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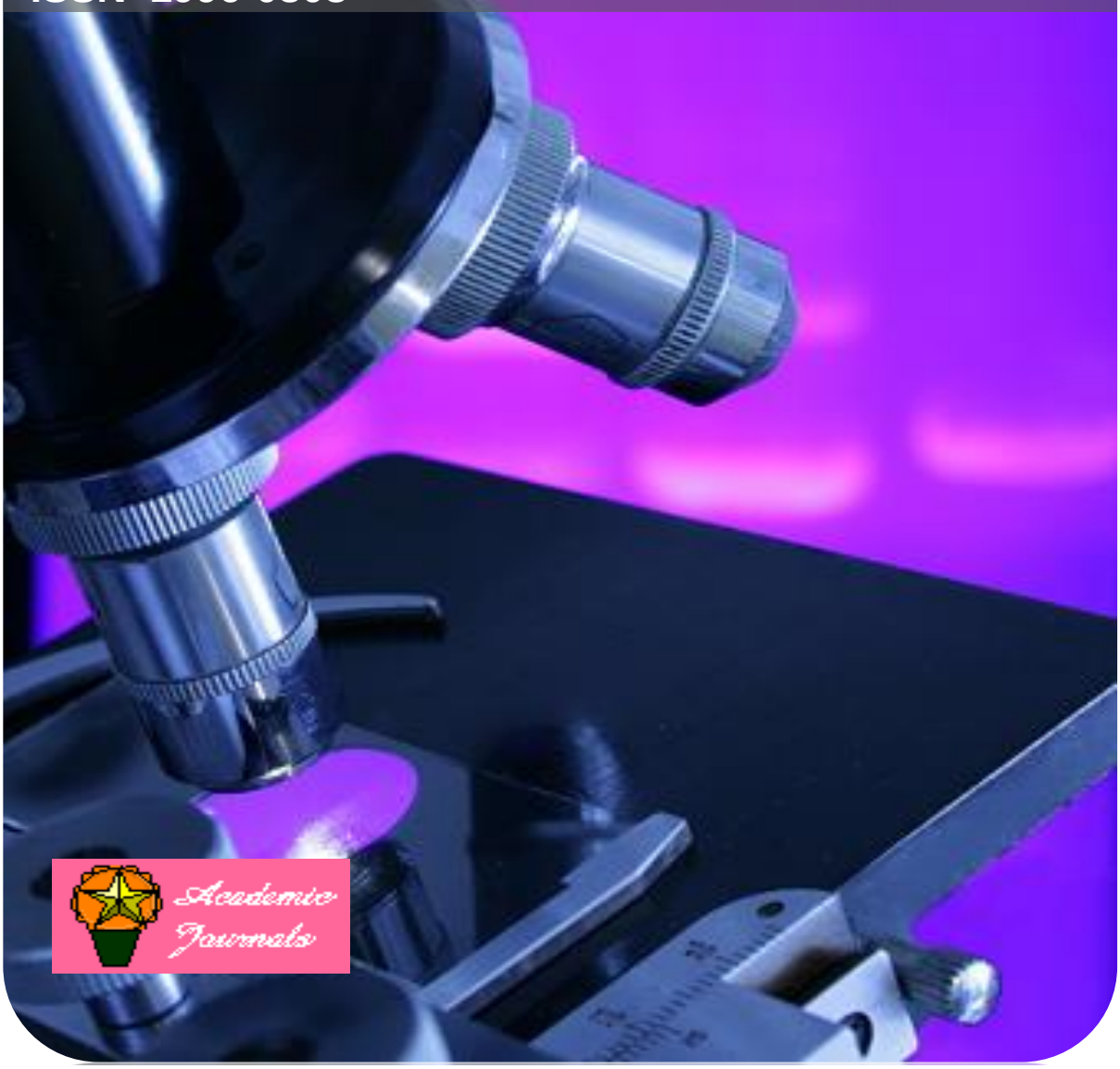

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Phytochemical screening and biological activities of *Garcinia kola* (bark, leaves and seeds) collected in Benin

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Garcinia kola **is a tropical plant which grows in moist forest. It seeds are edible and are consumed for their multiple property. This study aimed at making phytochemical screening and evaluating some biological activities of *G. kola* organs (bark, leaves and seeds) extracts. The selected organs powdered were used for phytochemical screening and extractions. The antibacterial activity was tested on 10 references strains and 28 meat isolated *Staphylococcus* strains by agar perforation method. The antifungal activity of three fungal strains was determined on the potato-dextrose agar medium mixed with the tested extract. The antioxidant activity was determined by both DPPH and ABTS methods. The results reveal that 40% of studied secondary metabolites were present in the seeds against 28.57% in the leaves as the bark. The reference and meat isolated strains inhibitory diameter ranged from 28 ± 2.3 mm (*S. haemolyticcus*) to 7.5 ± 0.70 mm (*E. coli*). All extracts had a minimum inhibitory concentration (MIC) lower than 20 mg/ml, while the minimum bactericidal concentration (MBC) was ≥ 20 mg/ml for some tested extracts. The lowest MIC and MBC was 0.039 mg/ml with *C. albicans*. The fungal strains susceptibility varied ($p < 0.001$) depending on extracts. The seeds ethyl acetate extract displays the better antioxidant activity with DPPH (25.03 ± 3.64 µg/µl) and ABTS (22.99 ± 1.34 µmol EqAA/g) methods. The LD_{50} of the extracts was greater than 0.1 mg/ml and not exhibiting toxicity. The leaves extract had the best bactericidal effects while the seeds extract presented better antifungal and antioxidant activity.**

Key words: Antifungal, *Staphylococcus*, food strains, antioxidant, cytotoxicity, Benin.

INTRODUCTION

The Benin forest agro-ecosystems shelter over 162 forest plant species, with social, religious and/or cultural meaning, used for many purposes such as domestic food and commercial (Codjia et al., 2009). Apart from feeding

and commercial uses, many of these species are currently used in traditional medicine all over the country. Among the plants uses in traditional medicines, we can cite *Garcinia kola* (*Clusisaceae*), a large tree with fattening base (Akoegninou et al., 2006). This species is present in tropical Africa, from Sierra Leone to the DR Congo (Adesuyi et al., 2012).

Nowadays, infectious diseases are responsible for a high morbidity and mortality rate and are consider as a public health problem because of their frequency and their severity (Bourgeois, 1999). For the treatment of these diseases, people often use synthetic drugs such as beta-1actamines. But, bacteria developed a resistance mechanism to fight against most of the synthetic family of antibiotics. There are four main mechanisms used by bacteria to resist beta-1actamines molecules: i) enzymatic inactivation, ii) the modification of the cellular target of the antibacterial agent, iii) the cellular efflux and, iv) the decrease or absence of penetration of the antibiotic in the bacteria. The combination of these mechanisms in a bacterium makes its multi-resistant to many antibiotics (Gangoue-Piéboji et al., 2004). Similarly, several cases of fungi resistant to conventional antifungal agents have been reported. Moreover, the production of free radicals in living organisms is a vital phenomenon for the cell regulated through various biochemical or enzymatic detoxification process (Salem, 2009). The free radicals are reported to be involved in many serious illnesses and constitute even aggressive form factors to DNA (Boumaza, 2009).

To face all these health problems, the formalization of endogenous knowledge would be a reliable asset in the control of not only resistant microbial strains but also diseases caused by free radicals. It is known that several medicinal plant synthesize a wide variety of phytochemicals which include alkaloids, tannins, flavonoids, steroids, saponins, and phenols, which have antimicrobial proprieties. Moreover, potential sources of antioxidant compounds have been searched in several types of plant materials such as vegetables fruits, leaves, barks, roots and crude plant drugs. Antioxidants are vital substances which protect the body from damage caused by free radical inducing oxidative stress (Ozsoy et al., 2008). Therefore, many plants were used as a source of traditional medicine to treat various diseases and conditions (Razali et al., 2008). *G. kola* belongs to the plant used in traditional pharmacopeia in Benin. This plant various parts extracts are reported to be used in the treatment of cough, sore heart, liver problems (Kanmegne and Ndoumou Omokolo, 2007), diarrhea (Braide, 1991) inflammatory diseases (Iwu and Igboko, 1982). *G. kola,* one of the preferred seed in West Africa is deemed to have an antidote power (Kabangu et al., 1987), anti-hepatotoxic properties (Akintowa and Essien, 1990), antinephrotoxic, antimicrobial (Adefule-Ositelu et al., 2004), hypoglycemic (Odeigah et al., 1999) and aphrodisiac effects (Ajibola and Satake, 1992). Other scientific studies of *G. kola* by other authors (Farshori et al., 2013; Okunji et al., 2002; Pietta, 2000) showed the importance of the plant. In Benin, there is few scientific work on this plant species. So, the aims of this study were to investigate the phytochemical on one hand and on the other hand evaluate some biological activities of *G. kola* collected in Benin.

MATERIALS AND METHODS

Collection of plant material

The bark, seed and leaves were collected in the village of Anagbo (commune of Adjarra: 6°29'35"N, 2°40'28"E) department of Oueme, southern Benin. The plants materials were air dried at 25 to 30°C for two weeks, ground and sieved into a bark powder. The smooth powder was stored in airtight glassware and kept in darkness at -20°C until use.

Phytochemical profiling

The phytochemical profiling of bark, seed and leaves powders of *G. kola* to determine the major constituents (nitrogenous, polyphenolic, terpenic compound and glycosides) was done according to the method describe by Houghton and Raman (1998).

Preparation of aqueous

The aqueous extract was obtained according to the method describe by Dah-Nouvlessounon et al. (2015). Briefly, the powder (50 g) of *G. kola* appropriate organ (bark, leaf or seed) obtained above was macerated into 500 ml of distilled water under magnetic agitator for 72 h at room temperature. The homogenate was then filtered two times on absorbent cotton and once on Whatman N°1 paper (125 mm ø, Cat No 1001 125). For the aqueous extract, the filtrate was dried in the oven at 40°C; the obtained powder is considered as the total extract ready to use for the biological activities. All extracts were stored in labeled sterile bottles and kept at -20°C until further use.

Preparation of ethanol and ethyl acetate extracts

These extracts were made using an adapted methods described by Sanogo et al. (2006) and N'Guessan et al. (2007). We macerated 50 g of *G. kola* powders (bark, leaf or seed) in 500 ml of 96% ethanol for 72 h. The obtained extract was filtered thrice using Whatman N°1 filter paper (125 mm ø, Cat No 1001 125). Half of the filtrate was directly dried at 40°C to obtain the ethanolic extract of *G. kola*. To the second half of the filtrate, 200 ml of H_2O and 100 ml of ethyl acetate were added. The solution was gently mixed and left settled until we obtain two phases (about 45 min). The lower phase was collected and dried as described above to obtain the ethyl

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acetate extract. The alcoholic and ethyl acetatic extracts were stored in labeled sterile bottles and kept at -20°C until further use.

Microorganism's cultures

The tested microorganisms include ten references, twenty height *Staphylococcus* meat isolated strains and three fungal strains (*Penicillium citrinum*, *Aspergillus tamarii* and *Fusarium verticilloides*). The three fugal strains were part of the microorganisms isolated in a Beninese traditional cheese wagashi by Sessou et al. (2012). The reference strains were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* T22695, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilus* A24974, *Micrococcus luteus* ATCC 10240, *Proteus vulgaris* A25015, *Streptococcus oralis*, *Enterococcus foecalis* ATCC 29212 and *Candida albicans* MHMR. The *Staphylococcus* strains used in this study were those isolated from three different meat products in Ivory Coast by Attien et al. (2013) and stored in the Laboratory of Biology and Molecular Typing in Microbiology (University of Abomey-Calavi, Benin).

Antimicrobial activity

Sensitivity test

The agar perforation method inspired of those described by Bauer et al. (1966) was used to screen the antimicrobial activity. For this method, four to five perforations were performed under aseptic conditions, on Mueller Hinton agar Petri dish previously flooded by the appropriate bacterial culture (adjusted to 0.5 McFarland standard). Twenty five microliter of extract solution (20 mg/ml) were aseptically lodged in the hole. These dishes were kept for 15-30 min at room temperature before incubation at 37°C for 24 and 48 h.

After the incubation period, the dishes were examined for inhibitory zones. Each sample was used in triplicate for the determination of antibacterial and antifungal activity.

Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations (MIC) of the plant crude extract was performed by macrodilution method (Saha and Rahman, 2008). First, the extracts were diluted in sterilized distilled water to the highest concentration of 20 000 µg/ml and then nine dilution were performed to obtain successively the concentrations of 10 000, 5 000, 2 500, 1 250, 625, 312.5, 156.25, 78.12 and 39.06 µg/ml in screw tube. To 1 ml of the above concentrations was added 1 ml of the bacteria inoculum (10^6 UFC/ml) to obtain 2 ml as a final volume. Culture medium without samples and others without micro-organisms were used in the tests as controls. Tubes were incubated at 37°C for 18-24 h and growth was indicated by turbidity. The MIC is the lowest concentration of the compound at which the microorganism tested does not demonstrate visible growth.

Determination of Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) of the tested microorganisms was determined by sub culturing the test dilutions onto a fresh solid medium and incubated further for 18-24 h. The highest dilution that yielded no bacterial growth on solid medium was taken as MBC (Farshori et al., 2013).

Evaluation of the cytotoxicity activity of *G. kola* extracts

The cytotoxic effect of the extracts was evaluated according to an adaptation of the method described by Kawsar et al. (2008). The tests were carried out twice on 72 h larvae of *Artemia salina* (ARTEMIO JBL GmbH D-67141 Neuhofem). Briley, a test was constituted of 16 *A. salina* larvae in a 2 ml solution containing 1 ml of the extract tested concentration and 1 ml of sea water. The number of surviving larvae is counted after incubation (24 h) and the LD_{50} was calculated using the regression line obtained from the surviving larvae according the extracts concentration representation.

Antifungal activity

The *in vitro* antifungal activity of the extracts was evaluated according to the method previously described by Dohou et al. (2004). The assay was performed on the Potato-Dextrose Agar medium. Briefly, the extracts use for the antifungal activity was dissolved with sterilize distillated water or if necessary with a water-ethanol mixture (60:40). One (1) ml of the dissolved extract (20 mg/ml) was thoroughly mixed with 10 ml of the sterilized potato-dextrose agar medium before it was transferred to sterile Petri dishes for solidification. After the medium solidification, a sterile 6 mm disc pretreated with fungal strain was placed in each Petri plate. Plates were incubated at 25 ± 1°C for five days. Each treatment was replicated twice. Fungal radial growth was measured by averaging the two diameters taken from each colony. Percentage growth inhibition of the fungal colonies was calculated using the formula:

$$\text{Inhibition Percentage } (\%) = \frac{\text{Control's growth} - \text{Treatment's growth}}{\text{Control's growth}} \times 100 \quad (1)$$

Antioxidant activity determinations

The antioxidant activity was measured using both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) methods.

The ABTS assay was conducted according to the method described by Re et al. (1999). The working solution of $ABTS^+$ (10 mg of ABTS, 2.6 ml of deionized water and 1.72 mg of potassium persulphate) was left to stand at room temperature for 12 h in the dark before use. This solution was diluted with ethanol until obtaining an absorbance of 0.70 ± 0.02 at 734 nm. Twenty µl of each extract sample (1 mg/ml) was diluted with a fresh prepared ABTS solution to a total volume of 1 ml. All the assays were performed in triplicates, the absorbance was read after 15 min in dark at 734 nm and the reference molecule was ascorbic acid. The concentration of compounds with a capability to reduce $ABTS^+$ radical cation is expressed as µmol equivalent Ascorbic Acid (µmol EqAA) per gram of dry extract using the following formula used by Guenne et al. (2011).

The DPPH method was conducted using an adapted method of Scherer and Godoy (2009). Practically, equal volumes (100 µl) of DPPH (50 µM) and plant extracts (200 µg/ml) were mixed in a 96 well microplate and allowed to stand in darkness for 20-30 min at room temperature. Then, the absorbance was read at 517 nm and the blank was a mixture of methanol and DPPH (v:v). The inhibitory percentage of DPPH radical indicating the antioxidant activity of extracts and BHA, gallic acid was obtain using the formula establish by Schmeda-Hirschmann et al. (2003).

The concentration providing 50% inhibition (IC_{50}) was determined graphically using a calibration curve in the linear range by plotting the extract concentration and the corresponding scavenging effect. Antioxidant Activity Index (AAI) was calculated according to the formula used by Scherer and Godoy (2009).

Statistical analysis

All experiment was done in triplicate and data thus obtained

Table 1. Phytochemical constituents of *G. kola* powdered organs samples.

Group of compounds	Class	Organ		
		Bark	Leaves	Seeds
Nitrogenous compound	Alkaloids	−	−	+
Poly-phenolics compound	Tanins	+	+	+
	Tanins catéchiques	−	+	−
	Tanins galliques	+	+	+
	Flavonoids	−	−	+flavone
	Anthocyans	−	−	−
	Leuco-anthocyanes	−	−	−
	Coumarin	+	−	+
	Quinonics derivate	−	−	−
Terpeniques compound	Triterpenoids	−	+	+
	Steroids	+	−	−
	Cardenolids	−	−	−
Heterosides	Cyanogenics derivate	−	−	−
	Saponosids (IM)	+ (500)	+ (111)	+ (200)
	Reducing compounds	−	+	+
	Free anthracénics	−	−	−
	O-heterosides	−	−	−
	O- heterosides at GR	−	−	−
	C-heterosides	−	−	−
	Mucilags	+	−	+

(+), Presence of secondary metabolite; (-), Absence of secondary metabolite; (IM), Index mouss.

reported as a mean ± standard deviation (SD). The logit model of stata v.12 software is used to qualitative analysis. The data were also analyzed using Graph Pad Prism 5 software for quantitative analysis. Differences of $p < 0.05$ were considered significant.

RESULTS

Phytochemical screening

The phytochemical analysis performed on three organs of *G. kola* revealed the presence of several secondary metabolites (Table 1). It was noted an uneven distribution of these metabolites from one organ to another. Indeed, 40% of the studied secondary metabolites were present in the seed against 28.57% in the leaves as in the bark.

Antimicrobial activity of *G. kola* extracts

Sensitivity test

The ability to inhibit microbial growth of the extracts evaluated on reference strains revealed that the results of extract deferred from an extract to another depending on the organs. Indeed, all bark extracts had no inhibitory effect on the reference strains. It is the same for the aqueous extract of the two others organs of the plant. In contrary, the ethanol extract of the leaves and seeds, as well as ethyl acetate extract of the seeds are active. Thus, the ethanol extract of the leaves and ethyl acetate seeds extract inhibits the growth of 90% (9/10) of the strains while the ethanol extract of the seeds inhibits the totality (100%) of the strains.

Qualitative analysis of the inhibitory capacity of G. kola extracts

The results of the qualitative analysis of data relating to the evolution of the diameter in time (24h and 48h) according to organ, type of extract and the type of microorganism, showed that the inhibition zones diameters of the sensitive strains vary from one species to another. The Husman test conducted for this purpose showed that the logit model premium on the probit model. With a predictive power of 86.11% sensitivity and specificity of 61.11% showing its reliability, the logit model shows in organs level that the probability that the

diameter increases from the leaves to the seed decreased of 0.13. Regarding the type of extract, logit model marginal effects show that the probability that the diameter increases from the ethanol extract with ethyle acetate extract increases of 0.30. Considering the type of microorganism, from Gram + and Gram- bacteria to yeast, the probability that the diameter increases with the time decreases of 0.08. Similarly passing reference strains for food strain this probability decreases by 0.003.

Quantitative analysis of the inhibitory capacity of G. kola extracts

Concerning the reference strains, the inhibition zones diameters of sensitive strains do not differ ($p > 0.05$) in the time (24 and 48 h) with the ethanol extract of the leaves (Figure 1a) and seeds ethyl acetate extract (Figure 1c). On the contrary with the seeds ethanol extract, a change ($p < 0.05$) of diameter is observed between 24 and 48 h for *E. foecalis* (Figure 2b). With the leaves, the largest diameter of inhibition (15 ± 0.00 mm) of the ethanol extract was obtained on *E. foecalis*, while the lowest (7.5 ± 0.70 mm) was recorded with *E. coli* after 48 h of incubation (Figure 1a). With the seeds, the larger diameter (25 ± 0.00 mm) and the lowest (5 ± 0.00 mm) were recorded with the ethanol extract respectively on *S. epidermidis* and *E. coli* (Figure 1b). The compared action with the same type of extract (ethanol) leaves and seeds showed a difference ($p < 0.0001$) of susceptibility according to the strains. During this, the ethanol extract of the seeds generally exhibits inhibition diameters greater than the same extract of the leaves (Figure 1a). The comparative effect of seeds' ethanol extract and ethyl acetate also shows a variation ($p < 0.0001$) of diameters depending on the strains and the type of extract.

On food strains, it is noted with the leaves ethanol extract a remanence effect for the strains *S. equorum*, *S. saprophyticus* ($p < 0.01$) and *S. haemolyticcus* ($p < 0.001$) after 48 h of incubation (Figure 1d). Similarly for the seeds the same phenomenon was observed for *S. simulans* ($p < 0.01$) strain and *S. lentus, S. haemolyticcus* ($p < 0.0001$) after 48 h of incubation with both the ethanol extract (Figure 1e), and ethyl acetate extract. Note that the ethyl acetate extract of the seeds was more active ($p < 0.0001$) with time with *S. lentus* (Figure 1f). The compared effect of ethanol extract of leaves and seeds show that the leaves has a broad spectrum of action by inhibition of 100% food strains while the seeds ethanol extract is active on 44.44% of the strains. Nevertheless the greatest inhibition diameter obtained with the leaves ethanol extract is 22 ± 0.3 mm (*S. lentus*), whereas that obtained with the seeds' ethanol extract was 28 ± 2.3 mm (*S. haemolyticcus*). Passing leaves to seed, the action of this ethanol extract is not different ($p > 0.05$) for *S. lentus* strains, *S. simulans,* and *S. sciuri*. The comparative action of seeds' ethanol and ethyl acetate extracts shows that the ethyl acetate extract is active on 90% of food strains with a greater inhibition diameter of 24 ± 1.3 mm obtained with *S. lentus*. Also we observed for both type of extract a difference of action ($p < 0.01$) using *S. xylosus* strain and ($p < 0.0001$) *S. equorum* and *S. saprophyticus*.

Minimum inhibitory (MIC) and bactericide (MBC) concentrations of G. kola extracts

Our results show that all extracts have a minimum inhibitory concentration below the starting concentration (20 mg/ml), while for the MBC, some extracts have a concentration ≥ 20 mg/ml (Table 2).

Considering the reference strains, the MICs obtained vary depending on the type of extract and organs. With seeds, a greater sensitivity of the strain *C. albicans* (0.039 mg/ml) is observed to the ethyl acetate extract and *E. foecalis* strain (0.078 mg/ml) to the ethanol extract. With the leaves, the smallest minimum inhibitory concentration (0.156 mg/ml) of the ethanol extract was obtained with *P. aeruginosa* strain. MBC are generally higher than the MIC, but in some cases is equal to the MIC, it is the case of the seeds ethyl acetate extract with *C. albicans* strain which has the greatest sensitivity (MBC = 0.039 mg/ml). Otherwise, to the tested dose, the leaves ethanol and seeds ethyl acetate extracts had no bactericidal effect (MBC > 20 mg/ml) on the *S. epidermidis* strain.

Like with the reference strains, the MIC of meat isolated strains vary depending on the type of extract (Table 2). A greater sensitivity of *S. equorum* to the seeds ethyl acetate extract with an MIC of 0.078 mg/ml was observed. On the contrary, the smallest MIC obtained with the leaves ethanol extract was 0.625 mg/ml (*S. saprophyticcus*) while it was 2.5 mg/ml (*S. sciuri* and *S. haemolyticcus*) with the seeds ethanol extract. In addition, the MBC vary from 0.078 mg/ml to a concentration greater than 20 mg/ml. Indeed, the smallest MBC (0.078 mg/ml) was obtained with the seeds ethyl acetate extract on the *S. equorum*. With the ethanol extract, the lower MBC leaves (1.25 mg / ml) was obtained with the *S. saprophyticcus*, while seeds' (10 mg/ml) was recorded with *S. lentus*, *S. . simulans* and *S. haemolyticcus*. The leaves ethanol extract had no bactericidal effect on *S. simulans* and *S. sciuri* at 20 mg/ml.

Evaluation of bactericidal and bacteriostatic effects of G. kola extracts

The ratio MBC/MIC shows the kind of effect exerted by the extracts on the tested strains. The results of this ratio show that both extracts have bactericidal and bacteriostatic effects on reference strains and on meat

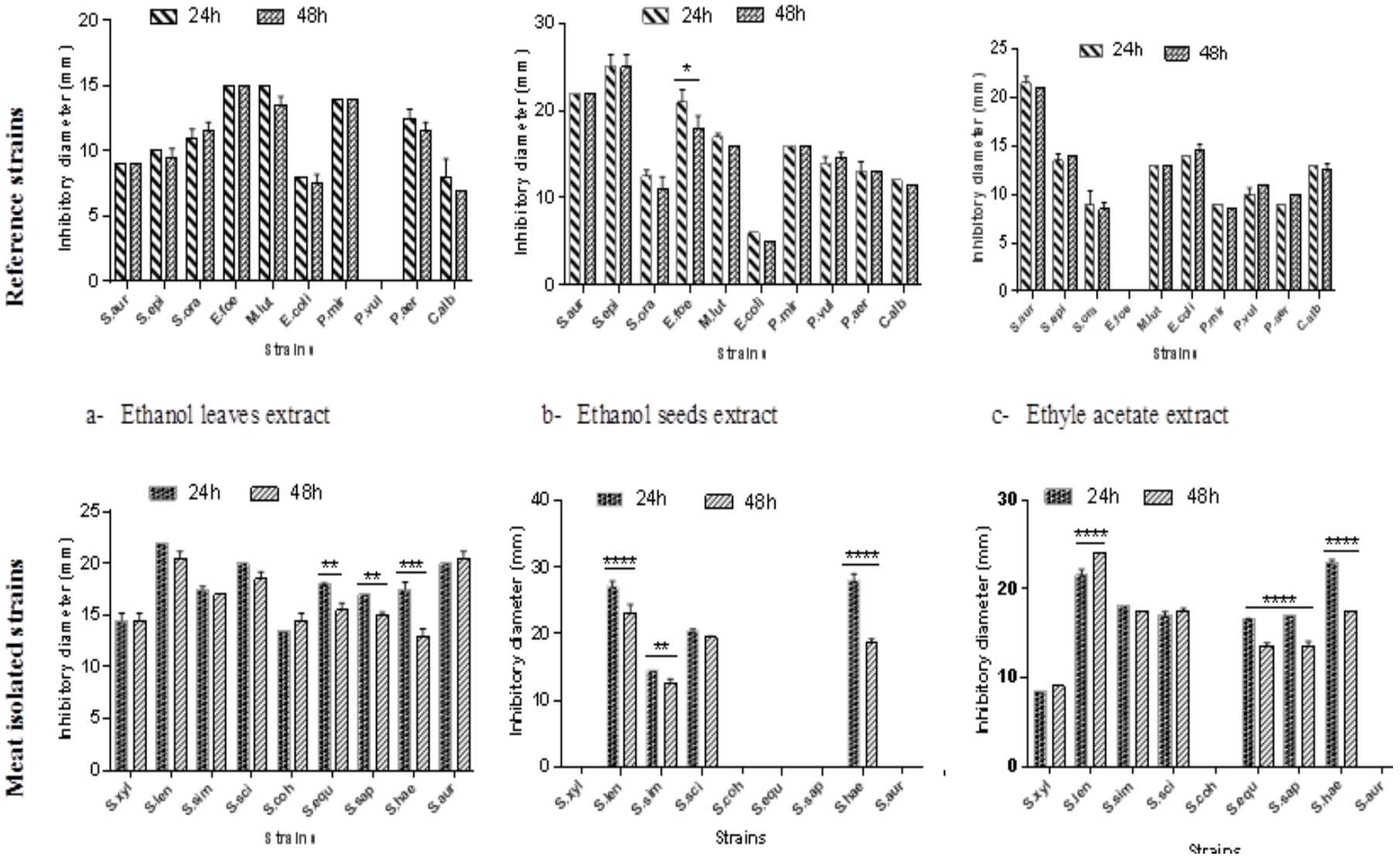


Figure 1. Medium inhibitory diameter zone of *G. kola* extracts on reference and meat isolated *Staphylococcus* strains after 24 and 48 h. For reference strains S. aur, *Staphylococcus aureus*; M. lut, *Miccrococcus luteus*; S. epi, *Staphylococcus epidermidis*; S. ora, *Streptococcus oralis*; P. aer, *Pseudomanas aeruginosa*; E.foe , *Enterococcus foecalis*; P. vul, *Proteus vulgaris*; *E. coli*, *Escherichia coli*; C. alb, *Candida albicans*; P. mir, *Proteus mirabilis ;* For meat isolated strains , S. sci*, S. sciuri*; S. aur*, S. aureus*; S. sim*, S. simulans;* S. xyl*, S. xylosus;* S. coh*, S. cohnii*; S. equ*, S. equorum*; S. sap*, S. saprophyticus*; S. hae*, S. haemolyticus* and S. len*, S. lentus.* *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Table 2. Minimum Inhibitory (MIC) and Bactericidal (MBC) Concentrations of *G. kola* extracts on reference strains and meat isolated *Staphylococcus* strains.

Strain	MIC (mg/ml)			MBC (mg/ml)		
	$EtOH_{leaves}$	$EtOH_{seeds}$	EAC_{seeds}	$EtOH_{leaves}$	$EtOH_{seeds}$	EAC_{gr}
Reference strains						
S.aur	0.312	0.312	0.152	0.625	1.25	2.5
S.epi	0.625	0.312	0.078	>20	1.25	>20
S.ora	2.5	5	2.5	5	10	10
E.coli	5	5	2.5	5	10	10
E.foe	0.625	0.078	-	1.25	2.5	-
M.lut	1.25	0.156	0.078	2.5	0.156	0.156
P.mir	0.625	0.312	0.625	20	10	2.5
P.vul	-	0.625	0.625	-	5	1.25
P.aer	0.156	2.5	0.312	10	20	5
C.alb	5	0.156	0.039	5	0.156	0.039
Meat isolated *Staphylococcus* strains						
S.xyl	2.5	-	0.625	2.5	-	10
S.len	1.25	5	2.5	2.5	10	20
S.sim	2.5	5	5	>20	10	20
S.sci	2.5	2.5	2.5	>20	20	5
S.coh	1.25	-	-	20	-	-
S.equ	1.25	-	0.078	10	-	0.078
S.sap	0.625	-	0.625	1.25	-	10
S.hae	1.25	2.5	0.625	5	10	10
S.aur	2.5	-	-	10	-	-

EtOH, ethanol extract; EAC, ethyl acetate extract. **For reference strains** , S. aur, *Staphylococcus aureus*; M. lut, *Miccrococcus luteus*; S. epi, *Staphylococcus epidermidis*; S. ora, *Streptococcus oralis*; P. aer, *Pseudomanas aeruginosa*; E.foe , *Enterococcus foecalis*; P. vul, *Proteus vulgaris*; E. coli, *Escherichia coli*; C. alb, *Candida albicans*; P. mir, *Proteus mirabilis ;* **For meat isolated strains** , S. sci*, S. sciuri*; S. aur*, S. aureus*; S. sim*, S. simulans;* S. xyl*, S. xylosus;* S. coh*, S. cohnii*; S. equ*, S. equorum*; S. sap*, S. saprophyticus*; S. hae*, S. haemolyticus* and S. len*, S. lentus.*

isolated *Staphylococcus* (Table 3). With reference strains, the bactericidal effect of extracts decreases as follows: leaves ethanol > seeds ethanol > seeds ethyl acetate. *M. luteus* and *C. albicans* strains were very sensitive to all the tested extracts. With the meat isolated *Staphylococcus*, leaves ethanol extract displays a bactericidal effect on three strains (*S. xylosus, S. lentus* and *S. saprophyticcus*). It can be noted that from these results, the antibacterial activity is more interesting with the leaves.

Antifungal activity of G. kola extracts

The antifungal activity of our extracts was evaluated at mycelial development stage of the three fungal strains. The extracts show an antagonism effect to the growth of the three tested fungal strains. Figure 2 shows the appearance of the plates on *F. verticillioides* after five days of incubation.

Extracts inhibition rate varies depending on the strain and shows that the interaction between the fungal strains and the extracts has a highly significant difference ($p < 0.0001$). The percentage inhibition of the various extracts varies between 38.3 to 78.1% (Figure 3). Considering the susceptibility of the strains toward tested extracts, only the seeds ethanol extract has a difference in action ($p < 0.001$) between *F. verticillioides* and *A. tamari*; the same difference was observed between the strains *F. verticillioides* and *P. citrinium* on the one hand ($p < 0.01$ for leaves ethanol extract and $p < 0.05$ for the seeds ethanol extract) and between *A. tamarii* and *P. citrinium* on the other hand.

Antioxidant activity of *G. kola* extracts

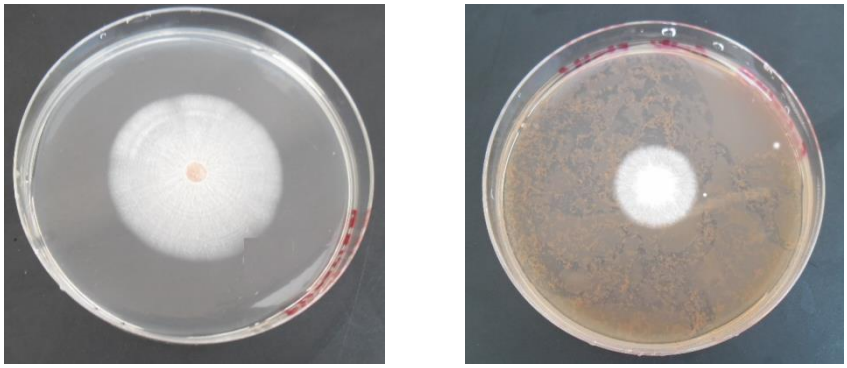
The antioxidant activity profiles using the DPPH radical and $ABTS^{+}$ cation obtained reveal that the extracts possess antioxidant dose-dependent activity (Table 4).

By DPPH method, the results show that seeds' ethyl acetate extract has the lowest concentration (25.03 ±

Table 3. Bactericidal and bactériostatic effect of *G. kola* extracts on reference and meat isolated *Staphylococcus.*

Strain	MBC/MIC		
	Leaves ethanol extract	**Seeds ethanol extract**	**Seeds ethyl acetate extract**
Reference strains			
Staphylococcus aureus	2.003*	4.006	16.44
Staphylococcus épidermidis	>20	4.006	>20
Streptococcus ora	2*	2*	4
Escherichia coli	1**	2*	4
Enterococcus foecalis	2*	32.05	-
Micrococcus luteus	2*	1**	2*
Proteus mirabilis	32	32.05	4
Proteus vulgaris	-	8	2*
Pseudomonas aeruginosa	64.10	8	16.02
Candida albicans	1**	1**	1**
Meat isolated *Staphylococcus* strains			
Staphylococcus xylosus	1*	-	16
Staphylococcus lentus	2*	2*	8
Staphylococcus simulans	-	2*	4
Staphylococcus sciuri	-	8	2*
Staphylococcus cohnii	16	-	-
Staphylococcus equorum	8	-	1*
Staphylococcus saprophyticus	2*	-	16
Staphylococcus haemolyticus	4	4	16
Staphylococcus aureus	4	-	-

With *= Bactericidal effects and without * = Bacteriostatical effects.



a. Culture plate without extract b. Culture plate with extract

Figure 2. Antifungal activity of leaves ethanol extract with *F. verticilloide.*

3.64 µg/µl) to inhibit 50% of DPPH radical, while the leaves ethanol extract displays the largest (lower scavenging activity) IC_{50} value (163.5 ± 14.84 µg/µl). The seeds ethanol extract meanwhile has an intermediate IC_{50} of 84.5 ± 13.43 µg/µl. Compared to reference molecules (gallic acid and the BHA), the antioxidant power of our extracts displays lower values. At the same dose, the antioxidant power of gallic acid is four times higher than BHA. Antioxidant power varies from one molecule to another.

With the ABTS method, the strongest antioxidant activity (22.99 ± 1.34 µmol EqAA/g) of the extracts was obtained with the seeds ethyl acetate extract, while the lowest activity (10.79 ± 2.25 µmol EqAA/g) was recorded with the leaves ethanol extract. The antioxidant ascorbic acid (35.02 ± 0.73 µmol EqAA/g) which is a pure

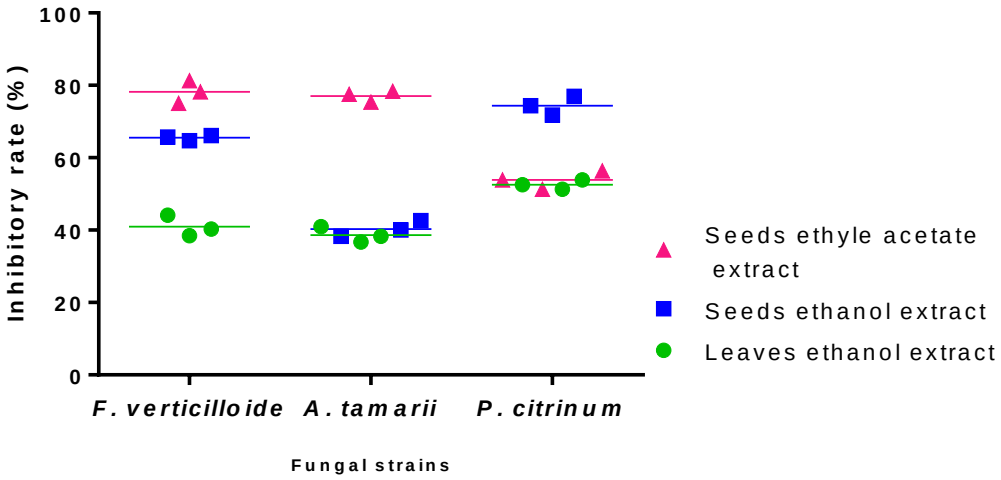


Figure 3. *G. kola* extracts inhibition rate of mycelia development.

Table 4. Free radical scavenging activity results by DPPH radical and $ABTS^+$ cation methods.

Extracts and reference molecules	DPPH		ABTS
	IC_{50} (µg/µl)	IAA	C (µmol EqAA/g)
Seeds ethanol extract	84.5 ± 13.43	0.59 ± 0.09	11.66 ± 0.38
Seeds ethyl acetate extract	25.03 ± 3.64	1.85 ± 0.10	22.99 ± 1.34
Leaves ethanol extract	163.5 ± 14.84	0.30 ± 0.02	10.79 ± 2.25
BHA	4.31 ± 1.10	12.07 ± 2.87	nd
Gallic acid	0.8 ± 0.07	62.74 ± 5.54	nd
Ascorbic acid	nd	nd	35.02 ± 0.73

nd, Not determined.

compound used in this case as a reference molecule is greater than that of our extracts.

However we should note that the antioxidant activity of the extracts follows the same order of effectiveness using the two methods of study and this activity is less high than that observed with the reference molecules.

Artemia salina larvae cytotoxicity test of *G. kola* extracts

The behavior of the larvae introduced in various concentrations of the tested extracts show that the survival of larvae respects a dose-response relation. The dose that induces the death of 50% (LD_{50}) of the larvae was determined from the regression line of each curve. Thus seeds ethyl acetate extract has the lowest LD_{50} of 8.83 mg/ml with a correlation coefficient R^2 = 0.873. This extract is followed by the seeds ethanol extract (LD_{50} = 10.38 mg/ml, R^2 = 0.758). The leaves ethanol extract has the highest concentration (LD_{50} = 10.60 mg/ml, R^2 = 0.655).

DISCUSSION

The phytochemical screening was performed on the powder of three organs (bark, leaves and seeds) in order to highlight the presence of 20 secondary metabolites belonging to four chemical groups. The results reveal, in the nitrogen compounds group, the presence of alkaloids in seeds. This observation is similar to those made in Cameroon by Lacmata et al. (2012). Concerning the phenolic compounds group, our study reveals the presence of tannins with a predominance of gallic tannins (found in the three organs). These results corroborate those of Adesuyi et al. (2012) in Nigeria from the seed of the same species. However, the seeds of *G. kola* were reported, in Nigeria, not to contain tannins (Ghamba et al., 2012). This difference could be explained by the maturity stage of the used fruit before their harvest. Indeed, during a study performed in Congo Brazzaville, Morabandza et al. (2013) demonstrated that the concentration of certain secondary metabolites in the mesocarp of *G. kola* fruit increases with the evolution of maturity. It should be noted that in the three organs, the tannins were

more present in leaves than in seeds and bark. We also note the absence of alkaloids and flavonoids in the leaves. In contrary to those found, Eleazu et al. (2012) showed the presence in a small proportion of alkaloids and flavonoids in the leaves of *G. kola*. This variation can be explained by the difference of those compounds detection method. Beyond tannins, we notice the presence of flavonoid (flovone) and coumarins respectively in the seeds and bark of this plant species. These results corroborate those of Cotterih et al. (1978) when they isolated *G. kola* seeds'; the bioflavonoids called GB1, GB2 and GB1a. Likewise, Okunji et al. (2002) reported that phytochemical compounds so far isolated from *G. kola* seeds are biflavonoids such as kolaflavone and 2-hydroxybi-flavonols. The cyanogenic derivatives absence is very important because they are the main causes of toxicity due to the production of cyanide ions and manifested by the subsequent mass poisoning from the ingestion of cyanogenic derivatives, acceleration and amplification of breathing, respiratory depression, dizziness, headache, disturbance of consciousness, coma (Bruneton, 1993). From these observations, and from the properties of the flavonoids according to Ortuno et al. (2006), the seeds of *G. kola* could therefore be more advantageously than other organs (bark and leaves) of the plant to be used in the case of inflammations, infections, superficial wound or burn and may allow a reduction in the cholesterol in the body.

Regarding the antimicrobial capacity of the three organs of *G. kola*, the bark extracts (aqueous, ethanol, ethyl acetate), and the aqueous extract of the seeds and leaves had no action on the tested reference microorganisms strains. Our observations are not the same as those made by Indabawa and Arzai (2011) when they showed in their study that the seeds aqueous extract of *G. kola* had antibacterial activity against *S. aureus* at 2000 µg/ml. In the same way, Ghamba et al. (2012) reported that the seeds aqueous extract of *G. kola* had an antimicrobial activity against clinical isolates of *S. aureus*, *P. aeruginosa* and *E. coli* at 50 mg / ml with a mean inhibition diameter of 3.66 ± 0.28 mm. This difference can be explained by the antimicrobial active ingredient concentration in the aqueous extracts of our seeds; these antimicrobial agents in the seeds are not concentrated enough in the aqueous extract, and this may also be due to the origin of the strains. Moreover Burger (1990) reported that no active substance showed its maximum activity in laboratory experiments and the activity can be recorded when a great concentration is used. The organic extracts (ethanol and ethyl acetate) of *G. kola* leaves and seeds have inhibited many microorganisms at 20 mg/ml. The same report has been done on other plants by other authors as Sharmila and Gomathi (2011) in India on *Crossandra infundibuliformis*. Also Bouzid et al. (2011) in Algeria on *Crataegus monogyna* noted that organic extracts (ethanol, methanol dichloromethane and others) were active while the aqueous extract was inactive. These observations can be explained by the solubilization capacity of phyto-molecules in solvents. The activity ratio (CMB/CMI) according to Berche et al. (1991) showed that the ethanol extract of the leaves and seeds has a higher bactericidal activity than the seeds ethyl acetate extract. This difference can be due to the affinity of antimicrobial molecules toward ethanol and ethyl acetate. Indeed during the liquid/liquid extraction, phyomolecules are distributed in the solvents according to their polarity (Cowan, 1999). We can then deduce that ethanol (protic and polar solvent) better dissolves active ingredients contained in the seeds of *G. kola* than ethyl acetate (aprotic and polar solvent).

Through the various extracts, the studied organ of *G. kola* exhibit varied antifungal activity depending on the strains. The comparative effect of the ethanol extract of the two organs (leaves and seeds) showed that the seeds ethanol extract has an inhibition percentage of mycelial growth higher than the leaves one on the three tested fungal strains. The hypothesis that can explains this difference would be the high percentage (50%) of polyphenolic compounds reported, during phytochemical screening, in the seeds in comparison to the leaves (10%). The evaluation of antifungal activity of the same organ extracts shows that the seeds ethyl acetate extract has better anti-fungal activity than the ethanol extract of the same organ on the strains *F. verticilloide* and *A. tamarii*. The large number of polyphenolic compounds extracted with ethyl acetate (Rohman et al., 2010) as well as glycosides (Cowan, 1999) would be a reliable explanation for these observations. Similarly Zareen (2006) showed that the ethyl acetate fraction of *Terminalia glaucescens* rich in polyphenol was more active than other fractions. All these observations come to confirm the previous assumption. Compared to the antifungal specificity, the results testify a difference between the responses of the three fungal strains opposite the extracts studied independently to the extraction organs. Nevertheless, there is a preferential antifungal activity of the extracts according to the strains and plant organs. No conclusions could be drawn from this observation since the difference may be related to the variation of fungi growth.

In this study, the various tested extracts from leaves and seeds showed varying antioxidant activities. The antioxidant activity of *G. kola* seeds has also been proven by Okoko (2009). Likewise Farshori et al. (2013) and Olatunde et al. (2004) report that *G. kola* contains natural antioxidants. Considering the Antioxidant Activity index according to Scherer and Godoy (2009), the results obtained with the extracts showed that any extract possesses a very strong antioxidant activity. Nevertheless of the two organs, the seeds exhibit a more interesting antioxidant activity than leaves. Compared to reference molecules, BHA and gallic acid which showed

potent antioxidant activity (with the respective IC_{50} and AAI of 12.07 ± 2.87 µg/µl and 62.74 ± 5.54 µg/µl); the activity of the extracts is less than that of the reference molecules. It must be remembered that the reference molecules are pure compounds set from the specific active ingredient concentration. In addition, the use of synthetic antioxdants like butylated hydroxy anisole (BHA) and butylated hydroxyl toluene (BHT) has been limited due to their toxicity and side effects. Therefore search for the novel sources of natural oxidants will be important for performing such research (Pourmorad et al., 2006). Thus, through its seeds, *G. kola* can be used in the treatment of diseases caused by oxidative stress.

Concerning the extracts cytotoxic test, by referring to the toxicity scale established by Mousseux (1995), extracts LD_{50} is greater than 0.1 mg/ml, value above which the extract is considered exhibiting no toxicity. It appears that all the extracts of the two organs of *G. kola* are not toxic to humans, because there is a correlation between cytotoxicity of shrimp larvae and cytotoxicity in human cells; notably lung cells on one hand and the cells colon on the other hand (José et al., 2002).

Conclusion

The aim of our study was to identify some chemical groups of secondary metabolites present in three organs of *G. kola* and evaluate some of their biological activities including antibacterial, antifungal, antioxidant and cytotoxic activity of the extracts. At the end of this study, it appears that the three organs contain important chemical constituents (flavonoids, tannins, saponins coumarins) and have many biological activities. The different extracts of leaves and seeds presented a broad antibacterial activity on the tested reference strains and some food strains. The ethanol extract of the leaves had the best bactericidal effects while the ethyl acetate extract of the seeds present better antifungal and antioxidant activity. Through their antioxidant property, these organs and particularly seeds may be useful to strengthen the body in oxidative stress situations and prevent various diseases that occur following a radical attack. LD_{50} obtained through the cytotoxic activity allow us to say that the extracts are not toxic.

Conflict of interests

The authors did not declare any conflict of interest.

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Evaluation of bacteriological quality and essential elements in commercially bottled/packaged water produced and marketed in Southeastern Nigeria

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The bacteriological quality and levels of essential mineral elements and organic acids were evaluated in 33 randomly selected bottled/packaged water samples produced and marketed in Southeastern Nigeria. The microbiological assay, essential mineral elements and organic acids were determined using American Public Health Association (APHA) standard methods. The distinguishing characteristics of the microorganisms identified were their psychotropic growth capabilities, motility at 37°C, growth in air, negative phenylalanine, deaminase, positive urease, catalase, oxidase and coagulase activities. Data obtained were compared with the accepted standards for safe drinking water as recommended by WHO/UNICEF. Some of the bacteria species recovered from the water samples included *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*. The results show that only 9% of the samples investigated met the WHO/UNICEF standard of zero microbial load, 73% of the samples fell within 10^3 - 10^4 colony forming units (CFU)/100 ml, while 18% had the poorest with 10^6 CFU/100 ml. Major essential minerals found were calcium, sulphate, chloride, nitrate, bicarbonate and phosphate. Furthermore, the levels of these essential minerals were within the acceptable range in 76% of the samples while in others (24%) they were below the standard range recommended by WHO/UNICEF/APHA. There is need to maintain the approved standard level of chlorination in bottled /packaged water produced and sold in Southeastern Nigeria. Moreover, routine bacteriological quality assay by companies producing bottled/packaged drinking water should be enforced in Nigeria in particular and other developing countries in view of the findings in our study.

Key words: Microorganisms, packaged water, psychotropic growth, bacteriological quality, essential minerals, organic acids.

INTRODUCTION

Commercially bottled/packaged water is mainly produced for human consumption. In Nigeria, it comes in two forms: they are either sealed in plastic containers or in small nylon sachets. The sachet forms are generally called

'pure water' by many Nigerians. 'Pure water' enjoys much better patronage from those in the low socioeconomic class (over half of the population of Nigeria) because they are much cheaper than those in sealed plastic containers. Except for safe fluorides, no other ingredients are added in commercially produced drinking water.

Adherence to quality control standards and international best practices by those who produce packaged water in Nigeria are doubtful for so many reasons. First, most of the producers lack appropriate technology to meet with the acceptable international standards. Second, Oyedeji and co-workers (2010) reported that some producers source their raw water from well water which is not a good source of portable drinking water. Third, findings from local regulatory authorities indicated that some producers of packaged water indulge in very dubious practices by producing under very unhygienic conditions, packaging of untreated water, use of unapproved premises to produce unregistered water, use of non-grade waterproof sachets and marketing of packaged water without production and expiry dates among other sharp practices (Edema and Atayese, 2010).

Unfortunately, the lack of safe municipal and portable water in Nigeria has recently increased the demand for these commercially bottled/sachet water. According to Gardner (2004) this is because of the impression that bottled/sachet water is safer and healthier. Sahota (2005) opined that lack of proper sanitation and unhygienic practices account for the major sources of microbial contamination of any portable water. Nigeria is a country with a population of over 160 million persons. The country is also a market destination for many West Africans and a lot of foreign visitors come to Nigeria for one reason or the other. Therefore, the indiscriminate sale and consumption of sealed water in plastic containers/sachets in Nigeria is of public health significance and this is the major motivation for our study. To the best of our knowledge, there is no study that has comprehensively evaluated the quality of commercially marketed drinking water in all of the Southeast States of Nigeria, an important business hub in West Africa. Southeast Nigeria is made up of five states: Enugu, Anambra, Imo, Abia and Ebonyi states (Figure 1). Furthermore, water-related diseases continue to be a major issue in many developing countries. The high incidence of dysentery, cholera, typhoid and diarrhea has been linked to the consumption of unsafe and non-hygienic drinking water and their production processes (Mead et al., 1999).

Some published studies have reported the detection of heterotrophic and coli form bacteria counts in bottled water (Hobbs, 1962; Craun, 1997; Bhareth et al., 2003). Adelegan (2004) also reported that the increase in the cases of Salmonellosis and typhoid fever in Nigeria is as a result of increased consumption of low quality sachet water. The refusal of some producers of water in plastic containers/sachets to mark production and expiry dates on their products means that the product can also deteriorate before it reaches the consumer (Da Silva et al., 2007).

Examination of water samples for pathogens are usually carried out because many workers have found positive correlation between high density viable counts, total and fecal coli forms and the presence of pathogens such as *Salmonella, E. coli, S. aureus* in drinking water (Hood et al., 1983; LeChevallier, 1990; Payment et al., 1993). Moreover, there have been reported cases of food and waterborne disease outbreaks in both children and adults in Southeastern Nigeria (Blum et al., 1987; Ogan, 1988). For example, it is most probable that the organisms causing these diseases must have been transmitted directly or indirectly to food and water through fecal contamination or by the urine of the carrier animal or man. The study was therefore, conducted to ascertain the quality of commercially marketed drinking water sold in Southeast Nigeria. The levels of microbial contamination and that of essential organic and inorganic ions in the randomly selected water samples were evaluated to determine their conformity with the acceptable reference standards approved by (WHO, 1998; APHA, 1998; UNICEF (2008).

MATERIALS AND METHODS

Sampling

Thirty three (33) water samples collected from thirty three companies producing commercially bottled and/or sachet water in Southeastern Nigeria were analyzed. Samples were collected from fresh stocks supplied to the sellers by the different producers. They were stored in a chilled thermo flask containing ice blocks to regulate the temperature. They were later transported back to the laboratory and processed immediately. The states that make up the Southeastern Nigeria are: Abia, Anambra, Ebonyi, Enugu and Imo. The companies chosen were randomly selected from different locations in the region. Based on the population of companies producing water in plastic containers and sachets in these states, samples were collected as follows: Anambra (8), Abia (7), Enugu/Imo/Ebonyi (6 each). The map of the area covered in the sample collection is shown in Figure 1.

The methods for detection, isolation and enumeration of bacteria in the water samples were determined according to the procedures described by American Public Health Association APHA (1998), Chigbu and Sobolev (2007) and Douterelo et al. (2014)

Presumptive total coli forms, fecal coli forms and *E. coli* detection and enumeration in the water samples

In this method, serial dilutions of the water samples were made and

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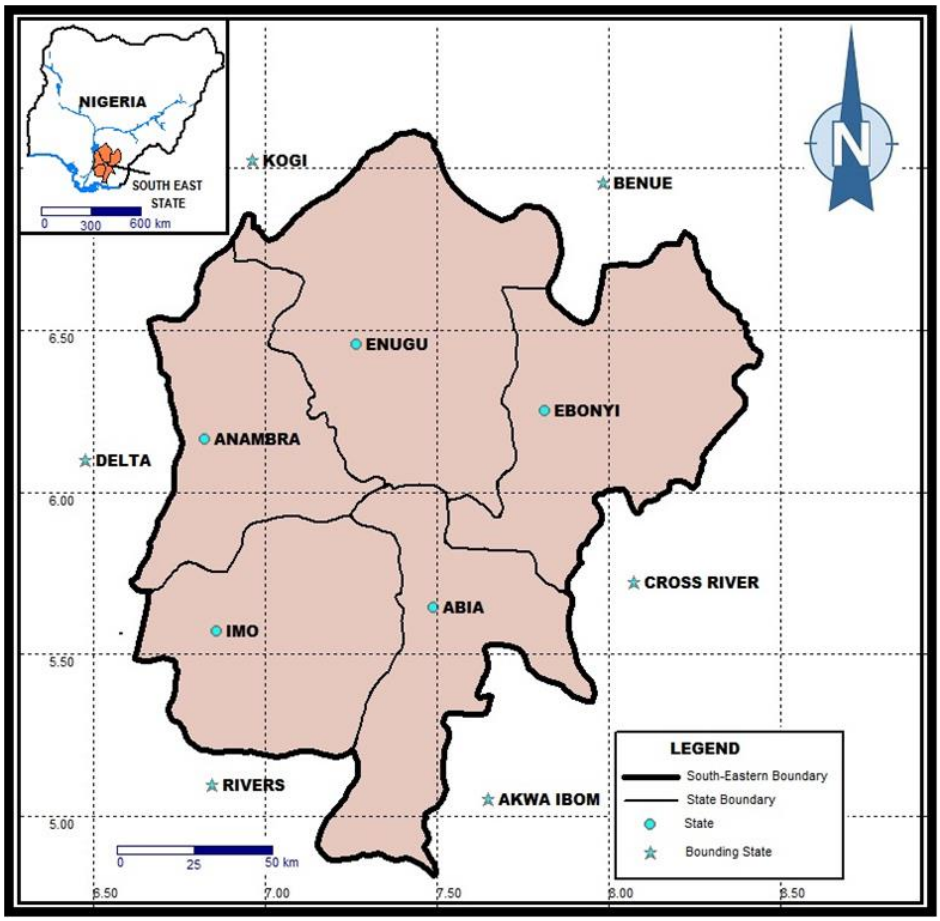

Figure 1. Map of Southeast Nigeria where samples were collected

inoculated into Laury Tryptose broth (LTB) growth media along with fermentation tubes with inverted vials for gas production. Samples were then incubated for 24h at 35°C and for an additional 24 h where there was no growth at the end of the first 24 h. Growth (turbidity), gas bubbles or acid in the tube was due to the fermentation of lactose and indicated the presence of coli form bacteria. A 10- tube most probable number (MPN) method (Multiple Tube technique) was used for the analysis of the water samples.

Confirmation test for the coli form bacteria

The brilliant green lactose bile broth (BGLBB) was used for the confirmation test for the coli form. After preparation, 10 ml of the medium was added into the fermentation tubes such that the media level covered the inverted tubes in the fermentation tubes. The final pH of the broth was 7.2 ± 0.1 after sterilization. All tubes showing growth, gas bubbles or acid reaction in the LTB test were transferred to the BGLBB tubes and incubated for 24-48 h at 35- 37 ± 2°C. Growth or gas production in the tubes showed confirmatory test for the coli form bacteria. The MPN of bacteria present in the sample was calculated using the number of positive BGLBB tubes and the MPN index table (Chigbu and Sobolev, 2007).

Confirmation test for *E. coli* in coli form positive samples

A mixture of the water samples and 4- methylumbelliferyl- b-D-glucoronide (MUG) was added to EC growth medium at a concentration of 50 ml/l. The test is based on the cleavage of MUG to free methylumbelliferyl moiety, which fluoresces in blue color when irradiated with the ultra violet (UV) radiation. EC medium was sterilized before use and the pH after sterilization was 6.9 ± 0.2. The EC medium was tested for fluorescence before use. The EC tube from positive BGLBB tubes were incubated in a water bath at 44.5 ± 2°C for 22-26 h. The inverted Durham tubes were omitted. A positive reaction for *E. coli* was indicated by the presence of blue fluorescence. A tube inoculated with a known positive culture and a negative culture were included for each batch to be tested to serve as a reference in order to eliminate false positives (Chigbu and Sobolev, 2007; Mossel and Vega, 1973).

Mineral and organic acids determination

The essential elements and organic acids in the samples were estimated in the water samples using wet digestion with nitric and perchloric acid to produce complete digestion. The acidified sample was evaporated to the lowest possible volume before precipitation. Nitric acid addition continued until a clear solution was obtained. The values were then read in atomic absorption spectrophotometer and gas chromatography, respectively. The principles of the two methods are based on the formation of colored compounds with appropriate and specific reagents. During the process, the radiant energy of a very narrow wavelength (visible or UV region) is selected from a source, and passed through the sample solution,

Table 1. Range of bacterial contamination of the water samples.

Bacteria/samples	Total viable counts (TVC) (CFU/100 ml)	Total coliform counts (TC) (CFU/100 ml)	Fecal coliform counts (FC)(CFU/100 ml)
MW_1	1.2 - 1.3 x 10^6	1.4 - 1.6 x 10^5	1.1 - 1.2 x 10^1
MW_2	1.6 - 1.7 x 10^4	1.8 - 2.0 x 10^3	1.8 - 2.2 x 10^1
MW_3	2.2 - 2.5 x 10^4	2.3 - 2.4 x 10^2	2.2 - 2.3 x 10^2
MW_4	1.5 - 1.6 x 10^4	1.3 - 1.4 x 10^3	2.1 - 2.2 x 10^1
MW_5	1.6 - 1.7 x 10^5	1.8 - 2.0 x 10^3	1.8 - 2.2 x 10^2
MW_6	1.4 - 1.6 x 10^6	1.6 - 2.2 x 10^4	1.7 - 1.9 x 10^2
MW_7	2.1 - 2.2 x 10^3	2.2 - 2.3 x 10^2	1.9 - 2.0 x 10^1
MW_8	2.2 - 2.4 x 10^4	2.2 - 2.4 x 10^2	1.8 - 2.0 x 10^2
MW_9	1.8 - 2.0 x 10^5	1.6 - 1.8 x 10^3	1.6 - 1.70 x 10^2
MW_{10}	1.6 - 1.8 x 10^4	1.86 - 2.2 x 10^2	1.2 - 1.4 x 10^2
MW_{11}	1.8 - 2.0 x 10^4	2.2 - 2.6 x 10^2	2.4 - 2.6 x 10^2
MW_{12}	1.8 - 2.0 x 10^6	2.2 - 2.6 x 10^4	2.4 - 2.6 x 10^2
MW_{13}	1.8 - 2.0 x 10^3	2.2 - 2.6 x 10^2	2.4 - 2.6 x 10^1
MW_{14}	1.8 - 2.0 x 10^5	2.2 - 2.6 x 10^3	2.4 - 2.6 x 10^2
MW_{15}	1.8 - 2.0 x 10^4	2.2 - 2.6 x 10^3	2.4 - 2.6 x 10^1
MW_{16}	ND	ND	ND
MW_{17}	1.8 - 2.0 x 10^6	2.2 - 2.6 x 10^4	2.4 - 2.6 x 10^2
MW_{18}	1.8 - 2.0 x 10^4	2.2 - 2.6 x 10^2	2.4 - 2.6 x 10^2
MW_{19}	1.8 - 2.0 x 10^4	2.2 - 2.6 x 10^2	2.4 - 2.6 x 10^2
MW_{20}	1.8 - 2.0 x 104	2.2 - 2.6 x 102	2.4 - 2.6 x 102
MW_{21}	1.8 - 2.0 x 105	2.2 - 2.6 x 103	2.4 - 2.6 x 102
MW_{22}	1.8 - 2.0 x 106	2.2 - 2.6 x 104	2.4 - 2.6 x 102
MW_{23}	1.8 - 2.0 x 104	2.2 - 2.6 x 103	2.4 - 2.6 x 101
MW_{24}	ND	ND	ND
MW_{25}	1.8 - 2.0 x 106	2.2 - 2.6 x 105	2.4 - 2.6 x 101
MW_{26}	1.8 - 2.0 x 103	2.2 - 2.6 x 102	2.4 - 2.6 x 101
MW_{27}	1.8 - 2.0 x 104	2.2 - 2.6 x 103	2.4 - 2.6 x 101
MW_{28}	ND	ND	ND
MW_{29}	1.8 - 2.0 x 103	2.2 - 2.6 x 102	2.4 - 2.6 x 101
MW_{30}	1.8 - 2.0 x 104	2.2 - 2.6 x 103	2.4 - 2.6 x 101
MW_{31}	1.8 - 2.0 x 103	2.2 - 2.6 x 102	2.4 - 2.6 x 101
MW_{32}	1.8 - 2.0 x 104	2.2 - 2.6 x 103	2.4 - 2.6 x 101
MW_{33}	1.8 - 2.0 x 103	2.2 - 2.6 x 102	2.4 - 2.6 x 101

*Means ± (SD) of 3 determinations. Samples on appropriate media incubated at 35°C - 44.5 ± 2°C as determined by MPN method. MW_1 - MW_{19} = water from different locations in the region. ND = Not detected.

which is contained in the quartz cell. The amount of radiation absorbed at a certain wavelength is proportional to the light absorbing chemical in the sample (Peldszus et al., 1996; Kuo, 1998; Jorge et al., 2007; Nachiyunde et al., 2013).

Statistical analysis

The analysis of variance (ANOVA) and Duncan's New Multiple Range Tests (DNMRT) were used to test the significance of the difference among means. (Steel and Torrie, 1980).

RESULTS

Bacteriological quality of the water samples

Table 1 presents the results obtained for the total viable counts, total coli forms and fecal coli forms. On the basis of total viable counts, it is evident that samples MW_{16}, MW_{24} and MW28 had the highest microbiological quality with no significant detectable bacteria counts ($p>0.05$) compared with the rest of the samples. On the basis of total coli forms and fecal coli forms, MW_1, MW_6, MW_{12}, MW_{17}, MW_{22} and MW_{25} had the poorest sanitary quality with the average counts of 10^6 CFU/100 ml. The values for the other samples ranged from 10^3 to 10^4CFU/100 ml. The overall ranking of the microbiological quality for the entire water samples studied is thus: MW_{16}, MW_{24}, MW_{28}>MW_2, MW_3, MW_4, MW_5, MW_7, MW_8, MW_9 MW_{10}, MW_{11}, MW_{13}, MW_{14}, MW_{15}, MW_{18} , MW_{19}, MW_{20}, MW_{21}, MW_{23}, MW_{26}, MW_{27}, MW_{29}, MW_{30}, MW_{31}, MW_{32}, MW_{33}

Table 2. Population of individual pathogenic organisms in the water samples (CFU/100 ml).

Samples/bacteria	MW_1	MW_2	MW_3	MW_4	MW_5	MW_6	MW_7	MW_8	MW_9	MW_{10}	MW_{11}
Escherichia coli	-	-	-	-	-	-	-	-	$5.4x10^3$	$2.1x10^4$	-
S. aureus	-	-	-	-	-	-	-	$3.5x10^3$	-	$4.1x10^3$	-
B. subtilis	-	-	-	-	$4.0x10^3$	$3.0x10^3$	$9.4x10^5$	$4.1x10^3$	-		$1.4x10^4$
Acinetobacter sp.	$4.7x10^3$	-	-	-	-	-	-		-		-
Salmonella sp.	-	-	-	-	-	-	-	-	-		-
Alcaligenes sp.	-	-	-	$1.3x10^4$	-	-	-	-	-		-
Pseudomonas sp.	-	-	-	-	$4.0x10^3$	-	-	-	-		-
Klebsiella sp.	-	-	$1.0x10^3$	-	-	-	-	-	-		-
Aerobacter sp.	-	-	-	-	-	-	-	-	-	$1.2x10^3$	$1.3x10^4$
Lactobacillus sp.	-	-	$1.0x10^3$	-	-	-	-	-	-	$1.3x10^3$	-
Leuconostoc sp.	-	-	-	$1.0x10^3$	-	-	-	-	$1.0x10^3$		-
Clostridumperfrigenes	-	-	-	-	-	-	-	-	-	-	-

*Means ± (SD) of 3 determinations. Samples on appropriate media incubated at 35°C - 44.5 ± 2°C as determined by MPN method. MW_{20} - MW_{33} = water from different locations in the region; ND = Not detected.

Table 2. Contd.

Samples/bacteria	MW_{12}	MW_{13}	MW_{14}	MW_{15}	MW_{16}	MW_{17}	MW_{18}	MW_{19}	MW_{20}	MW_{21}	MW_{22}
Escherichia coli	-	-	-	-	NS	-	-	-	$5.4x10^3$	$2.1x10^4$	-
S.aureus	$4.7x10^3$	-	-	-	NS	-	-	$3.5x10^3$	-		-
B. subtilis	-	-	-	-	NS	$3.0x10^3$	$9.4x10^5$	$4.1x10^3$	-		$1.4x10^4$
Acinetobacter	$4.7x10^3$	-	-	-	NS	-	-		-		-
Salmonella sp.	-	-	-	-	NS	-	-	-	-		-
Alcaligenes sp.	-	-	-	$1.3x10^4$	NS	-	-	-	-		-
Pseudomonas sp.	-	$1.0x10^3$	-	-	NS	-	-	-	-		-
Klebsiella sp.	-	-	$1.0x10^3$	-	NS	-	-	-	-		-
Aerobacter sp.	-	-	-	-	NS	-	-	-	-	$1.2x10^3$	$1.3x10^4$
Lactobacillus sp.	-	-	$1.0x10^3$	-	NS-	-	-	-	-	$1.3x10^3$	-
Leuconostoc sp.	-	-	-	$1.0x10^3$	NS-	-	-	-	$1.0x10^3$		-
Clostridumperfrigenes	-		-	-	NS-	-	-	-	-	-	-

*Means ± (SD) of 3 determinations. Samples on appropriate media incubated at 35°C - 44.5 ± 2°C as determined by MPN method MW_{12} - MW_{22} = water from different locations in the region. ND = Not detected.

>MW_1 , MW_6, MW_{12}, MW_{17}, MW_{22} and MW_{25}. The results were further subjected to Duncan's new multiple range test. On the basis of the total viable counts, all the samples were found to be significantly ($p<0.05$) different from each other. Similarly, on the basis of total coli forms and fecal coli forms, the samples were found to be statistically different at 5% level of significance. Furthermore, the results obtained on the basis of individual microorganisms (Table 2), indicated that some samples had pathogenic organisms notably, *E. coli, B. subtilis* and *S. aureus* with the average contamination of 10^3CFU/100 ml.

Table 3 presents the essential elements and pH of the water samples. The calcium ion contents of the samples varied. The values ranged from 6.00 mg/ml in MW_7 to 24.00 mg/ml in MW_{33} and the difference was significant ($p<0.05$). Sample MW_{10} had the highest sulphate concentration of 0.039 mg/ml compared with MW_1 with the least value (0.010 mg/ml). There were significant ($p<0.05$) differences in the chloride concentrations among the samples (Table 3). However, MW_{14} and MW_{28} had similar values (17.54 mg/ml). The nitrate ion levels of the samples ranged from 0.100 to 2.34 mg/ml with MW_4 having the highest value and MW_1 the least. Samples MW_2 and MW_6 had similar value (1.34 mg/ml). The bicarbonate ion concentrations of the samples varied. Sample MW_{22} had the highest value (20.24 mg/ml) while MW_4 had the least (7.45 mg/ml). The phosphate ion levels ranged from 5.56 mg/ml in MW_{21} to 20.26 mg/ml in MW_{33}. The logarithm of hydrogen ion concentration (pH) of the entire samples were comparable.

Table 4 presents the organic acid concentrations of the water samples. The oxalic acid ion ($HOOCCOO^{-}$) contents of the samples were of the order of 0.98 to 4.66

Table 2. Contd.

Samples/bacteria	MW_{23}	MW_{24}	MW_{25}	MW_{26}	MW_{27}	MW_{28}	MW_{29}	MW_{30}	MW_{31}	MW_{32}	MW_{33}
Escherichia coli	-	NS	-	-	-	NS	-	-	$5.4x10^3$	$2.1x10^4$	-
S.aureus	$4.7x10^3$	NS	-	-	-	NS	-	$3.5x10^3$	-		-
B. subtilis	-	NS	-	-	$4.0x10^3$	NS	$9.4x10^5$	$4.1x10^3$	-		$1.4x10^4$
Acinetobacter sp.	$4.7x10^3$	NS	-	-	-	NS	-		-		-
Salmonella sp.	-	NS	-	-	-	NS	-	-	-		-
Alcaligenes sp.	-	NS	-	$1.3x10^4$	-	NS	-	-	-		-
Pseudomonas sp.	-	NS	-	-	$4.0x10^3$	NS	-	-	-		-
Klebsiella sp.	-	NS	$1.0x10^3$	-	-	NS	-	-	-		-
Aerobacter sp.	-	NS	-	-	-	NS	-	-	-	$1.2x10^3$	$1.3x10^4$
Lactobacillus sp.	-	NS	$1.0x10^3$	-	-	NS	-	-	-	$1.3x10^3$	-
Leuconostoc sp.	-	NS	-	$1.0x10^3$	-	NS-	-	-	$1.0x10^3$		-
Clostridumperfrigenes	-	-	-	-	-	NS	-	-	-	-	-

*Means ± (SD) of 3 determinations. Samples on appropriate media incubated at 35°C - 44.5 ± 2°C as determined by MPN method. MW_{23} - MW_1 = water from different locations in the region; ND = Not detected.

Table 3. Essential mineral elements and pH of the water samples.

Parameters/samples	MW_1	MW_2	MW_3	MW_4	MW_5	MW_6	MW_7
Calcium (mg/l)	11.10^a±1.0	16.0^c±1.0	15.0^c±1.2	11.0^a±1.30	22.0^d±1.00	$11.0±1^a.20$	6.00^e±1.30
Sulphate (mg/ml)	0.010^d±0.001	0.013^d±0.001	0.037^c±0.002	0.010^d±0.001	0.110^d±0.001	0.015^d±0.002	0.07^e±0.001
Chloride (mg/l)	14.27^a±1.2	11.38^b±1.3	2.84^c±1.00	14.20^a±1.20	11.36^b±1.30	19.88^d±2.00	17.04^e±1.20
Nitrate (mg/ml)	0.100^a±0.001	1.34^b±0.002	0.105^a±0.001	2.34^c±0.010	0.31^d±0.002	1.34^b±0.001	0.664^e±0.020
Biocarbonate (mg/l)	19.00^a±2.00	7.77^b±1.20	20.00^a±2.00	7.45^b±1.20	17.00^c±1.20	16.00^c±1.20	15.90^c±1.50
Phosphate (mg/l)	11.00^a±1.20	13.60^c±1.50	14.00^c±1.00	10.00^a±2.0	20.20^d±2.00	12.50^b±1.00	6.00^e±0.20
pH	6.20^b	6.10^b	6.10^b	6.20^b	6.10^b	6.00^b	6.00^b

*Means ± (SD) of 3 determinations. Values on the same row with different superscripts are significantly different ($p<0.05$). MW_1 - MW_7 = municipal water from different locations in the region. WHO/UNICEF recommended values: pH (6.5-8.5), Phosphate (<20 mg/l), Bicarbonate (<20 mg/l), Nitrate (0.02mg/l, Chloride (250 mg/l), Sulphate (100 mg/l), Ca^{2+} (< 20 mg/l).

Table 3. Contd.

Parameters/samples	MW_8	MW_9	MW_{10}	MW_{11}	MW_{12}	MW_{13}	MW_{14}
Calcium (mg/l)	11.10^a±1.0	15.0^c±1.0	14.0^c±1.2	10.0^a±1.30	23^d±1.00	$11±1^a.20$	7.0^e±1.30
Sulphate (mg/ml)	0.012^d±0.001	0.013^d±0.001	0.039^c±0.002	0.011^d±0.001	0.110^d±0.001	0.014^d±0.002	0.007^e±0.001
Chloride (mg/l)	14.26^a±1.2	11.38^b±1.3	2.84^c±1.00	14.22^a±1.20	11.36^a±1.30	19.87^d±2.00	17.54^e±1.20
Nitrate (mg/ml)	0.100^a±0.001	1.32^b±0.002	0.106^a±0.001	2.43^c±0.010	0.33^d±0.002	1.34^b±0.001	0.664^e±0.020
Biocarbonate (mg/l)	18.66^a±2.00	8.22^b±1.20	20.00^a±2.00	8.20^b±1.20	17.00^c±1.20	15.00^c±1.20	16.10^c±1.50
Phosphate (mg/l)	10.00^a±1.20	11.50^b±1.50	14.20^c±1.00	10.00^a±2.0	20.10^d±2.00	12.20^b±1.00	6.22^e±0.20
pH	6.10^b	6.11^b	6.10^b	6.10^b	6.11^b	6.01^b	6.00^b

*Means ± (SD) of 3 determinations Values on the same row with different superscripts are significantly different ($p<0.05$). MW_8 - MW_{14} = municipal water from different locations in the region. WHO/UNICEF recommended values: pH (6.5-8.5), Phosphate (<20 mg/l), Bicarbonate (<20 mg/l), Nitrate (0.02 mg/l, Chloride (250 mg/l), Sulphate (100 mg/l), Ca^{2+} (< 20 mg/l).

mg/ml. MW_{33} had the highest value (4.66 mg/ml), while MW_{23} had the least (0.98mg/ml). There were significant($p<0.05$) differences amongst the samples. The formic acid ion ($HCOO^-$) levels of the various samples varied. The values ranged from 1.12 mg/ml in MW_{13} to 4.24mg/ml in MW_{33} and the differences were significant ($p<0.05$). The acetic acid ion ($CH3COO^-$) values ranged from 0.687 to 6.682 mg/ml. The acetic acid contents of MW_8, MW_9, MW_{20} and MW_{30} samples were similar (6.682 mg/ml) ($p>0.05$). Similarly, samples MW_{13} and MW_{24} had the same value (0.687 mg/ml).

DISCUSSION

Bacteriological analyses of the water samples revealed

Table 3. Contd.

Parameters/samples	MW_{15}	MW_{16}	MW_{17}	MW_{18}	MW_{19}	MW_{20}	MW_{21}
Calcium (mg/l)	10.10^{a}±1.0	16.20^{c}±1.0	16.10^{c}±1.2	11.0^{a}±1.30	21.60^{d}±1.00	$12.22±1^{a}$.20	6.10^{e}±1.30
Sulphate (mg/ml)	0.011^{d}±0.001	0.014^{d}±0.001	0.036^{c}±0.002	0.011^{d}±0.001	0.110^{d}±0.001	0.014^{d}±0.002	0.007^{e}±0.01
Chloride (mg/l)	14.24^{a}±1.2	11.34^{b}±1.3	2.86^{c}±1.00	14.22^{a}±1.20	11.32^{b}±1.30	19.78^{d}±2.00	17.24^{e}±1.20
Nitrate (mg/ml)	0.101^{a}±0.001	1.32^{b}±0.002	0.103^{a}±0.001	2.23^{c}±0.010	0.34^{d}±0.002	1.35^{b}±0.001	0.662^{e}±0.020
Biocarbonate (mg/l)	20.22^{a}±2.00	8.32^{b}±1.20	20.00^{a}±2.00	8.10^{b}±1.20	16.00^{c}±1.20	16.20^{c}±1.20	16.24^{c}±1.50
Phosphate (mg/l)	9.50^{a}±1.20	12.06^{b}±1.50	14.00^{c}±1.00	10.00^{a}±2.0	20.00^{d}±2.00	12.00^{b}±1.00	5.56^{e}±0.20
pH	6.00^{b}	6.10^{b}	6.10^{b}	6.00^{b}	6.10^{b}	6.00^{b}	6.00^{b}

*Means ± (SD) of 3 determinations.Values on the same row with different superscripts are significantly different ($p<0.05$). MW_{15} - MW_{21} = municipal water from different locations in the region. WHO/UNICEF recommended values: pH (6.5-8.5), Phosphate (<20 mg/l), Bicarbonate (<20 mg/l), Nitrate (0.02 mg/l, Chloride (250 mg/l), Sulphate (100 mg/l), Ca^{2+} (< 20 mg/l).

Table 3. Contd.

Parameters/samples	MW_{22}	MW_{23}	MW_{24}	MW_{25}	MW_{26}	MW_{27}	MW_{28}
Calcium (mg/l)	12.10^{a}±1.0	15.20^{c}±1.0	16.0^{c}±1.2	11.50^{a}±1.30	21.50^{d}±1.00	$10.10±1^{a}$.20	5.10^{e}±1.30
Sulphate (mg/ml)	0.011^{d}±0.001	0.012^{d}±0.001	0.038^{c}±0.002	0.010^{d}±0.001	0.111^{d}±0.001	0.015^{d}±0.002	0.066^{e}±0.001
Chloride (mg/l)	14.24^{a}±1.2	11.33^{b}±1.3	2.82^{c}±1.00	14.20^{a}±1.20	11.36^{b}±1.30	19.86^{d}±2.00	17.54^{e}±1.20
Nitrate (mg/ml)	0.100^{a}±0.001	1.32^{b}±0.002	0.106^{a}±0.001	2.31^{c}±0.010	0.32^{d}±0.002	1.32^{b}±0.001	0.664^{e}±0.020
Biocarbonate (mg/l)	20.24^{a}±2.00	8.00^{b}±1.20	20.24^{a}±2.00	8.00^{b}±1.20	19.47^{a}±2.00	15.50^{c}±1.20	16.20^{c}±1.50
Phosphate (mg/l)	10.11^{a}±1.20	12.40^{b}±1.50	14.23^{c}±1.00	10.10^{a}±2.0	20.20^{d}±2.00	12.24^{b}±1.00	6.10^{e}±0.20
pH	6.10^{b}	6.10^{b}	6.10^{b}	6.20^{b}	6.00^{b}	6.00^{b}	6.00^{b}

*Means ± (SD) of 3 determinations. Values on the same row with different superscripts are significantly different ($p<0.05$). MW_{22} - MW_{28} = municipal water from different locations in the region WHO/UNICEF recommended values: pH (6.5-8.5), Phosphate (<20 mg/l), Bicarbonate (<20 mg/l), Nitrate (0.02 mg/l, Chloride (250 mg/l), Sulphate (100 mg/l), Ca^{2+} (< 20 mg/l).

Table 3. cont'd.

Parameters/Samples	MW_{29}	MW_{30}	MW_{31}	MW_{32}	MW_{33}
Calcium (mg/l)	11.12^{a}±1.0	15.20^{c}±1.0	14.20^{c}±1.2	11.10^{a}±1.30	24.00^{d}±1.00
Sulphate (mg/ml)	0.011^{d}±0.001	0.013^{d}±0.001	0.032^{c}±0.002	0.011^{d}±0.001	0.110^{e}±0.001
Chloride (mg/l)	14.24^{a}±1.2	11.31^{b}±1.3	2.84^{c}±1.00	13.82^{a}±1.20	11.34^{b}±1.30
Nitrate (mg/ml)	0.101^{a}±0.001	1.23^{b}±0.002	0.106^{a}±0.001	2.32^{c}±0.010	0.35^{d}±0.002
Biocarbonate (mg/l)	20.23^{a}±2.00	7.55^{b}±1.20	7.82^{b}±1.20	8.20^{b}±1.20	7.92^{b}±1.20
Phosphate (mg/l)	10.01^{a}±1.20	12.22^{b}±1.50	14.20^{c}±1.00	10.22^{a}±2.0	20.26^{d}±2.00
pH	6.20^{b}	6.10^{b}	6.20^{b}	6.20^{b}	6.10^{b}

*Means ± (SD) of 3 determinations. Values on the same row with different superscripts are significantly different ($p<0.05$). MW_{29} - MW_{33} = municipal water from different locations in the region. WHO/UNICEF recommended values: pH (6.5-8.5), Phosphate (<20 mg/l), Bicarbonate (<20 mg/l), Nitrate (0.02 mg/l, Chloride (250 mg/l), Sulphate (100 mg/l), Ca^{2+} (< 20 mg/l).

the presence of pathogens and this is in agreement with the reports of LeChevallier (1990); Michiels and Moyson (2000); Chigbu and Sobolev (2007) that implicated these organisms in different waterborne disease outbreaks across the world. The public health significance of these organisms cannot be over-emphasized. A higher load over MID had been implicated in foodborne enteritis (Chigbu and Sobolov, 2007; Onoja et al., 2011), traveller's diarrhea (Gorbach et al., 1975) and water borne diseases (Mackenzie et al., 1994; APHA, 1998; Hunter and Fewtrell, 2001; Feng et al., 2002; Onoja et al., 2011). *E. coli* is a heat sensitive organism that cannot withstand the processing temperature hence, it is evident that its mode of entry must have been through handling and post process re-contamination and cross - contamination (LeChevallier, 1990). Furthermore, *E. coli* is solely an organism of intestinal origin, hence its presence in the water samples is an indication of

Table 4. Essential organic acids content of the water samples*

Samples/organic acids	Oxalic acid (mg/l)	Formic acid (mg/l)	Acetic acid (mg/l)
MW_1	$1.50^{a} \pm 0.020$	$1.22^{a} \pm 0.001$	$2.097^{a} \pm 0.020$
MW_2	$2.10^{a} \pm 0.10$	$1.22^{a} \pm 0.002$	$0.776^{b} \pm 0.020$
MW_3	$2.54^{b} \pm 0.20$	$2.20^{b} \pm 0.100$	$1.043^{c} \pm 0.020$
MW_4	$1.55^{a} \pm 0.10$	$1.42^{a} \pm 0.010$	$1.045^{c} \pm 0.010$
MW_5	$3.64^{c} \pm 0.20$	$2.21^{b} \pm 0.020$	$1.314^{c} \pm 0.010$
MW_6	$3.04^{d} \pm 0.30$	$1.23^{a} \pm 0.010$	$1.084^{c} \pm 0.020$
MW_7	$2.44^{b} \pm 0.20$	$2.20^{b} \pm 0.010$	$2.623^{d} \pm 0.020$
MW_8	$3.22^{d} \pm 0.40$	$3.20^{c} \pm 0.002$	$6.824^{e} \pm 0.400$
MW_9	$2.44^{b} \pm 0.20$	$2.44^{b} \pm 0.001$	$6.824^{e} \pm 0.500$
MW_{10}	$2.42^{b} \pm 0.10$	$1.66^{a} \pm 0.002$	$3.159^{f} \pm 0.100$
MW_{11}	$4.64^{e} \pm 0.20$	4.22 ± 0.002	$3.675^{f} \pm 0.020$
MW_{12}	$1.55^{a} \pm 0.020$	$1.20^{a} \pm 0.001$	$2.094^{a} \pm 0.020$
MW_{13}	$1.96^{a} \pm 0.10$	$1.12^{a} \pm 0.002$	$0.687^{b} \pm 0.020$
MW_{14}	$2.52^{b} \pm 0.20$	$1.77^{b} \pm 0.100$	$1.045^{c} \pm 0.020$
MW_{15}	$1.64^{a} \pm 0.10$	$1.43^{a} \pm 0.010$	$1.039^{c} \pm 0.010$
MW_{16}	$3.62^{c} \pm 0.20$	$2.22^{b} \pm 0.020$	$1.313^{c} \pm 0.010$
MW_{17}	$3.22^{d} \pm 0.30$	$1.23^{a} \pm 0.010$	$1.084^{c} \pm 0.020$
MW_{18}	$2.42^{b} \pm 0.20$	$2.11^{b} \pm 0.010$	$2.524^{d} \pm 0.020$
MW_{19}	$2.66^{b} \pm 0.40$	$3.23^{c} \pm 0.002$	$5.823^{e} \pm 0.400$
MW_{20}	$2.40^{b} \pm 0.20$	$2.44^{b} \pm 0.001$	$6.820^{e} \pm 0.500$
MW_{21}	$2.46^{b} \pm 0.10$	$1.64^{a} \pm 0.002$	$3.145^{f} \pm 0.100$
MW_{22}	$4.64^{b} \pm 0.20$	$4.22^{d} \pm 0.002$	$3.574^{f} \pm 0.020$
MW_{23}	$0.98^{a} \pm 0.020$	$1.22^{a} \pm 0.001$	$2.092^{a} \pm 0.020$
MW_{24}	$1.66^{a} \pm 0.10$	$1.22^{a} \pm 0.002$	$0.687^{b} \pm 0.020$
MW_{25}	$2.56^{b} \pm 0.20$	$2.44^{b} \pm 0.100$	$1.045^{c} \pm 0.020$
MW_{26}	$1.66^{a} \pm 0.10$	$1.44^{a} \pm 0.010$	$1.048^{c} \pm 0.010$
MW_{27}	$3.66^{c} \pm 0.20$	$2.24^{b} \pm 0.020$	$1.314^{c} \pm 0.010$
MW_{28}	$2.64^{d} \pm 0.30$	$1.24^{a} \pm 0.010$	$1.073^{c} \pm 0.020$
MW_{29}	$2.44^{b} \pm 0.20$	$2.34^{b} \pm 0.010$	$2.524^{d} \pm 0.020$
MW_{30}	$3.44^{d} \pm 0.40$	$3.22^{c} \pm 0.002$	$6.682^{e} \pm 0.400$
MW_{31}	$2.44^{b} \pm 0.20$	$2.46^{b} \pm 0.001$	$6.622^{e} \pm 0.500$
MW_{32}	$2.44^{b} \pm 0.10$	$1.66^{a} \pm 0.002$	$3.146^{f} \pm 0.100$
MW_{33}	$4.66^{b} \pm 0.20$	$4.24^{d} \pm 0.002$	$3.664^{f} \pm 0.020$

*Means ± (SD) of 3 determinations. Values on the same column with different superscripts are significantly different ($p<0.05$). MW_1 - MW_{11} = water from different locations in the region. WHO/UNICEF recommended values: oxalic acid (<5 mg/l), formic acid (<4 mg/l), acetic acid (<6 mg/l).

contamination by fecal matter of human and animal origin. Also there is copious evidence that poor handling and sanitation would result in the increased proliferation of microorganisms and this will exacerbate the bacterial populations including pathogens. For example, it has been reported that water used for drinking and domestic uses in Nigeria have been found to be heavily contaminated with fecal matter (Blum et al., 1987; Ogan, 1988).

The possible explanation for the absence of microorganisms including pathogens in some samples could be that proper hygienic processing and handling conditions were maintained thereby, preventing post process re-contamination and cross-contamination. This observation is in agreement with the report of ICMSF (1980), Jay (1986) reported that proper handling after processing could prevent bacteria re- entry into the processed products. The potential health risks associated with these pathogens is that of healthy carriers, especially individuals with nasal carriers and boils. When such persons are involved in handling food items including water, both before and after processing, such individuals would constitute transmission vectors. The above observations are in agreement with the reports of WHO (2011); Hunter and Fewtrell (2001). Hence, people hand-

ling foods including drinking water should be made to pass microbiological tests in order to detect such healthy carriers.

Although *Salmonella* species was not detected in the samples, it could have been due to possible limitations in the analytical procedures since other associated organisms such as *E. coli, S. aureus, Alcaligenes, Acinetobacter, B. subtilis* and *Pseudomonas* were present. Moreover, the frequent case of reported typhoid fever in the region is a proof of the above assertion (Blum et al., 1987; Ogan, 1988). The presence of *S. aureus* in some samples could be due to re- contamination.

The low level or absence of *S. aureus* in some samples is because *S. aureus* is not usually of fecal origin and that its presence in the samples could have been associated with post processing contamination due to bottle/sachet leakage. The public health significance of these microorganisms can hardly be over-emphasized. They have been shown to cause cholera, typhoid fever, hepatitis, diarrhea, gastroenteritis and some emerging strains of *E. coli* have been reported to cause septicemia and urinary tract infections especially, in immuno-compromized individuals (Todar, 2002; Bik et al., 2010).
Table 3 presents the inorganic ions and pH values of the samples. It is evident that their values were within the acceptable levels. The robust Ca: P ratio in the samples is of nutritional importance as low Ca: P ratio has been shown to cause osteoporosis in animals (Orish et al., 2006; Jorge et al., 2007; Nachiyunde et al., 2013).

Although, the in-organic ion concentrations were within the standard limits, the low level or absence of some ions in some samples could be that some must have been used by the organisms to obtain necessary growth nutrients for their proliferation (Barrell et al., 2000).

Table 4 gives the essential organic acids in the water samples. Although organic acids are not regulated in drinking water, short-chain organic acids are formed as by-products during ozonation from natural organic matter present in the water (Chigbu and Sobolev, 2007). Ozonation has been used effectively as drinking water treatment technique for disinfection, destruction of taste and odor compounds and color removal amongst others (WHO, 1993; 1998, WHO/UNISEF, 1996; White, 1999). However, ozonation process should be followed by biological filtration to remove biodegradable organic compound such as organic acids so as to provide biological stable water and prevent bacterial re-growth and water borne disease outbreaks (LeChevallier, 1990; Stenstrom, 1994). The pH values of the water samples show that the levels are within the permissible acceptable standards by NAFDAC, the local regulator of food, water and drug marketed in Nigeria.

Conclusion and recommendations

The results show that although the levels of organisms in most of the water samples were within the acceptable limits, it should however, be a source of concern considering the limitations in the identification, type of organisms identified, and risk of post- treatment contamination and cross - contamination. The essential elements present in the samples were within the acceptable limits. Calcium ion was present in the highest concentration compared to other ions and the Ca/P ratio was good. Based on the findings it is, therefore, recommended that the mandatory standard level of chlorination should be adhered to, and the need for public enlightenment on the frequent routine microbiological checks due to cross- contamination and re-contamination through bottles/sachets leakage be encouraged. Moreover, further studies that should provide insights into the new emerging pathogens such as *E.Coli* 015:H7, *Helicobacter sp*, and *Caliciviruses* should employ the use of molecular methods coupled with throughput parallel processing, bio-informatics and *Cryptosporidium oocysts* tests in the analysis of drinking water. Finally, there is need to correlate bottled/packaged water micro flora with the source flora.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Optimization of bioprocess for enhanced production of alkaline protease by a *Bacillus subtilis* SHmIIIa through Plackett-Burman design

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Optimal conditions for the maximum production of alkaline protease by *Bacillus subtilis* SHmIIIa were evaluated by Plackett-Burman design. Nine process parameters namely, pH, temperature, agitation, inoculum, glucose, peptone, KH_2PO_4, $FeSO_4$ and tween 20 at two levels were selected for the design. Out of nine selected parameters, six parameters (pH 9.75, agitation 225 rpm, inoculum 3.5%, glucose 5.5 g/L, peptone 3.5 g/L and KH_2PO_4 1.5%, have shown significant influence on alkaline protease production. Optimization has resulted in spectacular enhancement in alkaline protease yield from 966 to 36000 EU/mg/ml with 37.26 fold increase.

Key words: *B. subtilis*SHmIIIa, alkaline protease, optimization, design of experiments, Plackett-Burman Design.

INTRODUCTION

Proteases (EC: 3.4. 21-24, 99) are one of the important groups of industrial enzymes with a share of more than 60% of the total global enzyme production (Beg et al., 2003). Alkaline proteases have several applications in a variety of industries such as detergents, foods, textile, pharmaceuticals, leather and in diagnostic reagents, recovery of silver from used x-ray films, wastewater treatment and clinical and medical applications (Kaur and Satyanarayana, 2005). International share market is expected to reach US $4.4 billion by 2015 (Oskouie et al., 2008). In view of the market potential, throughout the globe, the search for a potent alkaline protease producing bacteria which cater the needs of the current industrial sector is being taken up (Sen and Satyanarayana, 1993). Studies conducted so far suggest that microbial extracellular protease production is significantly influenced by media components including carbon, nitrogen sources and other environmental factors. A balance between the various medium components in production media is necessary for the enhanced protease production. The goal of any industrial process professional is to identify the factors that show a

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significant influence on the production. In any industrial setup, development of the cost effective fermentation process is necessary and also a challenging job. Optimization helps by minimizing the amount unutilized components. Statistical approaches have been made to identify the key factors to enhance the yield and reduce the cost of production to approximately 30-40% making the industrial process economically sustainable (Kaur and Satyanarayana, 2005). One-factor-at-a-time design approach has been quite popular with the researchers, mainly due to its simplicity of use. However the drawbacks are: it is time-consuming, requires more experimental runs and also cannot examine the interaction between factors. In this regard, the Plackett-Burman Design (PBD) is an efficient mathematical approach widely applied (Plackett and Burman et al., 1946). These experimental designs very clearly analyze the effects of variables on the response and pick up the essential factors). In light of the ever increasing demand for proteases, in the present study an attempt was made to maximize the alkaline protease production by *Bacillus subtilis* SHmIIIa mutantstrain using PBD in submerged and shake culture conditions.

MATERIALS AND METHODS

Microorganism

The bacterium used in the present studies was isolated from the slaughter house waste contaminated soils of Warangal (latitude 17° 58' N, longitude 79° 40' E and altitude 302 m (990 feet) located in Telangana state, India. The organism was isolated and tested for prospective alkaline protease production on alkaline skimmed milk agar (ASMA; Poly peptone 5 g/l; yeast extract 5 g/l; glucose 10 g/l; KH_2PO_4 1.0 g/l; $MgSO_4$ 0.02 g/l; skimmed milk 2 g/l; agar 18 g/l; Na_2CO_3 10 g/l) at pH 11.0, Protease production was readily observed by the formation of zone of clearance around the colony (Hegde et al., 2002).The culture of the mutant strain was maintained on Horikoshi basal medium (HBM) at pH 11 (Horikoshi, 1971).

Molecular identification

The efficient strain was tentatively identified as *Bacillus* sp based on morphological and biochemical characteristics and based on16S rRNA sequence homology and distance matrix, the organism was further identified as *Bacillus subtilis* sub sp. *Subtilis* strain DSM 10.

Mutagenesis with HNO_2

$NaNO_2$ with 0.1-0.3 M solution was prepared in carbonate buffer (0.02M pH 9.5). To one milliliter of the bacterial suspension 5 ml of the $NaNO_2$ was added. The solution was shaken thoroughly (Carlton and Brown, 1981). The one milliliter solution was withdrawn and diluted with 5 fold phosphate buffer (0.2 M, ph 7.1) to stop the reaction. A control was maintained by replacing $NaNO_2$ in acetate buffer with sterilized saline water. After fixed time interval, the tubes were spun at 6000 g for 15 min. The supernatant was discarded to remove nitrous acid from the pellet and ten milliliter of sterilized phosphate buffer was added to make the bacterial suspension. The tubes were respun for the three times to remove traces of nitrous acid. After washing the pellet was suspended in the same buffer. One milliliter of the bacterial suspension was plated on the ASMA medium and incubated at 30^{o}C for 48 h.

Enzyme production and assay

A single colony obtained on modified HBM with 1% skimmed milk was activated twice before the starter culture was prepared for seed inoculation. The starter culture was prepared by raising a cell suspension in a 500 ml baffled flask containing 100 ml of HBM medium (pH to 11.5±0.5) and incubated for 24 h on a shaker at 200 rpm. The starter absorbance was adjusted to 0.3 at 600 nm with modified Horikoshi medium. About 100 ml of ASMA medium with varying concentrations of the carbon, nitrogen and inducer sources was taken in flasks. Incubation was carried out for 24 h under the defined conditions. The cell-free supernatant recovered by centrifugation (10000 g, 10 min) was used as enzyme source. Extracellular alkaline protease activity was determined by the modified method of Kunitz (1947) and Yang et al. (1994).

About 0.1 ml of enzyme source was mixed with 0.9 ml of glycine-NaOH buffer (0.1M, pH 9) that contained 5 mg Hammerstein casein. Reaction mixture was incubated at 55□C for 10 min and later 2 ml of 5% trichloroacetic acid (TCA) was added to terminate the reaction. The contents were passed through No.2 filter paper (Whatman) to remove denatured proteins, filtrate was centrifuged at 10000 g for 10 min. The supernatant fraction was read at 275 nm. One unit of protease activity was defined as the amount of enzyme required to produce an increase 0.001 in the absorbency at 275 nm per min under the assay conditions. Specific activity was expressed as enzyme units per mg protein. Simultaneously, a blank without enzyme was used for comparison purpose.

Evaluation of cultural parameters for growth

The optimal growth conditions that is, pH, temperature, incubation time and inoculum levels for wild strain *B. subtilis* SH2 were evaluated by using MHBM broth.

Evaluation of nutritional parameters using OVAT method

The influence of various nutrients such as carbon and nitrogen sources was studied by one-variable-at-a-time method (OVAT method). Carbon sources (glucose, fructose, sucrose, maltose, lactose and starch) at the concentration of 1% (w/v) were evaluated while other components were maintained constant in the HBM composition. Similarly different nitrogen sources (tryptone, soy peptone, peptone, skim milk, yeast extract, beef extract) were analyzed at 0.5% levels while keeping other constituents at constant level.

Simulation of the Plackett-Burman Design

In order to identify the important variables affecting the enzyme production, PBD design with a set of two-level fractional factorial design with 12 experiments was employed. In this design, no main variables were mixed with any other variable, therefore, it efficiently achieves the goal and reduces the number of experiments required (Zhi and Feng et al., 2001; Zhng et al. 2013). PBD model was checked by F - test and goodness of fit by multiple regression analysis (De Coninck et al., 2000).The Design of experiments (DoE) methodology was utilized by employing the statistical analysis system (SAS) software version 9.0 (U.S.A).

Table 1. Optimized bioprocess parameters by OVAT method for *Bacillus subtilis* SHmIIIa.

Process variable	Mutant SHmIIIa	
	Formulated medium 1	
	Optimized level	EU/mg/ml
pH	9.56	3656
Incubation	66±6 h	
Temperature	43	
Inoculum	3%	
Rpm	200	
C	Glucose 5%	
N	Peptone 3%	
P&K	KH_2PO_4 1%	
Metal ion	$FeSO_4$0.2 mM	
Surfactants	Tween20 0.4%	

Validation of the experimental model

A random set of experiments were simulated to study the production of alkaline protease under the defined experimental conditions. The model was statistically validated with respect to all responses within the design space (Sasikumar and Viruthagiri, 2008). The conditions optimized from the model were further confirmed for accuracy by using the first order polynomial equation $Y= \beta_0 + \Sigma \beta_i X_i$ (i = 1, …. k) Where, *Y* is the estimated target function, *β0* is a constant, *β1 is* the regression coefficient, *X* is independent variable and *k* is the number of variables.

RESULTS

Microorganism

Based on the morphological, physiological and biochemical characteristics the bacterial isolate was tentatively identified as *Bacillus* sp. Further authentication was made by the 16S rDNA gene sequence analysis. The isolate SH2 showed 99% sequence homology with *B. subtilis* sub sp. *subtilis* strain DSM 10. Subsequently the isolate was mutated with HNO_2.

Evaluation of bioprocess parameters by OVAT methodology

In order to identify the essential process parameters and influence of various environmental, physiological and nutritional parameters on alkaline protease production OVAT methodology was adopted and the results are presented in Table 1. Our preliminary investigation revealed that FMI was found superior to HBM. Hence, the FM1 was considered as ideal for improved protease yield under the defined experimental conditions for the mutant strain.

Plackett-Burman design

Optimization of the fermentation conditions to maximize enzyme production was performed by the Plackett-Burman Design. A total of nine parameters were selected based on the OVAT method. The PBD simulation consisted 12 experiments at a resolution of 3. Each independent variable was investigated at a high (+1) and a low (-1) level which represent two different nutrient concentrations as shown in Table 2. The yield of the alkaline protease production by the present strain in defined fermentation conditions was studied and the results are précised in the Figure 1. Theyield of the enzyme was determined based on the t- values of the significant ingredients (Devendra and Pravin, 2010).

Determination of significance of each variable

The *p*-value serves as a tool for identification of significance of each variable and measures the probability magnitude coefficient of the process variability. The components were screened at the confidence level of 95% on the basis of their effects. Table 3 represents the results with respect to *t*-value, *p*-value and confidence level of each component. The present PBD model suggests that the componentsX1 pH; X3 agitation; X4 inoculum; X5 glucose; X6 peptone and X7 $KH_2PO_4$are significant or effective in alkaline protease production (Figure 1).

Validation of the model terms

The model was assessed using multiple regression equation (Myers and Montgomery, 2002) (Table 4). The model has shown 99.90 of the correlation between the model terms and the alkaline protease production. Hence, the model terms and alkaline protease production were strongly correlated (Palvannan and SathishKumar, 2010).

DISCUSSION

In general, the requirements of different bacterial strains for fermentation process differ. This usually depends upon the adaptive nature of the organism (Moorthy and Baskar et al, 2013; Pathak and Deshmukh, 2012). The regulatory mechanism behind the enhanced enzyme production is not yet understood. However, the contribution of the every physical and nutritional factor for optimal response should be known by performing OVAT method (Jeevan Chandra, 2013).Therefore, the role of individual factor has been taken into consideration for building a model process for optimized response (Moon and Parulekar, 1991; Periasamy et al., 2013). Through statistical analysis of the above model it is evident that out of nine parameters tested only six viz., pH; agitation; inoculum; glucose; peptone and KH_2PO_4 have shown a significant influence on enzyme production. The

Table 2. Placket Burman Design details of variables for production of alkaline protease by *Bacillus subtilis* SHmIIIa

Run	pH	Temp	Agitation	Inoculam	Glucose	Peptone	KH_2PO_4	FeSO4	Twntwnty	Protease
1	10	43	250	3	5	3	2	0.4	0.4	25734
2	10	45	200	4	5	3	1	0.4	0.4	1673
3	9.5	45	250	3	6	3	1	0.2	0.4	1243
4	10	43	250	4	5	4	1	0.2	0.2	26371
5	10	45	200	4	6	3	2	0.2	0.2	1435
6	10	45	250	3	6	4	1	0.4	0.2	21826
7	9.5	45	250	4	5	4	2	0.2	0.4	16363
8	9.5	43	250	4	6	3	2	0.4	0.2	1503
9	9.5	43	200	4	6	4	1	0.4	0.4	1232
10	10	43	200	3	6	4	2	0.2	0.4	21935
11	9.5	45	200	3	5	4	2	0.4	0.2	20435
12	9.5	43	200	3	5	3	1	0.2	0.2	1373

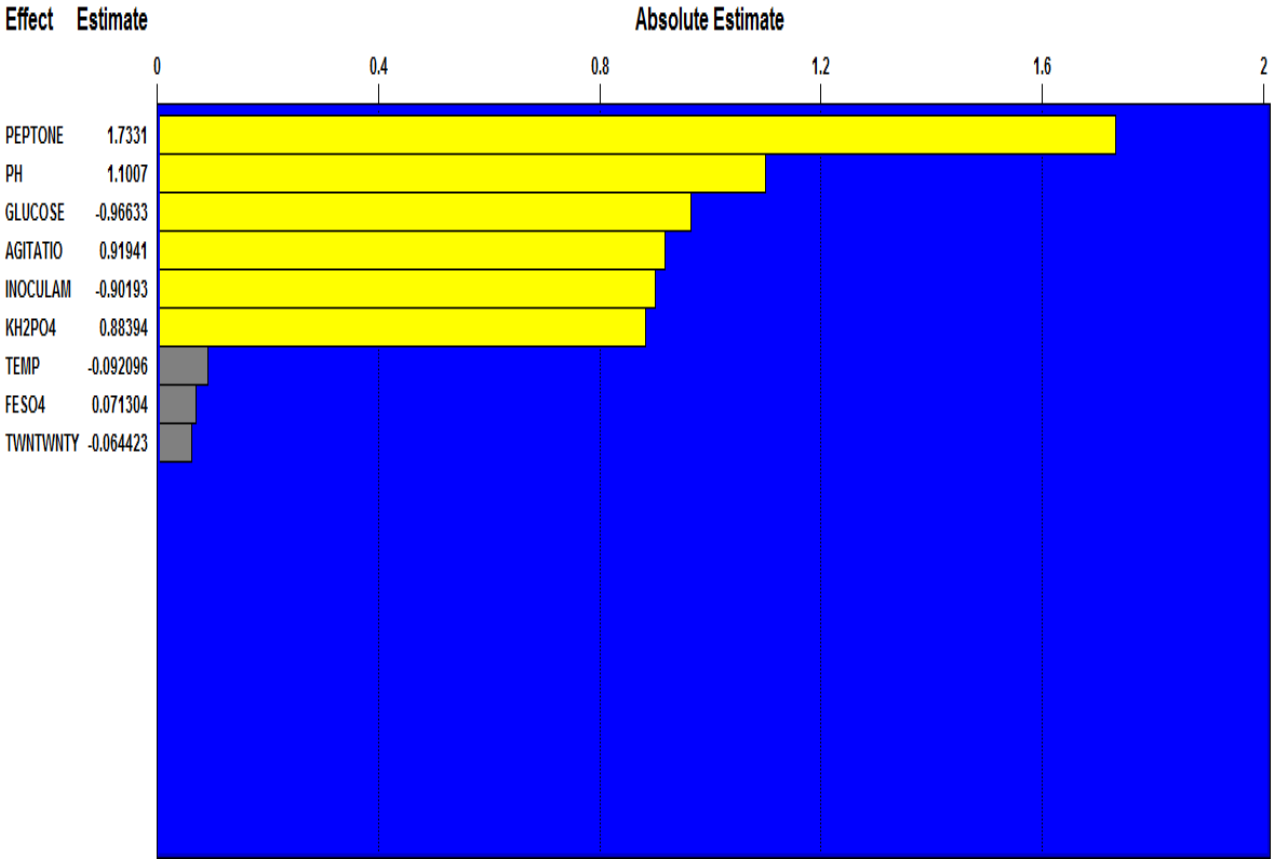

Figure 1. Pareto chart showing the important effects on alkaline protease production by Plackett-Burman Design for*Bacillus subtilis* SHmIIIa.

probability *P* value for lack of fit (0.022395) indicated that the experimental data obtained with the model is very good. In addition, the parameters have shown a direct influence on enhanced production by native strain in shake cultures (Cazetta et al., 2007).

Physiological adaptation of the strain shows a direct influence on the cellular activity of enzyme secretion. pH has strong influence in many enzymatic processes and transport of several molecules across the cell membrane. Difference in media pH alters acid-base equilibria and

Table 3. ANOVA of the Plackett-Burman Design for alkaline protease production by *Bacillus subtilis* SHmIIIa.

	Master model					Predictive model				
Source	**DF**	**SS**	**MS**	**F**	**Pr > F**	**DF**	**SS**	**MS**	**F**	**Pr > F**
PH	1	3.634563	3.634563	324.5909	0.003067	1	3.634563	3.634563	240.5611	0.0001
Temp	1	0.025445	0.025445	2.272418	0.270698					
Agitation	1	2.535926	2.535926	226.4753	0.004386	1	2.535926	2.535926	167.8456	0.0001
Inoculum	1	2.440452	2.440452	217.9488	0.004557	1	2.440452	2.440452	161.5264	0.0001
Glucose	1	2.801387	2.801387	250.1827	0.003973	1	2.801387	2.801387	185.4156	0.0001
Peptone	1	9.011211	9.011211	804.7618	0.00124	1	9.011211	9.011211	596.4257	0.0001
KH2PO4	1	2.344055	2.344055	209.3399	0.004743	1	2.344055	2.344055	155.1462	0.0001
FESO4	1	0.015253	0.015253	1.362167	0.36349					
Twntwnty	1	0.012451	0.012451	1.111953	0.40224					
Model	9	22.82074	2.535638	226.4496	0.004404	6	22.7676	3.794599	251.1534	0.0001
Error	2	0.022395	0.011197			5	0.075543	0.015109		
Total	11	22.84314				11	22.84314			

Table 4. Validation of the PBD model for alkaline protease production by *Bacillus subtilis* SHmIIIa

Analysis of coeffient of variance	Master model	Predictive model
RMSE	0.105818	0.122917
R-square	99.90%	99.67%
Adjusted R-square	99.46%	99.27%
Coefficient of Variation	1.227784	1.42619

fluxes of various nutrients in cell interior. The common optimum pH range for protease production among alkaliphilic and halo alkaliphilic organisms lies between 9 to 10 (Patel et al., 2006). The present investigations suggest that the test organism grows profusely at pH 9.75, which markedly specifies the alkaliphilic nature of the strain. Present observations are in agreement with the earlier studies carried out by Bhaskar et al. (2007).Krulwich (1995) has studied the facultative alkaliphile *Bacillus firmus*OF4 and pointedthat Na1/H1 anti-porter enables cells to adapt to shift in pH and to maintain the external pH in the most alkaline range of pH for growth.

The agitation rate has indirect relation with the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by means of variations in the aeration rate and agitation speed (Chu et al., 1992). The variation in the agitation speed influences the extent of mixing in the shake flasks and distribution of the substrates evenly in the medium makes them available for the growth and protease production (Moon and Parulekar, 1991*).*Hence, considerable improvement in the production of the alkaline protease can be achieved with the proper maintenance of the agitation. In the present investigation, 225 rpm of the speed agitation was found to be ideal for the enhanced protease production. These results concur with the earlier studies made by Sudhir and Ashis (2010).

A high inoculum size improves biomass production, while low inoculum size is generally unsupportive for the growth as well as enzyme secretion. A moderate inoculum size was suggested to the increased protease production, which may be due to the higher surface area to volume ratio resulting in increased protease production (Michalik et al., 1995).In the present investigations, the alkaline protease production steadily increased with increase in inoculum size and reached maximum at 3.5% level of inoculum production. Similar observations were made by Reddy et al. (2008).

Nutritional factors are the key contributors with a great extent of capacity for enhanced production of alkaline protease. The alkaline protease comprises 15.6% nitrogen and thus its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole et al., 1988). In the preset study, peptone and glucose have shown a significant influence on protease production and are considered as the prime factors of the entire process in the master model. For alkaline protease production, the complex nitrogen sources are usually preferred over the simple inorganic. This is just because of the feedback inhibition imposed by simple nitrogen sources. The same situation was

Table 5. Factors optimized through PBD for enhanced alkaline protease production by *Bacillus subtilis* SHmIIIa.

Factor	Setting	EU/mg/ml
pH	9.75	
Temperature	44°C	
Agitation	225 rpm	
Inoculum	3.5%	
Glucose	5.5 g/l	36000
Peptone	3.5 g/l	
KH_2PO_4	1.5 g/l	
$FeSO_4$	0.3 mM	
Tween20	0.3%	

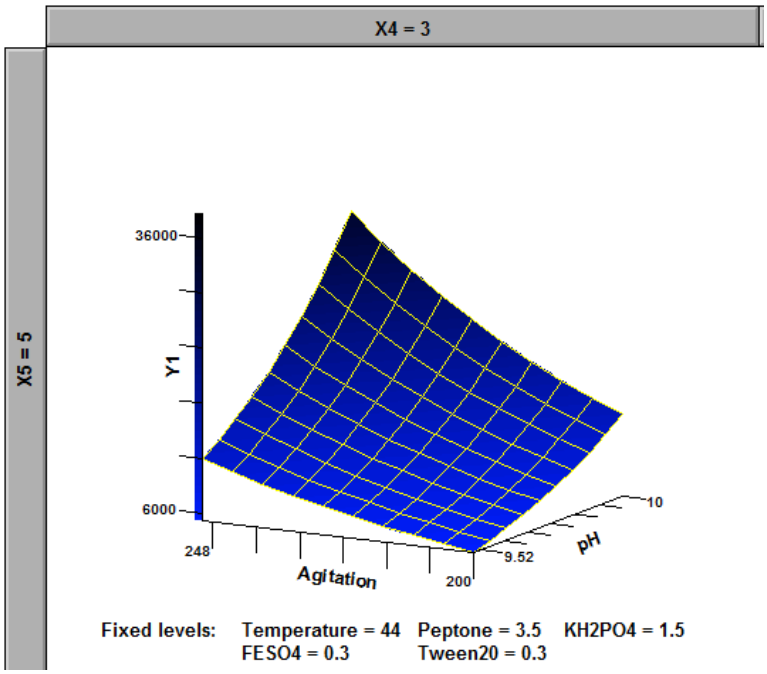

Figure 2. Response surface plot for production of alkaline protease by *Bacillus subtilis* SHmIIIa.

encountered with carbohydrates. Low levels of alkaline protease production were reported in the production medium (Chandrasekaran and Dhar, 1983). Present observations are similar to those of the studies made by Chaphalkar and Dey (1994) and Sen and Satyanarayana (1993).

Validation of the predicted model for enhanced alkaline protease production

The t-values of the process variables are 18.01641, 15.0491, 28.36832, 14.46858 and 1.167119 for pH, agitation, peptone, K_2HPO_4 and $FeSO_4$ respectively, showing a significant improvement. These findings indicate that these variables are very important for optimization of alkaline protease production. The R^2 and adjusted R^2 are 99.90 and 99.46% respectively which indicate a high correlation between the observed values of master model and the values of predicted model. This specifies that regression model provides an excellent correlation between the independent variables (factors) and the response (alkaline protease production) (Table 5).

The wild strain (SH2) at primary screening level has shown only 966 EU of alkaline protease production, however on mutagenesis it has increased to 3688 EU of enzyme production with 3.8 fold increase. The same bacterial strain in the final phase of optimization through PBD has shown a dramatic enhancement to 36000 EU with 37.26 fold increase (Figure 2).

Conclusions

The present investigations were primarily aimed at identifying the important factors which contribute to the enhanced protease production. The PBD has allowed the evaluation of main variables of the enzyme bioprocess. The PBD model adopted in the present investigations explained the effect of pH, agitation, inoculum, glucose, peptone and KH_2PO_4 on alkaline protease production by *B. subtilis* SHmIIIa. Further, investigations on characterization and stability of the alkaline protease under harsh industrial conditions are under progress.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Virulence factors expressed by *Mycobacterium ulcerans* strains: Results of a descriptive study

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***Mycobacterium ulcerans* is a slow-growing mycobacterium responsible for Buruli ulcer. The pathogenic virulence of *MU* is being linked to the expression of toxin called Mycolactone, whose form varies according to the origin of the productive strains. Forms A and B are secreted by African and Malaysian strains, C by Australian strains and D by Asian strains. Forms E and F of mycolactone are secreted by animal mycolactone producing mycobacteria strains. The genes for the biosynthesis of Mycolactone are located on a plasmid called pMUM001. We investigated the circulation of Mycolactone in body fluids coupled to the detection of virulence factors in *MU* strains. Suspicious BU patients and healthy subjects (negative controls) were selected in three Ivorian endemic areas. Exudates, fine needle aspiration (FNA) and blood samples were collected. Microscopy by Ziehl-Neelsen-staining, culture and PCR diagnostics using IS2404 and KR were performed in patient samples. The Mycolactone detection by HLPC coupled to MS was performed in patient and control samples. PCR using IS2404, IS2606, KR and ER were also performed in *MU* strains. Ziehl-Neelsen-microscopy detected acid-fast bacilli in 19% of samples while PCR were positive in 76.2% for IS2404 and 52.4% for KR. Mycolactone A/B was detected in 31% of exudates and in 42.8% of sera. No Mycolactone was detected in control subjects. 17 strains isolated from exudates possessed both IS2404 and IS2606. 70.6% of those strains were positive for KR and ER gene. The study shows that Mycolactone A/B was actually present in most of BU patients selected in three Ivorian endemic areas. With the methods used we detected very low concentrations in patient fluids. Plasmid and ER gene were found in the majority of *MU* strains. But they were not found in about 30% of strains. Mycolactone was detected only in patients infected by strains in which plasmid was found.**

Key words: Buruli ulcer, *Mycobacterium ulcerans*, insertion sequence, virulence factor, Mycolactone.

INTRODUCTION

Mycobacterium ulcerans (MU) is a slow-growing mycobacterium responsible for Buruli ulcer (BU). The cutaneous mycobacteriosis is mostly seen in tropical and subtropical countries (Anne-Caroline et al., 2013; Emmanuelle et al., 2007; Hong et al., 2005; Laurence et al., 2009; Mac et al., 1948). The pathogenicity of *MU* is known to be linked to the secretion of a toxin named mycolactone (Caroline et al., 2009; Emmanuelle et al.,

2007; Kathleen et al., 1999; Sarojini et al., 2005; Yoshito, 2011). The forms of Mycolactone are classified according to the origin of the productive strains. Forms A and B are secreted by African and Malaysian strains; C by Australian strains and D by Asian strains (Kathleen et al., 1999; Sacha et al., 2005). Forms E and F have been found in mycobacteria strains isolated from animals (Armand et al., 2005; Brian et al., 2006; Hong et al., 2008; Sylvain et al., 2008). Mycolactone is responsible for the tissue necrosis and the diverse immune reactions of the host (Armand et al., 2003; Emmanuelle et al., 2007; Kathleen et al., 1999; Hurtado et al., 2009; Phillips et al., 2006; Sarojini et al., 2005; Travis et al., 2001). The biosynthesis of Mycolactone is linked to some genes located on a giant circular 174 Kb plasmid named pMUM001 (Mac et al., 1948; Timothy et al., 2004; Timothy et al., 2005; Timothy et al., 2005). More than half of the plasmid is devoted to six genes among which three encode the synthesis of four types of polyketide synthetases (PKS). The molecular diagnosis of BU is performed by PCR targeting insertion sequences of *MU* (IS2404, IS2606). This search can be coupled by detection of the plasmid which carries the genes encoding the synthesis of Mycolactone (Elise, 2011). Sarfo et al. (2011) showed in 2011 that Mycolactone circulates actually in BU patients at various stages of antibiotic therapy as recommended by WHO. However, in other infected people of that cohort, Mycolactone was found, neither in the exudates nor in the serum (Sarfo et al., 2011). In this study we sought to understand why Mycolactone was not detected in all BU patients. The first objective was to characterize Mycolactone in body fluids of patients who had got no antibiotics treatment by rifampicin and streptomycin. The second objective aimed at detecting the virulence factors in clinical samples and in *MU* isolated strains.

MATERIALS AND METHODS

Patient cohorts

Buruli ulcer suspected cases and healthy subjects (negative controls) were selected in three endemic areas of Côte d'Ivoire. Some patients were recruited in the last quarter of 2011 by local health workers in two endemic areas (Tiassalé and Djékanou), where there is a high prevalence of BU. The others were recruited in January 2012 by a mobile medical team actively screening the district of Abidjan, a hypo-endemic area. Patients were recruited if they met the WHO clinical case definition of Buruli ulcer disease (nodule, plaque, edema or ulcer). The control subjects were selected according to the following criteria: to have no suspicious lesion, to have never been infected, to have no apparent progressive pathology and to be from the same area as the patients. All subjects provided written informed consent (thumb print of parent or legal tutor in the case of children). The study protocol was approved by the National Ethic Committee.

Sampling procedure

Samples were collected on each lesion (nodule, plaque, edema and ulcer). Four swabs were performed on each ulcer after cleaning with normal saline. Two swabs were discharged into a tube containing 2 ml of Middlebrook 7H9 medium supplemented by Cetylpiridium chloride (0.5%) to achieve the isolation of *MU.* The other two were discharged into a tube containing 1 ml of pure ethanol for the extraction of lipids. A fine needle aspiration (FNA) was collected from pre-ulcerative lesions after application of a topical antiseptic. Each FNA was discharged into two tubes: one containing 2 ml of Middlebrook 7H9 medium and the other one 1 ml of pure ethanol. 10 ml of venous blood were also collected from each patient and control subject.

Diagnosis of Buruli ulcer disease in patients

Exudates and FNA samples were centrifuged at 3000 rpm for 20 min. The pellet was re-suspended in 2 ml of saline buffer to test three diagnostic methods. Four tubes of Löwenstein-Jensen medium were inoculated with 100 µl of each homogenate and incubated at 32°C. A daily supervision was instituted till the appearance of colonies. Microscopic examination to detect Acid-fast bacilli was performed by using dried smears made with 10 µl of homogenate and staining by the Ziehl-Neelsen technique (Adalbert et al., 2000). 1 ml of homogenate was washed twice by adding 1 ml of sterile DNA-free water. The mixture was centrifuged at 14000 rpm for 3 min, and the pellet was suspended in 400 µl of lysis buffer containing 20 mM Tris, 2 mM EDTA, 150 mM NaCl, 50 mM NaOH, 1% SDS and 20 µg/ml Proteinase K. The mixture was incubated at 37°C for 24 h. The phenol/chloroform method was used to extract and purify DNA. DNA was eluted in 50 µl sterile DNA-free water and stored at -20°C for molecular diagnosis. PCR was performed using the *GoTaq Flexi DNA polymerase* reagents Kit (Promega, Germany). The previous protocol was used to detect the IS2404 and KR in samples (Table 1). The reaction (25 µl) contains 5 µl of DNA, 0.3 µM of each primer, 0.25 µM of labeled probe (for real time PCR), and PCR-Mix. PCR consisted of 35 cycles of melting at 95°C for 5 s, aling and extension at 60°C for 1 min. The 7300 real-time PCR machine (Applied Biosystems, USA) was used and the fluorescence of FAM was measured to determine the amplification threshold cycle (Ct). Classical PCR were performed in 9700 PCR system Thermocycler (Applied Biosystems, USA) to confirm *MU* in isolated strains with the following targets: IS2404, IS2606 and ER. Negative controls were performed with 5 µl of nuclease free-water. Positive controls DNA were tested in duplicate.

Mycolactone extraction and analysis by HPLC

Lipids were extracted from serum or from exudates by the method previously described by Sarfo et al. (2011). Briefly the samples were dipped in 1 ml of ethanol immediately after the sampling and stored in polypropylene tubes at -20°C, protected from light. The samples were concentrated by drying, using a Speed Vacuum and were suspended in 500 µl of ethanol. The lipids were extracted by sequential addition of 4/1 MeOH (v/v), 1/1 $CHCl_3$ (v/v), and 3/1 H_20 (v/v). The aqueous phase was eliminated and the organic phase was transferred into a new tube containing 3 ml of methanol. A double centrifugation was performed and the soluble organic phase was transferred each time into a new tube. It was dried and

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Table 1. Primers used in this study.

Target	Sequence (5'-3')		References
IS2404	Mu5	GAT CAA GCG TTC ACG AGT GA	Stinear and coll., 1999
	Mu6	GGC AGT TAC TTC ACT GCA CA	
IS2606	Mu7	CCGTCACAGACCAGGAAGAAG	Stinear and coll., 1999
	Mu8	TGCTGACGGAGTTGAAAAACC	
Enoyl- reductase	ER-R	GAG ATC GGT CCC GAC GTC TAC	Mve-Obiang and coll., 2005
	ER-F	GGC TTG ACT CAT GTC ACG TAA G	
Keto-reductase	KR-F	TCACGGCCTGCGATATCA	Fyfe and coll., 2007
	KR-R	TTGTGTGGGCACTGAATTGAC	

Table 2. Results of methods applied to samples of patients and controls. Microscopy, culture and PCR methods were applied to exudates and FNA samples. HPLC was applied to samples of patients (exudates, FNA, sera) and controls (sera).

Clinical samples		ZN Microscopy	IS2404 Detection	KR Detection	Culture of *MU*	HPLC (Mycolactone)
Batch A (N=42)	FNA (10)	2	10	5	7	5
	Exudates (32)	6	22	17	10	8
Batch B (N=42)	Sera (42)	-	-	-	-	18
Batch C (N=8)	Sera (8)	-	-	-	-	0
Total	92	8	32	22	17	31
Percentage (%)	-	19	76.2	52.4	40.5	36.9

suspended in 500 µl of ethanol to achieve a Shimadzu HPLC. Fractions of interest were collected in glass tubes and analyzed on a QSTAR XL, AB-MDS-SCIEX mass spectrometer. The data were collected and processed through the Analyst QS 1.1 software from AB-MDS-Sciex.

RESULTS

Microbiological and molecular tests of clinical samples

42 patients and eight control subjects were selected in three Ivorian endemic areas. 25% of these patients had pre-ulcerative lesions (10/42) and 75% had ulcers of various size (32/42). From all subjects 32 exudates, 10 samples of FNA and 50 sera were collected for the study. The microbiology tests were cell culture and microscopy of the clinical samples.

Acid-fast bacilli were found in 19% of exudates and FNA samples. 76% of those samples were positive for genomic target IS2404 and 52% for plasmid located KR target. *MU* was isolated in 40% and Mycolactone was detected in 36% of patient samples. No Mycolactone was detected in control subjects (Table 2).

Mycolactone detection in clinical samples

Total lipids of 92 samples were extracted and analyzed using HPLC method. The analysis by HPLC detected some peaks corresponding to mycolactone in 31% of exudates (13/42) and in 42.8% (18/42) of the sera of patients. The detection of the mycolactone was more consistent when the sample was from a recent lesion (non-ulcerative form or early ulcer). At this stage, the toxin has been detected in 50% of exudates and in 60% of the sera of patients. Conversely, no mycolactone was found in the control serums (0/8). The concentrations of mycolactone A/B in samples were relatively weak compared to those of positive controls (Figure 1A).

The fractions of interest analyzed by Mass spectroscopic (MS) showed in each case the characterizing spectrum of the ion 765, in harmony with the presence of intact structure of the mycolactone A/B in the samples (Figure 2). In the serum of controls, this specific ion and the isotope mass were negative, while the spectrum obtained with a serum of patient from an active Buruli ulcer showed specific ion with a molar mass of 765.4 $g.mol^{-1}$, with generated ions at 359.2, 429.3, 565.4 and 659.3 $g.mol^{-1}$ respectively (Figure 2B).

Molecular identification of isolated strains

To identify isolated strains, a PCR was performed targeting Insertion Sequences (IS2404, IS2606), and plasmid (KR, ER). All *MU* strains were 100% positive for targets IS2404 and IS2606, whereas only 70% were

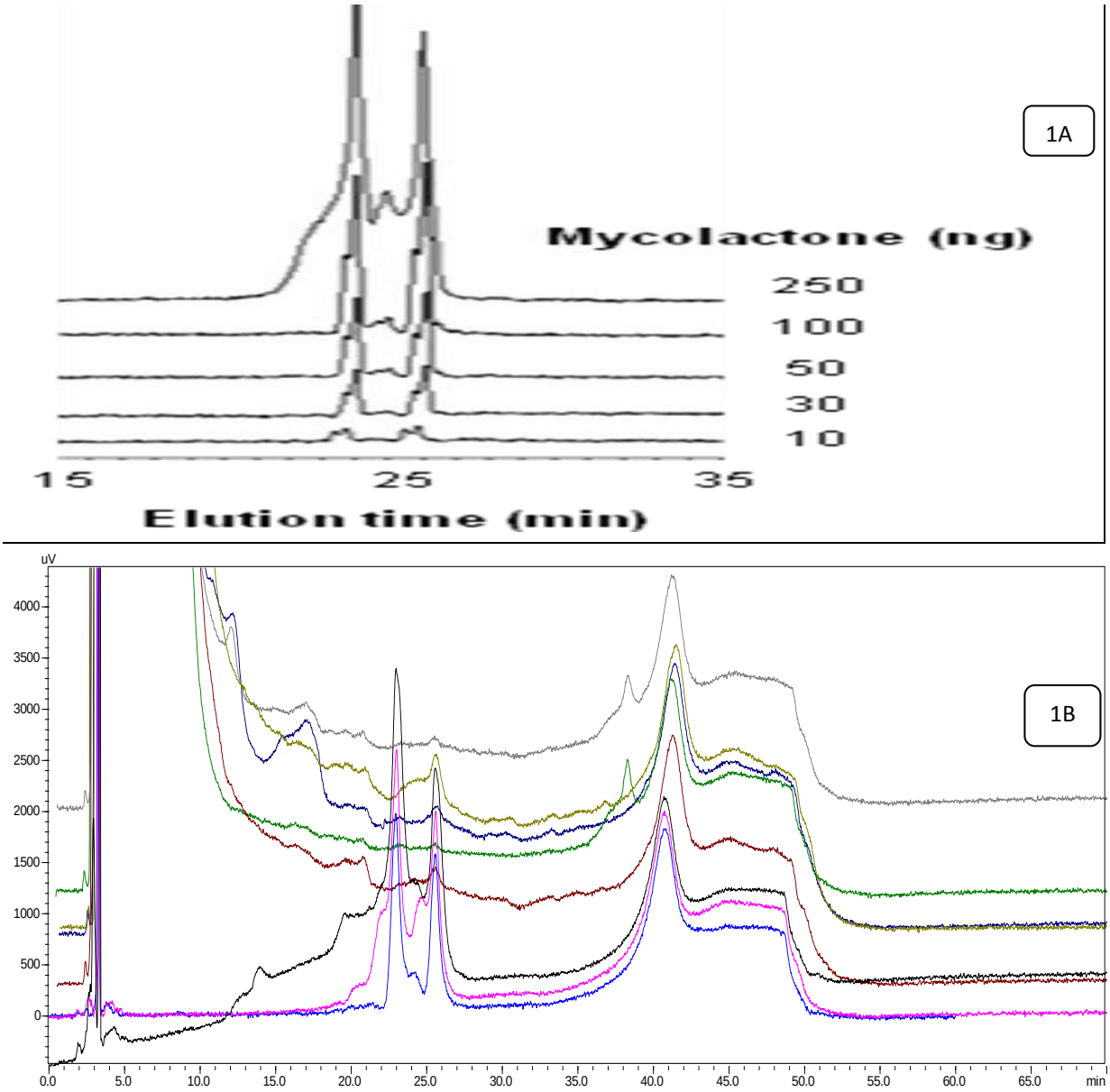

Figure 1. Chromatographic profiles of purified mycolactone (Figure 1A) and mycolactone in clinical samples (Figure 1B). **A**. Corresponds to the spectrum of 5 dilutions of the purified Mycolactone A/B. **B.** Shows signals obtained from clinical samples (exudates/sera). From bottom to top, the first three signals correspond to three concentrations of purified Mycolactone (50 ng; 100 ng; 250 ng). The curves 4 to 8 designate the signals given by the samples. Their mycolactone concentrations were respectively 20, 15.5, 26, 48 and 15 ng per ml. It is shown by weak amplitudes, compared to those of the dilutions of the purified Mycolactone.

positive for targets ER and KR (Table 3).

DISCUSSION

This study follows a previous one carried out on samples from Ivorian and Ghanaian patients. We had pointed out that mycolactone was present in body fluids at various stages of *MU* infection. Those results were obtained by 2 chemical approaches using extracts of total lipids (TLC-Fluo and HPLC/MS). The weak performance of the TLC-Fluo had been accounted by a link between the mycolactone and some biomolecules that could prevent the access to organic solvent during the chemical extraction of Mycolactone. The HPLC coupled by mass spectroscopic permitted to bring out some Mycolactone A/B at all the stages of the infection with *MU* (before, during and after the treatment by antibiotics) (Sarfo et al., 2011;

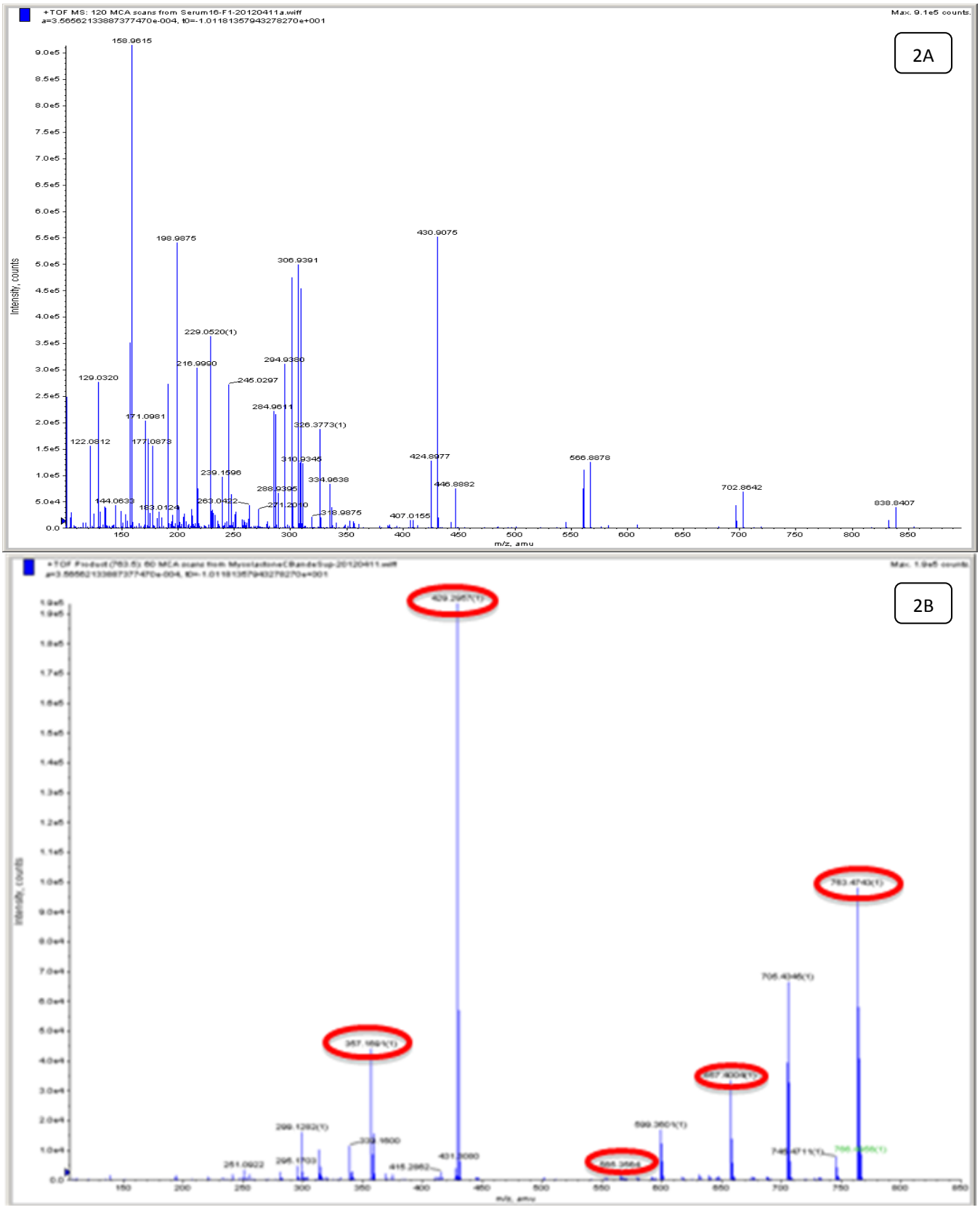

Figure 2. Mass spectrometry (MS) analysis of Acetone soluble lipids from fractions collected in the elution interval with HPLC in negative control (A) and in clinical sample for Buruli ulcer patient (B). The hydrolysis products of mycolactone and intact mycolactone can been identified at m/z 765.4.

Table 3. Molecular tests of isolated M.ulcerans strains.

Origin of strains	Quantity	Target detection		
		IS2404	IS2606	ER/KR
FNA	7	7	7	5
Exudates	10	10	10	7
Total	17	17	17	12
Percentage (%)	-	100%	100%	70.6%

Fred et al., 2014).

Our results show the high positive rate of molecular tests for Buruli ulcer in all samples collected in endemic areas. We used IS2404 and KR targets to detect *MU* in clinical samples. The combination of those targets permitted to confirm BU disease in patients and to determine if plasmid was present. The results show that 76.2% of samples contained IS2404 and plasmid was present in 52.4%. IS2404 and IS2606 have demonstrated the higher positivity by 100% for isolated strains. The high copy number of IS2404 (205-209 copies) and IS2606 (91 copies) in genome explain the sensitivity of PCR using IS2404 or IS2606 (Stinear et al., 2007). Fyfe et al. (2007) have demonstrated the identical sensitivity of PCR-IS404 and PCR-IS2606 (Fyfe et al., 2007).

Concerning PCR using targets KR/ER located on the virulence plasmid, the positivity rate was at 70.6% for isolated strains of *MU* and at 52.4% for clinical samples. The evidence of the plasmid lost or poor DNA extraction efficiency and PCR inhibition can explain our results. Molecular detection of *MU* in clinical and in environmental samples in multicenter external quality control program has demonstrated the limitations and the progress of the diagnosis (Eddyani et al., 2009). The lower positivity of culture by 40.5% is similar with the results of (Eddyani et al., 2008; Miriam et al., 2014). We demonstrated that the isolation in solid medium was more sensitive (82.3%) when the samples were taken from non-ulcerative lesions or from early ulcers (unpublished data). In this study, Mycolactone was detected in 31% exudates/FNA and 42.8% in sera but not in control subjects. The rate of Mycolactone detection varied according to the stage of the lesion. Mycolactone was more frequently detected in the liquid from non-ulcerated lesions (50%) and in the exudates from early ulcers (45.5%).

In contrast, the performance of HPLC was very weak in larger lesions (less than 18%). Similar result was observed in the sera, but the frequency was high for larger ulcers (40%). Our results confirm the evidence that serum was the appropriate biological fluid to follow the kinetics of secretion of the mycolactone during the *MU* infection (Fred et al., 2011; Fred et al., 2014).

The characterization by MS pointed out a predominant secretion of entire molecule of mycolactone A/B (93.6%) and 6.4% of Mycolactone A/B with loss of an ion of “hydrogen”. Our results are similar with the results of Portaels et al. (2008) for mycolactone A/B from *MU* isolated from environmental (Portaels et al., 2008). The results confirmed that isolated strains of *MU* belong to African strains with typically expression of virulence toxin, Mycolactone A/B.

Among the strains, 70.6% had the plasmid and the Enoyl-reductase gene. We compared the results of gene detection to those of the mycolactone detection in body fluids. The analysis showed that in patients infected by *MU* strains having a plasmid, the mycolactone was detected in body fluids. In contrast, when the plasmid was not found in *MU* strains, no mycolactone was detected in patients. These results could be explained by three factors: the first factor could be related to limits of extraction methods used in this study (poor DNA extraction efficiency and PCR inhibition). The second factor could be related to the loss of plasmid by *MU* during the culture process. The third factor could be related to the circulating in endemic areas of *MU* strains with plasmid and those without plasmid.

Conclusion

This study shows that Mycolactone A/B was actually found in most of BU patients selected in three Ivorian endemic areas. With the methods used we detected very low concentrations of mycolactone in patient fluids. Plasmid and ER gene were found in the majority of *MU* strains. But they were not found in about 30% of strains. Mycolactone was detected only in patients infected by strains in which plasmid was found. Further investigations, including the study of the virulence in laboratory animals and analysis of the entire genome of *MU* strains will certainly clarify these issues.

Conflict of interests

The authors did not declare any conflict of interest.

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Prevalence of *Brucella* antibodies in marketed cow milk in Benue State, Nigeria

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Brucellosis is an endemic disease in the animal population in Nigeria and of great public health importance, particularly among livestock owners and workers who are ignorant of the risk of *Brucella* infection. A total of 102 milk samples comprising 62 from cattle herds and 40 from milk vendors in Benue State, Nigeria were screened for *Brucella* antibodies by the milk ring test (MRT). The herds for the study were randomly selected within randomly selected local governent areas from the three geopolitical zones of the state. All herds sampled had no history of vaccination and cows with mastitis were excluded. 17.7% of milk samples from herds and 12.5% samples from milk vendors were positive for *Brucella* antibodies. The overall prevalence was 15.7%. The result shows significantly (P<0.05) higher prevalence in herds where fresh bulk herd milk was collected than those from milk vendors who sold fermented milk. However, prevalence within the geopolitical zones was not significantly different (P>0.05). These findings are of public health significance as about 90% of the milk sold to the public within the study area is neither pasteurized nor boiled. Local milk producers and milk vendors should be educated on the importance of pasteurization or boiling of milk. Adequate pasteurization or boiling of milk before sale and consumption must be enforced. These regulations should be mandatory for milk intended for human consumption.

Key words: Prevalence, *Brucella* antibodies, unpasteurized milk, milk ring test, Benue State Nigeria.

INTRODUCTION

Brucellosis is a zoonosis caused by *Brucella* species. The disease in animals causes abortion, infertility, neonatal mortality and hygroma, and is spread by materials contaminated by body fluids including milk. In humans, brucellosis presents as a febrile flu-like illness and is common among pastoralists in Africa (Nicoletti, 1984; Chukwu, 1987a). It is therefore a disease of great economic importance and public health significance.

Humans usually acquire brucellosis by consumption of raw milk and milk products such as cheese. Brucellosis is also recognized as an occupational hazard for livestock farmers, veterinarians, and workers in the meat industry within areas with enzootic *B. abortus*. Farmers and workers in the meat industry may contract brucellosis

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percutaneous, conjunctiva,or by nasal mucous membrane infection. Veterinarians may become infected when handling aborted foetuses or apparently healthy calves born to infected cows and by performing gynaecological and obstetric manipulations, or rectal examination of infected cattle (Schnurrenberger et al., 1975; Alausa, 1980). The most important means of humans acquiring brucellosis is through the consumption of raw milk. The prevention of brucellosis infection in humans is a major reason for the advocacy of milk pasteurization worldwide. The risk of infection by milk borne brucellosis is the reason for public health regulations which discourages the informal milk markets that sell unpasteurized milk. This is because of the risk it poses to human health. However, these regulations are not implemented in many developing countries including Nigeria. Over 90% of milk sales in Nigeria is in the hands of pastoral farmers who do not believe that milk could be a potential source of infection to humans and are not prepared to subject their milk to any form of treatment (Bertu et al., 2010). Despite the existence of regulations that require milk pasteurization, over 75% of the milk marketed in many developing countries is sold raw through local informal pathways (Staal et al., 2000). This is the scenario in Benue State, Nigeria as most of those involved in milk marketing are the Fulani milk maids and the milk is consumed raw or fermented as *fura* and *nono*. These informal milk markets thrive because they are immediate source of money and are of financial benefit to these pastoralists and milk agents.

Previously, only the Milk Ring Test (MRT), with a sensitivity of about 80% (Hunter and Allen, 1972) was available for detection of *Brucella* antibodies in fresh milk. A more accurate indirect Enzyme Linked Immunosorbent Assay (ELISA) with sensitivity = 95% and specificity = 99% for testing *Brucella* antibodies in milk has since been improved and validated (Kerkhofs et al., 1990; Nielsen et al., 1996). The milk ELISA is more sensitive than Rose Bengal test (RBT), MRT, and Complement Fixation Test (CFT) (Sutherland et al., 1986; Kerkhofs et al., 1990; Nielsen et al., 1996; Kerby et al., 1997) and reportedly able to detect antibodies in dilutions of up to 1:100. However, the MRT with a sensitivity of 87.5% and specificity of 98.6% (Bertu et al., 2010) was used in this study to determine the prevalence of *Brucella* antibodies in milk samples as the indirect milk ELISA kits were not readily available.

The MRT is cheap, easy, simple and quick to perform. It detects lacteal anti-*Brucella* immunoglobulin M (IgM) and IgA bound to milk fat globules. However, it tests false positive when milk that contains colostrums, milk at the end of the lactation period, milk from cows with mastitis are tested (Alton et al., 1988).

The risk to human health from these informal marketing of milk is the main objective of this study. This paper presents results of a study on the occurrence of *Brucella* antibodies in informally marketed milk in Benue State, Nigeria and examines the health risks from consumption of raw or untreated milk.

MATERIALS AND METHODS

The study area

Benue State is one of the 36 States of the Federal Republic of Nigeria and it is referred to as the Food Basket of the Nation. It was created on February 3, 1976 with Makurdi as the capital city. The State has 23 Local Government Areas (LGAs) with a population of 4,219,244 (Daily Trust Newspaper, Wednesday, 10th January 2007). The state occupies a landmass of 30,955 square kilometres. The major ethnic groups in the state include Tiv, Idoma and Igede in that order (Benue State Diary, 2007). Benue State is located between latitudes 6°30'N and 8°10'N and between longitudes 7°30'E and 9°50'E. It is located in the middle belt of Nigeria and derives its name from River Benue which is the second largest river in Nigeria. The state lies in the transition belt between the tropical rain forest of Southern Nigeria and the open grassland savannah vegetation of Northern Nigeria. It is divided into three geopolitical zones A, B and C (Benue State Diary, 2007). Benue State experiences a typical tropical climate with two distinct seasons, the rainy season and the dry season. The rainy season lasts from April to October with annual rainfall in the range of 150-180 mm. The dry season begins in November and ends in March while mean monthly temperatures fluctuate between 23 and 30°C in the year.

Benue State is endowed with abundant agricultural resources. About 80% of the population depends on agriculture for their sustenance and livelihood. The state has favourable climatic conditions and fertile soils conducive for the rearing of animals and cultivation of virtually all crops grown in Nigeria. Most prominent among the animals reared are pigs, goats, poultry and cattle. Major crops cultivated include: yam, cassava, rice, soya beans, sesame, maize, citrus, mangoes, vegetables and sugarcane. The State has high migrant populations of nomadic Fulani pastoralists whose main occupation is raising cattle.

Sample collection

About 10 ml of bulk herd milk was collected immediately after milking from 62 randomly selected herds in nine randomly selected Local Government Areas in the three geopolitical zones of Benue State, Nigeria (3 LGAs from each geopolitical zone). None of the herds had record of vaccination against brucellosis. Also another 10 ml of milk from each milk seller (40 in total) at marketing points in the Local Government areas were collected (Table 1). Before samples were collected, the herdsmen and milk vendors were asked whether they were aware of the need to boil or pasteurize fresh milk before marketing. The samples were collected into labelled 10 ml sterile plastic tubes with screw caps. The samples were then taken to the laboratory where they were held under refrigeration for 48-72 h and tested thereafter. The duration of sample collection was 12 months (April 2010 to March 2011).

Laboratory testing

The MRT works on the principles that lacteal antibodies essentially agglutinins to *Brucella* attach themselves to fat globules which rise to the surface of the milk and cluster at the cream layer. When haematoxylin stained *Brucella* antigen is added to the milk (Hunter and Allen, 1972), *Brucella* antibodies in the milk if present, form a complex. The complex attaches to the fat globules in the milk forming a blue ring at the cream layer leaving the normal white

Table 1. Summary of milk samples collected from herds and milk vendors for milk ring test.

Zone	LGA	No. of herds	No. of milk vendors
A	K/Ala	9	6
	Ukum	6	4
	V-ikya	4	3
Subtotal		19	13
B	Gboko	11	6
	Gwer East	5	4
	Makurdi	10	6
Subtotal		26	16
C	Ogbadibo	6	3
	Oju	3	2
	Otukpo	8	6
Subtotal		17	11
Grand Total		62	40

skimmed milk below, indicating a positive result. If *Brucella* antibodies are absent, the whole mixture remains homogenously bluish-white, indicating a negative result.

Prior to testing of milk samples by the milk ring test, the pH for each sample was determined using a pH meter (Hanna Instruments USA, www.clarksonlab.com). The test was done using *Brucella abortus/melitensis/suis* Milk Ring Test (MRT) antigen form the Veterinary Laboratories Agency (VLA) – Weybridge, Surrey, UK as described by Alton et al. (1988). The test was carried out by pipetting 1 ml of milk sample to be tested (thoroughly mixed to disperse the cream evenly) into a 5 ml sterile glass tube. One drop (0.03 ml) of the ring test antigen was added and while closing the top of the tube with the index finger of gloved hands, mixed gently by shaking and inverting the tube several times. The index finger was rinsed and dried between samples. The mixture was allowed to stand for about a minute and examined to make sure that the antigen is thoroughly mixed with the milk. The mixture was then incubated at 37°C for 1 h. The test result was the read using uniform light source. If the intensity of the blue colour in the cream layer is deeper than the skim portion (forming a blue ring), the test was considered positive. If the intensity of the blue colour in the cream layer was less than in the skim portion, the test was considered negative.

Statistical analysis

Data were analyzed using SPSS version 19. Statistical analysis to test for associations between variables was done using the chi-square test and the test level of statistical significance was set at 5% (95% level of confidence, $P < 0.05$).

RESULTS

Of the 62 fresh bulk milk samples collected from herds and tested using MRT, 11 (17.74%) were positive for *Brucella* antibodies while 5(12.50%) of the 40 samples from milk vendors were positive to the MRT. This gives an overall prevalence of 15.69% (16/102) (Table 2).

There was significant difference ($\chi^2 = 0.51$; $P< 0.05$) in prevalence of *Brucella* antibodies in fresh bulk herd milk and milk from vendors. However, prevalence of *Brucella* antibodies in milk was not significantly different between the geopolitical zones of the state ($\chi^2 = 0.38$; $P> 0.05$).

The pH of fresh bulk herd milk samples ranged from 6.5 to 6.8 while that of sour (fermented) milk sold by vendors ranged from 4.5 to 4.8. Of the total 102 herdsmen and vendors interviewed, 72 (70.59%) agreed that they were aware of the need to boil or pasteurize milk before sale and consumption but emphasized that they do not do so to avoid additional costs and time. Only 30 (29.41%) respondents agreed that they usually boil fresh milk collected before sale, but revealed that boiling of a consignment of milk do not usually prevent them from adding fresh milk to it.

DISCUSSION

Of the 62 fresh milk samples from herds tested, 17.74% were positive for *Brucella* antibodies while 12.5% of the 40 milk samples collected from milk sellers (hawkers) tested positive to *Brucella* antibodies. This gave an overall prevalence of 15.69%. Since the herds sampled in the study were not vaccinated, the result is indicative of response to *Brucella* infection and not due to vaccination. Although the serological diagnosis of brucellosis requires the use of more than one test, other tests such as RBPT, SAT and CFT are used only for testing serum samples. The indirect milk ELISA (Kerkhofs et al., 1990; Nielsen et al., 1996) recommended by OIE (2011), could not be carried out due to non-availability of the test kits.

The result showed a significantly higher prevalence of *Brucella* antibodies in fresh milk samples (17.74%) than in milk from vendors (12.5%), which are mainly fermented milk. The overall prevalence of 15.69% agrees with 13.5% reported by Bertu et al. (2010) for Jos and environs and 18.61% reported by Cadmus et al. (2008) for trade cows at Bodija abattoir, Ibadan, South-western Nigeria. However, the result obtained was lower than the 25.5% milk seropositivity reported by Junaidu et al. (2011) from individual lactating cows in Sokoto State, Nigeria. Alton et al. (1988) stated that ring tests carried out on undiluted milk samples from individual cows may give false-positive results shortly after parturition, near the end of lactation, and when mastitis is present.

All the samples collected were from bulk herd milk from various herds and milk sellers each representing a herd. The prevalence obtained is therefore herd prevalence rather than individual animal prevalence. The infected animals serve as sources of infection to healthy animals within the herds as well as other neighbouring herds. This is because grazing of animals is unrestricted and contact between different herds is usual. The herdsmen and their families are also at risk of infection as they handle and milk these animals on daily basis. They can easily get

Table 2. Milk ring test results for *Brucella* antibodies in cattle in different areas of Benue State, Nigeria.

Zone	Area	No. of herds tested	MRT positive (%)	No. of vendor milk tested	MRT positive (%)	Zone Total MRT positive (%)
	K-Ala	9	2(22.22)	6	1(16.67)	
A	Ukum	6	0(00.00)	4	0(00.00)	
	V-ikya	4	1(33.33)	3	0(00.00)	
Subtotal		19	3	13	1	4(12.5)
	Gboko	11	2(18.18)	6	1(16.67)	
B	Gwer East	5	1(20.00)	4	0(00.00)	
	Makurdi	10	2(20.00)	6	1(16.67)	
Subtotal		26	5	16	2	7(16.7)
	Ogbadibo	6	1(16.67)	3	1(33.33)	
C	Oju	3	0(00.00)	2	0(00.00)	
	Otukpo	8	2(25.00)	6	1(16.67)	
Subtotal		17	3	11	2	5(17.9)
Grand Total		62	11(17.74)	40	5(12.5)	16(15.7)

Type of milk. χ^2=0.51; P<0.05 Geopolitical zones (A, B and C). χ^2=0.38; P>0.05.

infected because of close contact with the animals (Chukwu, 1987b). Megersa et al. (2011) stated that brucellosis in traditional livestock husbandry practices certainly poses a zoonotic risk to the public, in consequence of raw milk consumption, close contact with animals and provision of assistance during parturition. Adherence to traditional farming practices, preference for fresh dairy products and contact with animals have been reported to be risk factors for human exposure (Kassahun et al., 2006; Meky et al., 2007). Adesokan et al. (2013) in a survey for knowledge and practices related to bovine brucellosis transmission among livestock workers in South-western Nigeria revealed that consumption of unpasteurized milk, uncooked meat and its products, co-habitation with animals, and poor hygiene were significant risk practices in the transfer of *Brucella* infection from animals to humans among these workers. Previous reports indicated that about 90% of milk consumed in sub-Saharan Africa is raw or soured (Walshe et al., 1991; Mfinanga et al., 2003). The consumption of unpasteurized dairy products has been identified as a brucellosis risk factor for humans (Kang'ethe et al., 2000; Cadmus et al., 2008). Regassa et al. (2009) reported that 85.7% of cases of human brucellosis in Ethiopia resulted from the consumption of raw milk emphasizing the gravity of the problem.

Most of the milk sold to the public in major settlement areas of Benue State does not undergo any form of treatment such as pasteurization or boiling. The Fulani believe that milk is wholesome and could even be taken directly from the cow. Milk being sold by Fulani milk maids is either fresh or soured (fermented). This means that milk being consumed by the public in the study area is mostly not boiled and therefore a potential health risk to the consumers. It is a point of public health concern that this work uncovered the unusual practice of adding fresh milk to one already boiled. This practice nullifies the importance and usefulness of boiling milk in the first instance and all efforts must be put in place through education to stop this practice.

Concern about human health risks from these market pathways needs to be addressed in the context of consumer practices such as boiling to reduce or eliminate potential infection by milk-borne pathogens without discouraging the markets through which the smallholders sell their milk (Kang′ethe et al., 2000). Boiling of raw milk achieves higher temperatures and duration than those attained through pasteurization (Kang′ethe et al., 2000). This means that if these milk sellers ensure regular boiling of milk before it is sold to the public, such milk could be said to be safe, as most infectious agents in the milk might have been destroyed during boiling.

In this work, the pH of fresh bulk herd milk ranged from 6.5 to 6.8 while that of fermented milk ranged from 4.5 to 4.8. There is the belief that traditionally fermented milk known as *nono* is safe since it has undergone fermentation. Fermentation (souring) only lowers the pH of milk from about 6.8 to about 4.5. Under this acidic pH, *Brucella* organisms are only mildly affected (Farrell, 1996). Minja (1999) found that the low pH level in sour milk only destroyed *Mycobacterium bovis* after 66 hours. This implies that homemade fermented milk could be a possible source of infection with brucellosis to humans. Although the herdsmen, milk sellers and consumers do not believe that they could get infected by drinking raw milk or non boiled fermented milk, it is a major source of infection with brucellosis.

Despite the existence of pasteurization, most of the

milk marketed by traditional milk maids is raw and sold through informal channels. Boiling is not also considered a beneficial means of treating milk to make it safe for human consumption. Milk is a nutritious food for animals and humans and must be free of pathogenic organisms. The risk of infection by milk borne brucellosis is the reason for public health regulations. Adequate pasteurization or boiling of milk before consumption must be enforced. This is the only means by which milk can be made safe for human consumption. A prevalence of 25% in lactating cows was earlier reported in Nigeria by Junaidu et al. (2011) and 80.7% in Pakistan by Soomro (2006). This is of public health importance particularly for those Fulani observed to be drinking raw milk directly from the udder of the cow, since *B. abortus* has been isolated from raw and sour milk of Fulani cattle in Nigeria (Bale and Kumi-Diaka, 1981 and Eze, 1978). Brucellosis remains one of the most common zoonotic diseases worldwide with more than 500,000 human cases reported annually (Seleem et al., 2010). In this area, milk is usually preserved by souring, which does not destroy brucellae as they are preserved in milk fat (Eze, 1978). Unfortunately, many farmers do not take measures to protect them against brucellosis and are quite willing to drink unpasteurized milk. Infected farmers with symptoms of undulating fever and joint pains rarely seek medical help and when they do, the fever is usually ascribed to malaria or typhoid, therefore human brucellosis is likely to be greatly under diagnosed (Njoku, 1995; Mai et al., 2012). Although human brucellosis is essentially an occupational hazard, many people can become infected through the consumption of raw milk or milk products. Milk producers and the general public need to be educated on the danger of drinking raw milk. Adequate pasteurization or boiling of milk before sale and consumption must be enforced. Further work on the isolation and characterization of *Brucella* from marketed milk is suggested.

Conflict of interests

The authors did not declare any conflict of interest.

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