain bioactive compounds as well as plant derived substances with antimicrobial potentials [5,6], with strong emphasis on effective combinations that can combat the problem of bacterial resistance [7]. The combination approach is based on the concept that the more target a drug has on microorganism the more difficult the cell develops resistance [8]. The use of medicinal plants as natural care remedies is a common tradomedicinal practice in Asia, Latin America and Africa [9]. The untapped Nigeria forest reserve is a rich source medicinal plants that can be used to prevent and cure bacterial infections. *Millettia aboensis* and *Gongronema latifolium* is a concoction mixture claimed by tradomedicine in South Eastern Nigeria as a remedy for bacterial infections.

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Millettia aboensis of family Leguminosae, commonly known as “uturuekpa” in South Eastern Nigeria, is a small tree of 30-40 feet high with a rust-hairy leaves and purple flowers. In the rain forest zone of Nigeria and Cameroon it is characterized by streaked dark reddish or chocolate colored wood [1,10]. A decoction of the leaf, stem and root is used in combination with other plant species in the cure of venereal diseases such as gonorrhea and syphilis [11] and it exhibits antimicrobial activities on pathogenic microorganisms when used alone [12]. *Gongronema latifolium*, locally called “utazi” in South Eastern Nigeria, is a tropical rain forest plant belonging to the family Asclepiadeceae [13]. The plant produces white latex and yellow flowers [14]. The essential oil and extracts of *Gongronema latifolium* possess moderate inhibitory activity that is comparable to ampicillin against *E.coli*, Salmonella specie, Shigella specie, Staphylococcus specie, *Klebsiella pneumonia*, *Onchrobactrum antropi* and *Candida albican* microorganisms isolated from blood stream of HIV patient [15]. Methanolic extract of the plant leaves produces inhibitory activity against Salmonella species such as *Salmonella enteriditis*, *Salmonella cholerasius* and *Salmonella typhimurium*, while the aqueous extract produces inhibitory activity against *E. coli* [16].Considering the vast potentiality of plants as alternative sources to synthetic antibacterials, an investigation on interactive antibacterial activity of *Millettia aboensis* and *Gongronema latifolium* leaf extracts against *E. coli* and *Salmonella kintambo* typed cultures was deemed worthwhile.

MATERIALS AND METHODS

Source and Authentication of Plant Samples

Fresh matured leaves of *Millettia aboensis* and *Gongronema latifolium* were collected from a garden in Okigwe, South Eastern Nigeria in the morning hours between 9 – 10am. The plant samples were identified and authenticated in the Herbarium of Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria, were voucher specimens were deposited for reference purpose with identification numbers, UPH/P/1470 and UPH/P/1471 for *Millettia aboensis* and *Gongronema latifolium* respectively.

Consumables and Reagents

Muller Hinton Agar (Biolab, Budapest, Hungary), Muller Hinton Broth (Biolab, Budapest, Hungary), MacConkey Agar (Highflow Biotech, UK), Salmonella/Shigella Agar (Titan Biotech, India), Nutrient Broth (Titan Biotech Ltd, India), Nutrient Agar (Titan Biotech Ltd, India), Barium Chloride (Super Tek Chemical, Germany), Dimethylsulphoxide, Tetraoxosulphate VI acid (Hi Media Laboratories Pvt Ltd, India) Hydrochloric acid (Nice Laboratories Reagent, Kevala, India), Ferric Chloride (Super Tek Chemical, Germany), Sodium Hydroxide (Rankem Mumbai, India), Sodium tetraoxocarbonate IV (Sigma Aldrich Chemie, Germany), Glacial acetic acid (Sigma Aldrich Chemie, Germany), 96% Ethanol (Gungsdong Guandgua Chemical Factory ,China), Ethylacetate (Rankem, Mumbai, India).

Collection and Confirmation of the Test Organisms

This study used two typed cultures: *Escherichia coli* (ATCC, 11775) and *Salmonella kintambo* (SSRL, 115). They belong to the family Enterobactriaceae and were obtained from Bioresources Development and Conservation Project Centre, Nsukka, Nigeria. Using the procedure described by [17], the cultures were purified by repeated sub-culturing and isolation using various selective media (MacConkey Agar for *E. coli* and Salmonella/Shigella Agar for *S. kintambo*), and then confirmed by Gram staining reaction and biochemical tests to observe distinct cell characteristic and morphology. The organisms were stored appropriately on agar slant at refrigeration temperature throughout the study and used as stock cultures.

Preparation of Plant Materials

Fresh matured leaves of the plants were washed and rinsed severally with clean tap water to remove extraneous matters. Water remnants on the leaves were removed by placing in hot-air oven for about five minutes. Thereafter, the leaves were air-dried under shade for about two weeks to a constant weight. About 3.0kg of each of the plants leaves was blended into powder and stored in air-tight containers until when needed.

Proximate Composition Analysis

Using the procedure described by [18], the powdered plant materials were analyzed for ash value, moisture content, fat, fibre, protein and carbohydrate.

Extraction of Plant Material

For each of the plant materials, about 300g of dry powder was wrapped in Whatmann number 1 filter paper and placed in the holding chamber of reflux extractor. Extraction was carried out with about 600ml of 80% ethanol using reflux method at $40^{o}C$ for 48 hours period. Thereafter, the extracts was concentrated using rotary evaporator to expel the ethanol. The concentrated (dried) residues were kept in air-tight containers labeled “MAE” for *Millettia aboensis* extract and “GLE” for *Gongronema latifolium* extract, until when needed.

Phytochemical Analyses

Qualitative and quantitative phytochemical analyses were conducted on the plant extracts using the procedures described by [19, 20, 21].

Standardization and Reconstitution of Plant Extracts

A 10g amount of each of the plant extracts was initially dissolved with 5ml Dimethylsulphoxide (DMSO) in a measuring cylinder. Thereafter, the resulting solution was made up to 10ml to give a stock concentration calculated as:

Weight of cylinder alone = 46.68g
Weight of cylinder + plant extract = 56.68g
Weight of plant extract = 10.0g
10ml DMSO + 10.0g plant extract = 10g/10ml = 1000mg/ml

Preparation of MacFarland Standard

MacFarland standard was prepared based on principle that by mixing 0.05ml of 1.0% w/v $BaCl_2.2H_2O$ with 9.95ml concentrated H_2SO_4, with constant stirring gave Barium sulphate ($BaSO_4$) suspension that corresponded to 0.5 MacFarland turbidity standard of approximately $1.5x10^8$ cfu/ml of bacterial suspension. The matching test was done by viewing side by side the tubes that contained bacterial suspension and Barium sulphate standard against a white background. Before each use, the Barium sulphate was agitated vigorously and observed for appearance of uniform turbidity across the entire solution.

Preliminary Antibacterial Susceptibility Screening

This test was done to ascertain the susceptibility of the test organisms (*E. coli* and *S. kinambo* typed cultures) to the plant extracts (MAE and GLE), and also to determine the concentration of the extracts that would produce antibacterial activity. Agar well diffusion technique as described in our previous work [22] was used, where sterile agar plates were seeded with each of the test organisms and thereafter, a cork borer (8mm diameter) was used to bore wells on the agar plates. Different concentration of MAE and GLE were introduced into triplicate well, allowed for one hour for the extract to diffuse into the agar and then incubated at $37^{o}C$ for 24 hours. The zones of inhibition diameter (mm) were measured and mean zone inhibition (MZI) calculated.

Determination of Minimum Inhibitory Concentrations (MICs) of the Extracts on Test Organisms

The MICs of MAE and GLE for each strain of test organisms were determined using two fold serial broth microdilution method described by [23]. In this method, 160ul of Muller Hinton Broth (MHB) was initially distributed to 2nd – 12th well of a 96-well microtitre plate. From the previously prepared stock concentration (1000mg/ml) of each plant extract, 320ul was added to the first well of each row of the microtitre plate from where two-fold serial dilution was performed to the 11th

well. From the 11th well of each row, 160ul was discarded. To the 2nd – 12th well of each row (with exception of 11th well), 40ul of bacterial suspension adjusted at 0.5 MacFarland standard turbidity ($1.5x10^8$ cfu/ml) were added to make a final volume of 200ul. The final concentration of the extracts in the wells ranged between 64 – 0.063mg/ml for MAE and GLE. The 11th well served as negative control (with antibacterial; without bacteria) while the12th well served as positive control (without antibacterial; with bacteria). The experiments were performed in triplicates and the plates were incubated at 37°C for 24 hours. After incubation, 5ul of resazurin was added to assess bacterial growth and MIC was interpreted as the highest dilution or the least concentration that prevented a change in resazurin color [23]. Bacterial growth is detected by color change of resazurin from blue to pink (resorufin).

Determination of Combination Interaction by Checkerboard Method

According to [6, 24, 25], checkerboard method of assessing combined interactive inhibition is based on determining the fractional inhibitory concentration index (FICI) value using 96-well microtitre plates. Based on previously obtained MICs of MAE and GLE, this assay was performed by preparing six concentrations (4MIC, 2MIC, MIC, 1/2MIC, ¼ and 1/8MIC) of each extract. Along horizontal axis across the checkerboard, 160ul of each MAE concentration was added into each well, while on the vertical axis 160ul of each GLE concentration was added (figure 1). On both axes, the final concentration of 2MIC, MIC, 1/2MIC, 1/4MIC, 1/8MIC and 1/16MIC were obtained after 2-fold dilution. Positive and negative control tests were also conducted. The plates were sealed and incubated at 37°C for 24 hours. Wells containing inoculated MHB without extracts (antibacterials) served as positive control, while wells containing extracts (antibacterials) without inoculums (bacteria) served as negative control. The experiments were carried out in triplicates 37°C for 24 hours. After incubation, 5ul of resazurin was added to each well to assess bacterial growth. The combination MIC was interpreted as the least concentration at which there was no change in resazurin color. Fractional inhibitory concentration index (FICI) value for each organism was calculated from fractional inhibitory concentration (FIC) of the two extracts using the formula [26]

$$FICI = FIC_{MAE} + FIC_{GLE}$$

Where:

$$FIC_{MAE} = MIC_{MAE} \text{ in } \frac{\text{Combination}}{MIC_{MAE} \text{ alone}}$$

$$FIC_{GLE} = MIC_{GLE} \text{ in } \frac{\text{Combination}}{MIC_{GLE} \text{ alone}}$$

The FICI values were interpreted as follows [8,27,28,29]:

≤ 0.5 = Synergism
> 0.5 to ≤ 1.0 = Additive
> 1.0 to ≤ 4 = No interaction (Indifference)
> 4.0 = Antagonism

RESULTS

Proximate Analysis

The result of proximate analysis shown in table1 reveals that MAE and GLE contain basic nutrients. The most abundant nutrient is carbohydrate, 36.6 and 41.8 for MAE and GLE respectively. MAE has very low moisture content (0.7), while GLE contains no moisture.

Phytochemical Analyses

The result of qualitative phytochemical analysis of MAE and GLE (table 2) shows the presence of seven and eight classes of secondary metabolites (phytochemicals) respectively. Phenol and sterol were found to be absent in MAE while anthraquinone was tested absent in GLE.

However, the quantification of identified phytochemicals shows that MAE contains highest amount of alkaloids (4.05mg/g), while glycosides is the most abundant (4.18mg/g) in GLE.

Antibacterial Susceptibility Screening

The result of antibacterial susceptibility screening (table 4) shows that all concentrations used in this study produced significant ($p<0.05$) zone of inhibition. At 50mg/ml MAE and GLE respectively produced highest mean zone inhibition diameter (mm) of 19.6 and 17.2 on *E. coli*. Also at 50mg/ml, MAE and GLE respectively produced highest mean zone inhibition diameter (mm) of 18.5 and 15.8 on *S. kintambo*.

MICs of Extracts Alone and in Combination

AS shown in table 5, the MICs of MAE and GLE individually on *E. coli* are 64mg/ml and 16mg/ml respectively. On *S. kintambo*, MAE and GLE produced MICs of 32mg/ml and 16mg/ml respectively. The combined MICs of MAE and GLE on *E. coli* are 32mg/ml and 16mg/ml respectively. On *S. kintambo*, the combined MICs for MAE and GLE are 16mg/ml and 8mg/ml respectively. The FICI values were calculated as 1.5 and 1.0 for *E. coli* and *S. kintambo* respectively

Table 1. Proximate composition (g/100g dry matter) of *Miilettia aboensis* and *Gongronema latifolium* leaves

Component	*M. aboensis*	*G. latifolium*
Moisture	0.7 ±0.01*	-
Carbohydrates	36.6±0.02*	41.8±0.01*
Proteins	23.2±0.01*	20.5±0.03*
Fat	11.4±0.01*	8.3±0.01*
Fibre	14.6±0.01*	16.9±0.01*
Ash	13.3±0.01*	12.5±0.01*

*Value represents mean ±SEM of n=3

Table 2. Qualitative Phytochemical Analysis of *Millettia aboensis* and *Gongronema latifolium*

Phytochemicals	*M. aboensis*	*G. latifolium*
Flavonoids	+	+
Tannins	+	+
Saponins	+	+
Alkaloids	+	+
Glycosides	+	+
Phenols	_	+
Anthraquinones	+	_
Glucose	+	+
Sterol	_	+

+ = present; – = Absent

Table 3. Quantitative Phytochemical estimation (mg/g) of *Milletia aboensis* and *Gongronema latitolium* leaf Extracts

Phytochemicals	*M. aboensis*	*G. latifolium*
Flavonoids	1.32	1.09
Tannins	1.46	2.71
Saponins	2.66	3.10
Alkaloids	4.05	3.98
Glycosides	3.06	4.18
Phenols	-	2.22
Anthraquinones	1.68	-
Glucose	4.11	3.84
Sterol	-	1.74

– = Not detected

Table 4. Result of Antimicrobial Susceptibility Test of Extracts

		Mean Zone Inhibition (mm)	
Extract	Concentration (mg/ml)	*E. coli*	*S. kintambo*
	12.5	10.3±0.16*	8.7±0.10*
MAE	25.0	13.8±0.14*	15.1±0.17*
	50.0	19.6±0.19*	18.53±0.21*
	12.5	7.4±0.11*	9.4±0.18*
GLE	25.0	11.3±0.22*	12.6±0.15*
	50.0	17.2±0.13*	15.8±0.20*

*Value represents mean ±SEM of triplicate plates at p<0.05 significance

Table 5. MICs of extracts alone and in combination and outcome of antibacterial interaction of *Millettia aboensis* and *Gongronema latitolium* extracts combination on typed culture organisms

Organisms	Extract	MIC (mg/ml)		FIC	FICI	Observation
		Alone	Combination			
E.coli (ATCC 11775	MAE	64	32	0.5		
	GLE	16	16	1.0	1.5	Indifference
S.Kintambo (SSRL 115)	MAE	32	16	0.5		
	GLE	16	8	0.5	1.0	Additive

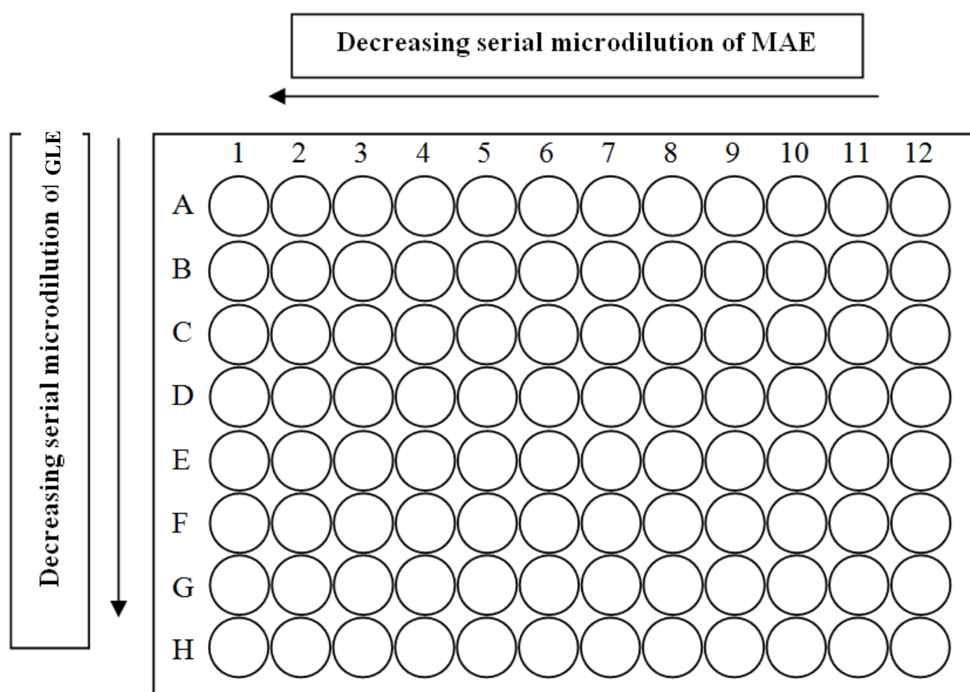

Fig. 1. Checkerboard method for determination of antibacterial interaction of combined *M. aboensis* and *G. latifolium* extracts against test cultures

DISCUSSION

Globally, particularly in Sub-Saharan Africa, *Escherichia coli* and Salmonella specie are critical source of food borne illness and a threat to public health [30]. Recent expansion of bacterial resistance in human and animal to currently used antibiotics has triggered immense interest to search for novel antibacterial agents of plant origin that can be effectively combined [31]. It is noteworthy to mention that about 80% of world population, particularly the rural dwellers, relies principally on medicinal plants for their health care needs [32]. Based on these, this study deemed it worthwhile to investigate on interactive antibacterial activity of *Millettia aboensis* and *Gongronema latifolium* leaf extracts against *Escherichia coli* and *Salmonella kintambo* typed cultures. The result of proximate analysis in table 1 shows that *Millettia aboensis* and *Gongronema latifolium* possess nutrients such as carbohydrate, protein, fats and fibre, This finding conforms with the report by [33] that in addition to mineral and vitamin contents, plants are richly endowed with nutrients. Nature is a unique source of diverse phytochemicals that possess interesting antibacterial activities and other medicinal properties. In this study phytochemical analysis of MAE reveals the presence of flavonoids, tannins, saponinis and glycosides. This finding is similar to the report by [1] on the same plant. Similarly, the finding in this study which identifies the presence of every tested phytochemical (except anthraquinone) conforms to our previous report on *Gongronema latifolium* ethanol leaf extract [34]. In table3, it is shown that MAE and GLE contain different secondary metabolites (phytochemicals) in varying amounts. This finding is in accordance with the report by [35] that secondary metabolites occur in varying concentrations in different plants or in different parts of the same plant, hence, giving each plant or a plant parts unique medicinal property.

These secondary metabolites can work individually or together with other active compounds or phytochemicals of the same plant or other plants [35]. Judging also from the result in table 3, the quantitative yield of the phytochemicals is low. This finding agrees with the report that biologically active compounds usually occur in plants in low amounts [36]. Given the fact that a variety of drugs were obtained from medicinal plants [32] because of their secondary metabolites [37], their combination can increase or decrease their assimilation into biological system (such as microbial cell), toxicity and effectiveness [38]. The result of antibacterial susceptibility screening (Table 4) shows that MAE and GLE exhibit concentration-dependent bacterial growth inhibition. Similar observation was reported by [1] on *Millettia aboensis* against pathogens. In this study, the higher degree of bacterial growth inhibition observed with increasing concentration of extracts might be due to increasing availability of active antibacterial compounds in the medium. The benefit of combination therapy depends on both the property of the drugs and dose ratio [39]. Antibacterials from natural origin can act on different target molecules and processes on susceptible microorganisms to inhibit colonization and replication, inactivate bacterial toxins, modulate metabolic pathways, or reduce rate of protein synthesis [40,41]. Plant extracts when used alone may not produce much effect against bacteria, but report by [42] has shown that the new approach of combining them can enhance their antibacterial activities. A combination is synergistic, additive or antagonistic when the effect is greater than, equal to, or less than summed effect of partner drugs. Based on the result of this investigation, MAE and GLE exhibit no interaction (i.e. indifference) on *Escherichia coli*, and additive interaction on *Salmonella kintambo*.

Conclusion

Based on checkerboard method and FICI interpretation in this study, combination of *Millettia aboensis* and *Gongronema latifolium* produced no interaction (i.e. indifference) against *E. coli*(ATCC, 11775) while additive interaction was observed against *S. kintambo*(SSRL, 115). Further clinical investigations should be carried out on interactions of medicinal plants in order to have effective alternative antibacterials against pathogens and their resistance. Salmonella specie is a common organism implicated in food and drug spoilage. Therefore food and pharmaceutical industries can further explore the potential additive interaction of *Millettia aboensis* and *Gongronema latifolium* in food and drug preservation.

Conflict of interest: The authors declare no conflict of interest

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