

ANTI-BACTERIAL ACTIVITY OF *GARCINIA KOLA* (*CLUSIACEAE*) SEEDS ON THE *IN VITRO* GROWTH OF BROAD SPECTRUM BETA LACTAMASE PRODUCING STRAINS

Abstract:

Antibiotic resistance is nowadays the major concern of many clinicians. This phenomenon is at the origin of a therapeutic impasse with high rates of morbidity and mortality. This calls for alternative care alternatives. The present study was initiated with the aim of evaluating the antibacterial activity of the aqueous and ethanolic extract 70% of *Garcinia kola* seeds on the *in vitro* growth of β -lactamase producing strains. The methodology consisted of extracting the drugs with a 70% hydroalcoholic solvent and distilled water. The diffusion methods in agar medium and in liquid medium were used for the sensitivity test and the determination of the MIC and the CMB. Inhibition zone diameters varied from 10 to 15.66 mm, however, the MICs of the extracts ranged from 3.12 mg / mL to 25 mg / mL and the CMBs ranged from 6.25 mg / mL to 100 mg / mL. The lowest value of MIC and CMB was observed with *E. coli* ATCC 25922 while the largest value of these same parameters was obtained on *S. flexneri* and *S. typhi*. The ethanol extract 70% and the oxacillin (reference molecule) exerted a bactericidal action on all the strains whereas, the aqueous extract recorded a bactericidal activity on the strains of *E. coli* and a bacteriostatic activity on *S. flexneri* and *S. typhi*. In addition, the antibacterial activities of *Garcinia kola* seeds highlighted in this study justify their use in the treatment of various infectious diseases in traditional environment.

Key words: antibacterial activity, β -lactamase, *Garcinia kola*, enterobacteraiceae, CMI,CMB

Introduction:

Antibiotics are one of the most effective forms of therapy in medicine. Their use has revolutionized the treatment of infectious diseases (Sylvie, 2009). This added value is due to several molecules but particularly to β -lactams. These molecules are widely used in medicine because of their broad spectrum of action, their low toxicity, their effectiveness, as well as their low cost for certain molecules (Livermore, 1995). Unfortunately, over the last 30 years, the effectiveness of β -lactams has been compromised by an increasing number of antibiotic-resistant pathogens (Jun et al 2015). Resistance to antibiotics and particularly to β -lactams has become a major concern for many clinicians. This phenomenon of resistance to conventional antibiotics is at the origin of a therapeutic impasse guaranteeing high rates of morbidity and mortality (Aboya 2013, Jun *et al* 2015). Indeed, bacteria have developed several mechanisms to counteract β -lactams (Pitout *et al.*, 2004). Many of these microorganisms are responsible for infectious diseases. This is the case of *Enterobacteriaceae* which are the subject of this study. These bacteria are frequently encountered in both normal and pathogenic flora. They are involved in gastroenteritis, urinary tract infections (Larabiet *al.*, 2003). In Côte d'Ivoire, according to the work of Guessenndet *al.* (2008), the prevalence of expanded-spectrum β -lactamase producing enterobacteria (ESBL) increased from 5.3% in 2005 to 16.8% in 2009. The

emergence of these β -lactamase producing strains poses a real public health problem (Sylvie, 2009). This situation requires increased surveillance of pathogenic strains and to test new antibacterial molecules accessible to populations (Soroet *al.*, 2010). Among the routes explored is the use of medicinal plants. They are used by more than 80% of people in developing countries for their primary health care (Adamaet *al.*, 2010). Several researchers have also shown the importance of bioactive compounds isolated from plants as well as their antimicrobial activity without major side effects in humans (Rocio *et al.*, 2007, Rathet *al* 2009). Thus, to treat various types of inflammation or cirrhosis of the liver, Ivorian populations use *Garcinia kola* seeds as a remedy (Onayadeet *al.*, 1998). The ground and dried almonds of this plant can be mixed with honey to prepare a traditional paste against various symptoms and diseases, including coughs, diarrhea (Adebisi, 2004). The purpose of this study is to evaluate the antibacterial activity of *Garcinia kola* seeds on bacterial extended-spectrum β -lactamase producing strains.

Material and Methods:

1. Biological material:

- ✓ Plant material: The plant material used is the *Garcinia kola* seed harvested in ELIBOU
- ✓ Bacterial strains: The bacterial material consists of expanded spectrum β -lactamase producing strains.

2. Methods:

2.1. Preparation of vegetable powders:

The seeds of *G. kola* were harvested, washed, crushed and then dried in the dark at laboratory temperature (25-30 ° C) for two weeks. After drying, they were sprayed with an electric grinder and the resulting powder was used to prepare the various plant extracts (aqueous and ethanoic).

2.2 Preparation of the aqueous extract:

The total aqueous extract was prepared according to the method described by Ackahet *al.*, (2008). The powder (100g) of the seeds of this plant were macerated in 1000 mL of distilled water by homogenization using a Blender. The homogenate obtained is first filtered on white fabric and then filtered twice on hydrophilic cotton and once on Whatman n° 3 filter paper. The filtrate obtained was dehydrated with the aid of an oven at a temperature of 50 ° C. for 72 hours. A powder is then obtained which constitutes the total aqueous extract.

2.3. Preparation of the ethanol extract 70%:

The 70% hydroethanoic extract (30: 70, v / v) was also prepared according to the method described by Ackahet *al.*, (2008). One hundred grams (100 g) of vegetable powder were dissolved in 1000 ml of hydroalcoholic solvent by homogenization in a Blender. The homogenate obtained is first filtered on white fabric and then filtered twice on hydrophilic cotton and once on Whatman n° 3 filter paper. The filtrate obtained was dehydrated with the aid of an oven at a temperature of 50 ° C. for 48 hours. The powder obtained constitutes the total hydro-ethanoic extract 70%.

2.4 Yield:

Extraction yield is expressed as a percentage relative to the initial mass of the powder of the *G. kola* seed subjected to extraction. The percentage of the extraction yield is calculated according to the following formula:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of dried extract} \times 100}{\text{Mass of plant powderused}}$$

2.5 Preparation of bacterial inoculums:

The bacterial inoculum was prepared from an isolated 18-hour colony in 10 mL Mueller Hinton broth (MHB) and incubated for 3 to 5 hours at 37°C to obtain a pre-culture. A volume of 1 mL of the opalescent pre-culture broth of the bacteria was removed and then diluted in a tube containing 9 ml of saline (0.09% NaCl). This bacterial suspension is evaluated at about 10⁶ cells/mL and constitutes the 10⁰ dilution or pure inoculum.

2.6 Numeration of bacterial inoculums:

The bacterial inoculum was homogenized and diluted 10 to 10 until 10⁻⁴. Then, the bacterial inoculum at 10⁰ and the 4 successive dilutions were inoculated with a calibrated ensemencer of 2µL in Mueller Hinton agar plates, on streaks 5 cm long. This preparation constitutes the growth control box (denoted A).

2.7. Preparation of the concentration range:

They were prepared in test tubes (T) numbered from T₁ to T₈ by the double dilution method in liquid medium. This concentration range varies from 200 mg / mL to 3.12 mg / mL. To do this, 4 mL of sterile distilled water or 70% ethanol was put in the T₁ tube, and 2 mL in all the other test tubes. An amount (0.8 g) of plant extract was dissolved in the T₁ tube and then homogenized completely to give the concentration of 200 mg / mL. Half of the T₁ tube contents (4 mL) were transferred to the T₂ tube and then homogenized. This operation was repeated until tube T₈. Half of the contents of this last tube were rejected.

2.8. Antibacterial Activity:

2.8.1. Sensitivity test:

The Müller-Hinton agar plate, dried for 15 minutes at room temperature in the laboratory, was uniformly seeded with the calibrated bacterial suspension. Wells about 6 mm in diameter are then dug into the agar using a sterile punch (Koffiet *al.*, 2014). In each well, 75µL of the substance to be tested is deposited therein. The latter diffuses with the creation of a concentration gradient after a contact time between the latter and the microorganism (Vinod *et al.*, 2010). The agar was subsequently incubated in an oven at 37 ° C for 18 to 24 hours. The MIC is the smallest concentration that described the smallest zone of inhibition.

2.8.2.Determination of Minimal Inhibitory Concentration (MIC):

The different ranges of plant extract concentrations to be tested were added to all seven experimental tubes according to (Koffiet *al.*, 2014). Thus, 1 ml of the highest concentration extract was transferred to tube T₁, that of the next concentration in tube T₂, and so on until the last concentration in tube T₁₀. This approach led to a dilution of half, the concentration of the contents of the tubes (T₁ to T₁₀). Also two tubes (T_c and T_s) were used. In the T_c tube, 1 mL of sterile distilled water was added, which served as a growth control tube, whereas 2 mL of the sterile physiological saline (NaCl 0.09%) was put in the tube T_s, serving as sterility control tube. All of these tubes were incubated at 37 ° C for 24 hours. The MIC therefore corresponds to the concentration of the first tube from which no disturbance is observed with the naked eye.

2.8.3.Determination of Minimal Bactericidal Concentration (CMB):

Using a ensemencer calibrated at 2 µL, the contents of the tubes in which no trouble was observed, were transplanted to a new GMH without antimicrobial (noted Box B). Transplanting is done by the 5cm long parallel streak method, starting with the MIC tube. After 24 hours incubation in an oven at 37 ° C, the number of colonies on the streaks of the box B with that of the box A is compared. The CMB is equal to the lowest concentration of box B whose colony number is less than or equal to that of the 10⁻⁴ dilution of box A.

2.9.Statistical Analysis:

The data are presented on average ± SEM. All data were analyzed by unidirectional ANOVA and the differences between the means were evaluated using the Neuman-Keuls multiple comparison tests. The differences were considered significant at p <0.05. All operations were repeated 3 times and analyzes were performed using the Graph Pad software, version 5.01 (USA).

3.Results and Discussions:

3.1.Plant extraction yield:

Extraction yields, aspects and colors of the various extracts are shown in Table I. Yields varied between 6.36% and 7.00% per 100 g of *G. kola* seed powder. The highest yield (7.00%) was obtained with 70% hydroethanolic extract (EE70%), while the lowest yield (6.36%) was obtained with the total aqueous extract (ETA). This would mean that the chemical groups present in the *Garcinia kola* seed have more affinity for the water-ethanol binary solvent compared to water alone. Similar results were obtained by Bagréet *al.* (2007) and Touréet *al.* (2011) during the extraction of *Morindamorindoides* Baker (*Rubiaceae*). However, the extraction yield (6.36%) obtained with distilled water in this study is less than 8.8% reported by Yétéet *al.* (2015) during the aqueous extraction of *G. kola* seeds. This observed variation in yield could be related to several parameters. Indeed, some authors have reported that the extraction yield may depend on the time of harvest of the plant, the age of the plant, the drying procedure, the solvent, the pH, the temperature, the extraction time and sample composition (Quyet *al.*, 2014). These factors would justify the variation in extraction yield found between two seeds of the same plant species.

Table I: Yield and appearance of *G. kola* seed extracts

Vegetal	Extract	Aspect	yield (%)
	Aqueous (ETA)	Brown	6,36
Seeds	Hydro-éthanolique 70% (EE _{70%})	Brown	7,00

ETA : Total Aqueous Extract ; EE70 % : hydro-Ethanolique Extract 70 %

3.2. Sensitivity of the studied bacterial strains:

Tables II, III and IV summarize the diameters of the zones of inhibition of ETA, EE70% of *G. kola* and oxacillin respectively obtained on the *in vitro* growth of the bacterial strains studied. At concentrations ranging from 1.56 mg / mL to 12.5 mg / mL, ETA induced no inhibition diameter (0 mm) on the strains tested (Table II). Only concentrations between 25 mg / mL and 200 mg / mL inhibited the growth of strains with inhibition diameters ranging from 10 mm to 15.66 mm. With regard to Table III, results similar to those in Table II were obtained with the only difference that, unlike Table II, the concentration range which induced inhibitory effects on the growth of β -lactamases is wider ranging from 6.25 mg / mL to 200 mg / mL. This indicates that the strains studied were sensitive to the action of EE70% at lower concentrations than those of ETA. This would mean that β -lactamase strains were more sensitive to EE70% action than ETA. EE70% was therefore be more active than ETA. These results are in agreement with those of many authors who during their work indicated that ethanol concentrates the active principles of medicinal plants better than distilled water (Akinyemi&Ogundare (2014), Oluremiet *et al.*, (2010). As for the results of the usual antibiotic (Table IV) used in this study as a positive control, the inhibition diameters ranged from 16 to 36 mm for a range of concentrations ranging from 0.19 to 6.25 mg / mL. The strains studied were very sensitive to the effect of this molecule. Of very low concentrations less than 0.09 mg / mL, oxacillin does not induce an effect on the growth of strains of the test. thus suggesting that β -lactamase strains are resistant to the action of oxacillin at very low concentrations. The relationship between the tested concentrations and the antibacterial effect show a dose-dependent activity. The dose-dependent effect is also observed with the standard antibiotic. All of the ESBL and reference strains (ATCC) tested were sensitive to the action of the aqueous and ethanolic *G. kola* extracts in a dose-response relationship. A gradual increase in the inhibition zone is observed as the concentration of the extracts increases. Similar results were obtained by Konan *et al.* (2014) in the study of *Terminalia glaucescens* on strains producing ESBL. In general, at the concentration of 200 mg / mL, ETA and EE70% induced inhibition zone diameters with an average of 15 mm on the growth of the strains studied. However, oxacillin recorded an average of 33.6 mm, twice as much as that of crude vegetable extracts. These results translate according to Ponce *et al.* (2003) that the organisms studied are very sensitive to the action of ETA, EE70% and oxacillin. The difference observed between the inhibition diameters of the crude vegetable extracts and the reference molecule could be explained by the difference in the purity of the extracts.

Table II: Diameters (mm) of the zones of inhibition obtained with the aqueous total extract (ETA)

Bacterial strains	Concentrations (mg/mL)							
	200	100	50	25	12,5	6,25	3,12	1,56
<i>E. coli</i> 25922	15,33 ±0,57 ^a	13,66 ±0,57 ^a	12,66 ±0,57 ^a	11,33 ±0,57 ^a	0	0	0	0
<i>E. coli</i> 205C/19	13,66 ±0,57 ^b	12,33 ±0,15 ^b	11,66 ±0,52 ^b	10,33 ±0,57 ^a	0	0	0	0
<i>E. Coli</i> 206C/19	14,66 ±0,57 ^c	13,33 ±0,57 ^b	11,66 ±0,57 ^b	10,33 ±0,57 ^b	0	0	0	0
<i>S. flexneri</i>	14,33 ±0,62 ^b	12,66 ±0,51 ^b	10,33 ±0,47 ^b	08,33 ±0,37 ^b	0	0	0	0
<i>S. typhi</i>	13,66 ±0,25 ^a	12,33 ±0,57 ^b	11,33 ±0,72 ^b	09,33 ±0,57 ^b	0	0	0	0

Averages are expressed with standard deviations (\pm), values with different letters in the columns are statistically different at $p < 0.05$

Table III: Diameters (mm) of the zones of inhibition obtained with the hydro-ethanol extract 70% (EE70%)

Bacterial strains	Concentrations (mg/mL)							
	200	100	50	25	12,5	6,25	3,12	1,56
<i>E. coli</i> 25922	16,33 ±0,57 ^a	13,66 ±0,57 ^a	12,66 ±0,57 ^a	11,33 ±0,57 ^a	10,66 ±0,57 ^a	10,33 ±0,57 ^a	0	0
<i>E. coli</i> 205C/19	16,66 ±0,57 ^b	13,33 ±0,15 ^b	11,66 ±0,52 ^b	11,33 ±0,57 ^a	10,66 ±0,52 ^b	10,33 ±0,57 ^a	0	0
<i>E. Coli</i> 206C/19	14,66 ±0,57 ^c	13,33 ±0,57 ^b	12,66 ±0,57 ^b	11,33 ±0,57 ^b	10,66 ±0,57 ^b	09,33 ±0,57 ^b	0	0
<i>S. flexneri</i>	14,33 ±0,62 ^b	13,66 ±0,51 ^b	12,33 ±0,47 ^b	11,33 ±0,37 ^b	10,33 ±0,47 ^b	09,33 ±0,37 ^b	0	0
<i>S. typhi</i>	15,66 ±0,25 ^a	14,33 ±0,57 ^b	12,33 ±0,72 ^b	10,33 ±0,57 ^b	09,33 ±0,72 ^b	08,33 ±0,57 ^b	0	0

Averages are expressed with standard deviations (\pm), values with different letters in the columns are statistically different at $p < 0.05$

Table IV: Diameters (mm) of Zones of Inhibition Obtained with Oxacillin (Control)

Bacterial strains	Concentrations (mg/mL)							
	6,25	3,12	1,56	0,78	0,39	0,19	0,09	0,05
<i>E. coli</i> 25922	33 ^a	30 ^a	28 ^a	24 ^a	21 ^a	18 ^a	0	0
<i>E. coli</i> 205C/19	36 ^b	33 ^b	29 ^b	25 ^b	22 ^b	16 ^b	0	0
<i>E. Coli</i> 206C/19	34 ^c	31 ^c	27 ^c	24 ^a	20 ^c	20 ^c	0	0
<i>S. flexneri</i>	32 ^c	30 ^c	28 ^a	24 ^a	20 ^c	16 ^b	0	0
<i>S.typhi</i>	33 ^a	31 ^c	29 ^b	27 ^b	21 ^a	19 ^c	0	0

3.3. Antibacterial parameters of the different plant extracts:

The antibacterial parameters (MIC, CMB) and the CMB / MIC ratio of the action of the plant extracts and oxacillin on the *in vitro* growth of the strains tested are recorded in Table V. The analysis of the results of this table, indicates that ETA has a bactericidal action on *E. coli* strains with CMBs between 6.25 mg / mL and 25 mg / mL. The recorded CMBs were double the MICs (CMB = 2 MIC). In contrast to the *E. coli* strains, ETA recorded bacteriostatic action on *S. flexneri* and *S. typhi* with CMB = 100 mg / mL, which is 4 to 16 times higher than the MBC of *E. coli* strains. This assumes that ETA is 4 to 16 times more active on *E. coli* strains than *S. flexneri* and *S. typhi*. As for the action of EE70% and Oxacillin, on the strains of the present study. MIC values ranged from 1.56 to 25 mg / mL. The CMB / MIC ratios varied from 1 to 2, thus showing the bactericidal effect of the extracts and the reference molecule (Fauchere and Avril, 2002). According to these authors, a substance is said to be bactericidal when the CMB / MIC ratio is ≤ 2 , and bacteriostatic when this ratio is > 2 . Oxacillin was more active than plant extracts in recording MICs = 1.56 mg / mL. This situation is explained by the fact that the extracts are unpurified crude extracts whereas the commercial antibiotic is a pure molecule. These results are in agreement with those obtained by Soma, (2002) during the study of extracts of *Euphorbia hirta*. A similar study conducted with another ethanolic extract of *G. kola* seeds on various bacterial and fungal strains yielded MIC values ranging from 2.5 to 7 mg / mL (Akerlele et al. 2008). These authors obtained from the ethanolic extract seeds of *G. kola*, a MIC of 5 mg / ml against *E. coli*. This value is different from that argued in this study. Several reasons may justify the observed differences. Some authors have reported that the different components of plant extracts have different degrees of activity against Gram-negative and Gram-positive bacteria (Dorman and Deans, 2000) and that the chemical composition of the same extract may vary according to several intrinsic factors and extrinsic (Lahlou, 2004). In addition, the literature teaches that the same bacterial species do not also have the same sensitivity to an antimicrobial agent. Thus, in a given bacterial family, an individual difference in

sensitivity can be observed (Rath *et al.*, 2009). The chemical study of *G. kola* seeds by some authors revealed that these seeds contain high content of phenolic compounds including flavonoids and tannins (Ijomone *et al.*, 2012). The antimicrobial activity of these chemicals substance has already been shown by several authors (Jayasinghet *et al.*, 2003, Konaté *et al.*, 2012). Moreover, phenolic compounds are known to be toxic to microorganisms and would be targeted envelopes such as the cytoplasmic membrane and the wall. The antimicrobial effect is linked to their free hydroxyl groups, which allows good solubilization in the medium (Ben *et al.*, 2006). Thus, the antibacterial activity of *G. kola* seeds in the present study could be justified by these chemical compounds it contains.

Table V: Compared Antibacterial Parameters and Their Interpretation

Bacterial strains	Extracts	Antibacterial Parameters (mg/mL)		CMB/CMI	Effect
		CMI	CMB		
<i>E. coli</i> 25922	ETA	3,12	6,25	2	Bactericidal
	EE _{70%}	3,12	6,25	2	Bactericidal
	OX	1,56	1,56	1	Bactericidal
<i>E. coli</i> 205/19	ETA	6,25	12,5	2	Bactericidal
	EE _{70%}	3,12	6,25	2	Bactericidal
	OX	0,78	1,56	2	Bactericidal
<i>E. coli</i> 206/19	ETA	12,5	25	2	Bactericidal
	EE _{70%}	12,5	25	2	Bactericidal
	OX	0,78	1,56	2	Bactericidal
<i>S. flexneri</i>	ETA	25	100	4	Bacteriostatic
	EE _{70%}	6,25	12,5	2	Bactericidal
	OX	1,56	3,12	2	Bactericidal
<i>S. typhi</i>	ETA	25	100	4	Bacteriostatic
	EE _{70%}	12,5	25	2	Bactericidal
	OX	1,56	1,56	1	Bactericidal

ETA: Total Aqueous Extract; EE70%: Hydroethanol extract 70%; MIC: Minimal Inhibitory Concentration; CMB: Minimal Bactericidal Concentration.

Conclusion:

The *in-vitro* study of aqueous and 70% ethanol extracts of the seeds of *G. kola* made it possible to highlight the antibacterial properties of this plant on the growth of the *Enterobacteriaceae* studied. The results obtained reveal the presence of antibacterial active principles in the aqueous and ethanol extracts 70%. The results showed a bactericidal effect of the aqueous extract on the strains of *E. coli*, but bacteriostatic on those of *S. flexneri* and *S. typhi*. As for ethanol extract 70% and Oxacillin, they exerted a bactericidal action on all strains studied. This bactericidal effect observed is dose dependent. The sensitivity of these enteric strains to the aqueous and ethanolic extracts of *G. kola* seeds is of great importance in the treatment of pathologies associated with them. The present results justify certain ethnopharmacological uses of *G. kola* seeds. This study demonstrates that the seeds of this plant can be used to treat infectious diseases. In view of the results, it would be interesting to undertake studies in order to evaluate the toxicity then purify the most active ethanolic extract of the seeds of this plant and consider the development of improved traditional medicines (MTA).

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