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Quantification of Bactericidal Action of the Ethanolic Extract of *Garcinia kola* Seeds Alone, and in Combination with the Branded Antibiotic Septrin, on the Culture Isolates from Throat Irritation Patients by Bacterial Growth Kinetics

V. A. Ajibade*[a], I. G. Adanlawo[b], T. T. Akande[a]

[a]Science Technology Department, Federal Polytechnic, PMB 5351, Ado-Ekiti, Nigeria

[b]Biochemistry Department, University of Ado-Ekiti, PMB 5363, Ado-Ekiti, Nigeria

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Abstract. The relationship of growth rates of *Streptococcus pyogenes,* isolated from patients with protracted throat irritations and tonsillitis, with *Garcinia kola* extract and the branded antibiotic Septrin was investigated. A steady state was obtained shortly after the addition of Septrin, whereas a lag phase of about five generations elapsed for *G. kola*. The inhibitory effect of Septrin was about five times greater than the effect of *G. kola.* The inhibitory effect of *G. kola* was only bacteriostatic, whereas Septrin caused bacterial death if a certain threshold of concentration (1 mg) was passed, as evidenced by a decrease in the number of bacterial cells. Combination of Septrin and *G. kola* at concentrations where both acted merely bacteriostatically, led to effects considerably greater than would be expected from simple additivity. It is justifiable to conclude that the combination of *G. kola* extract and Septrin had a synergistic effect.

Keywords: *Garcinia kola*, antibiotic Septrin, bacterial growth kinetics, ethanolic extract, antibiotic synergism, antibiotic Septrin, *Streptococcus pyogenes*, throat irritation

Introduction

Garcinia kola (bitter kolanut), Family Guttiferae, is a native of Southwestern African countries where it grows wild. It has not attracted the due attention of plant breeders to cultivate it and improve the wild strain. The plant, however, has gained recognition in Western Africa, though it is not a crop of commerce (Irvine, 1963). Its fruit is normally about 7.5 cm in dia and contains 4 to 6 light brown seeds embedded in the pulp. *G. kola* is a popular seed, eaten on social and other occasions in most parts of Western African countries and is an important ingredient in medicinal preparations (Nwafor and Ogbeneaga, 1992). In some parts of Africa, however, the seeds are believed to cause impotency in man. The nuts are eaten raw as a stimulant to resist hunger and sleep (Adeyeye and Ayejuyo, 1994), and also used for the treatment and management of cough and asthma (Nwafor and Ogbeneaga, 1992). The root cuttings are used as chewing stick and for medicinal purposes, such as in the treatment of cough.

The antibiotic Septrin (30 mg trimethoprim and 400 mg of sulfamethaxazole) is effective against a wide range of gram-negative and gram-positive bacteria (Hitchings, 1967). Septrin is indicated for the treatment of bacterial infections of the respiratory tract, urinogenital tract including gonnorhea, prostatitis, gastrointestinal tract (typhus, paratyphus, cholera), skin and soft tissues, and those following surgical and stomatological interventions. A potentiating effect of Septrin on *Garcinia kola* (or *vice versa*) has been reported, presumably due to the sites of action of the inhibitors being at different steps in the same biosynthetic pathway (Nwafor and Ogbeneaga, 1992). Although the combination of Septrin with *G. kola* is extensively used, there are no reports on the quantitative biochemical effects of such uses, as related to chemotherapeutics. Earlier studies have shown the synergistic action of the drug combinations, using serial-dilution techniques, disc-sensitivity test, or experimental infections in animals (Burchall, 1969). In addition to the differences in the definitions of synergism and additivity of the drug-action among various authors/workers (Garrett and Brown, 1993; Garrett, 1978a), these methods do not seem practicable for the quantification of the chemotherapeutic effect. A linear relationship between inhibition and the inhibitor cannot be presumed and should not be expected to exist. In addition, the additivity of killing of the numbers of bacteria is not necessarily synonymous with an additivity rate of constants for the killing of bacteria. Diffusion of drugs in culture experiments may not be independent of other laboratory factors, thus resulting in a false presumption of synergy of antibacterial action (Toama *et al.,* 1978), while experiments with live-animals may perhaps relate best to the clinical effects of the combination, these may show synergy as a re-

*Author for correspondence; E-mail: jdawogbemi@yahoo.com

sult of pharmacologic interactions rather than antibacterial effects. For these reasons, it was felt appropriate to study the antibacterial action of the combination in an experimental design not subject to these limitations. Garrett (1978b) has outlined theoretical/mathematical models for the differentiation of drug synergism, antagonism and additivity. Experimental methods that use the kinetics of bacterial growth and the effects of antibiotic combinations have been developed. In studies with such methods, the activity of the drug is defined in terms of decrease in the growth rate caused by the drug, as a function of the concentration of the drug. Techniques for viable and total count have been used to quantify the decrease in the growth rates of *Escherichia coli* generations as a function of concentrations of the antibiotic, and to differentiate between inhibition, uninhibited growth simultaneous with killing, and inhibition of growth simultaneous with or followed by killing (Garrett and Miller, 1995; Garrett and Brown, 1993; Garrett, 1978b).

The aim of the present work, therefore, was to use this general approach to determine whether the combined action of *G. kola* and Septrin will be additive or more than additive, i.e., synergistic. However, for such studies to be meaningful, it was also necessary to quantify the relative potencies of the two test materials by the same methods, and in particular to determine if they were acting by pure inhibition or inhibition with killing. Thus, the growth dependency of isolates of *Streptococcus pyogenes,* from sputum and throat swabs in the presence of the individual drug/plant extract was also evaluated.

Materials and Methods

Specimens of sputum and throat swabs, obtained from fifteen patients (ten from patients at the State Specialist Hospital, Ado-Ekiti and five from the Federal Polytechnic Campus, Ado-Ekiti) were used for the research study. The ten samples from the hospital were clinical specimens of patients referred to the laboratory for sputum microscopy, while the other five were from students complaining of cough and throat irritations. The sputum was collected in sterile disposable plastic bottles. Each specimen was inoculated on an agar base medium with 5% lysed blood and incubated anaerobically at 37 °C for 24 h. The culture isolates were identified according to Edwards and Ewing (1962) using the gram-staining procedure, blood haemolysis, catalase test and antibiotic susceptibility.

Garcinia kola seeds were obtained from the local market at Ibadan, Oyo State, Nigeria. The dried, mature nuts were ground into powder. Active principles were extracted from 30 g of the ground samples by agitating with 10 ml ethanol. The mixture was allowed to stand for 24 h and filtered. The extract was completely dried by using vacuum rotary evaporator at 40 °C.

The Septrin tablets (marketed as Septran in several countries) were obtained from a pharmacy at Olorunsogo area, Ibadan, Nigeria. The tablets were powdered and stored in sterile plastic bottles. Seven g of the crude extract of *G. kola* and Septrin were separately dissolved in 50 ml distilled water. The mixtures were shaken vigorously to make the stock solutions.

Various concentrations of *G. kola* extract (0.5-400 mg/ml) were added to the bacterial culture isolates in the logarithmic-growth phase. Samples were taken at appropriate time intervals and the number of viable cells (plate count) and/or the total number of cells (coulter counter or turbidity measurements) were counted during incubation at 37 °C using spectrophotometre. Similar experiments were done in the presence of graded concentrationsof Septrin (0.125-4.0 mg/ml) alone and in combination with the *G. kola* extract samples. Because of the lag time in *G. kola* action (Ajibade and Adanlawo, in press), the extract samples were added to the bacterial culture isolates at different times of the logarithmic growth phase. Thus, *G. kola* extract was added to the bacterial culture isolates first, which was followed by the addition of Septrin 1.5 h later.

Results and Discussion

The bacterial culture isolates from the sputum of 15 patients were noted to be predominantly gram-positive cocci arranged in chains, with β haemolysis on blood agar, catalase activity negative, and having sensitivity to the antibiotic bacitracin. The bacterial culture isolates were identified as predominantly *Streptococcus pyogenes*. Other colonies were not substantial enough to cause infection, and therefore ignored for further investigations.The effect of *G. kola* extracts on the isolated bactrial culture on their viable count and total count are shown in Fig. 1 and Fig. 2. A lag phase of about five generations existed before there was any significant effect of any *G. kola* extract concentration on the growth of *S. pygonese* at 37 °C (Ajibade and Adanlawo, in press). This lag phase appeared to be independent of the specific activity of *G. kola.* Subsequently, the technique for viable count (Fig. 1) and total count (Fig. 2) showed, respectively, that at 400 mg/ml the bacterial population decreased from over 10^{10} in the control to less than 10^5 cells/ml in the viable count (Fig. 1), while the bacterial population decreased from $10^{8.5}$ in the control to 10^5 cells/ml at 100 mg/ml (Fig. 2). Apparent first-order generation rate constants were obtained (K_{app}/sec) from the slope of these new growth curves in accordance with the general expression rates as below:

$$\log N = \frac{K_{app} \times t}{2.303} + \text{constant} \quad (1)$$

where:

K_{app} = apparent first-order generation rate

N = bacterial population

t = time

The coincidence of experiments for total and viable count supports the well known theory that *G. kola* extract is bacteriostatic and not bactericidal. Even at very high concentrations of *G. kola,* the rate constants were positive, and no decrease in viable cells was observed. It is obvious that there was no linear logarithmic relationship among these two parameters.

A linear relationship, therefore, appears to exist with a Lineweaver-Burk type plot as below:

$$\frac{1}{K_o - K_{app}} = \frac{K_a}{C} + K_b \quad (2)$$

where:

control
0.5 mg/ml
3.0 mg/ml
4.0 mg/ml
5.0 mg/ml
100 mg/ml

= generation rate constant in the absence of *G. kola* and K_b = constants

= the measure of the molar activity of *G. kola* extract tested

= concentration of *G. kola* extract

If this equation (2) is multiplied by the concentration of the extract, the following is obtained:

$$\frac{C}{K_o - K_{app}} = (K_a + K_b) \times C \quad (3)$$

Fig. 3a is a plot of this type, showing a fit of the experimental data to equation (3).

The effect of Septrin (Fig. 3a) in contrast to *G. kola* (Fig. 3b) showed only a very short phase before the onset of inhibition was observed with Septrin. Apparently, depending on the culture conditions and concentrations of Septrin, the onset of inhibition was obtained almost immediately (Fig. 4). The reasons for this difference in the lag phase could be consistent with the sites of actions of the two inhibitors (Hitchings, 1967). Cellular division was required before the *G. kola* activity was observed. During the approximately five cellular divisions, before the onset of *G. kola* inhibition, the stored folates in the cells, such as dihydrofolic acid (Hitchings and Burchall, 1992), were depleted, while new synthesis was inhibited by the *G. kola* extract. Only after the stored folate was depleted did the

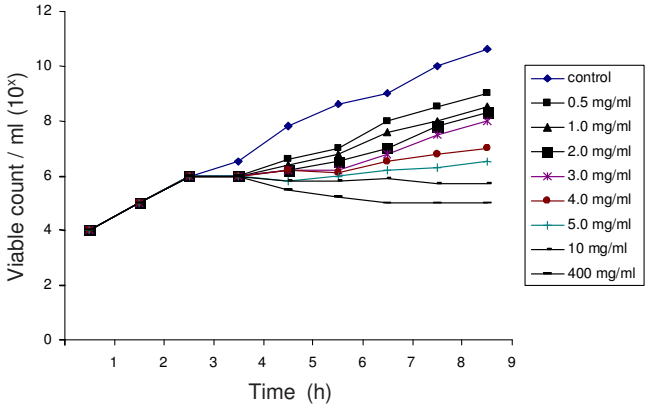

Fig. 1. Typical generation rate curves of *Streptococcus pyogenes* at 37 °C in the presence of various concentrations of *Garcinia kola* by the viable count method.

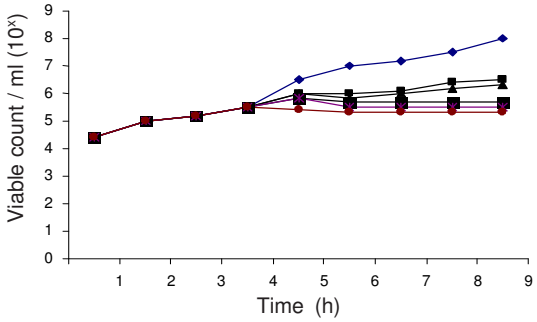

Fig. 2. Typical generation rate curves of *Streptococcus pyogenes* at 37 °C in the presence of various concentrations of *Garcinia kola* by the total count method.

inhibition of folate system became critical for cell division. On the other hand, Septrin blocked the systhesis of tetrahydrofolate directly at the level of dihydrofolic acid (Baker and Beng-Thong, 1994; Hitchings and Burchall, 1992). Apparently, Septrin blocked beyond the site of folate storage, and a relatively rapid onset of action was observed (Fig. 3a). After the short lag phase, cells grew in the presence of low concentrations of Septrin at reduced logarithmic rates. The same relationship was observed between the antibacterial activity and the rate constants for bacterial growth as for *G. kola* (Fig. 3b). From these results it appears that under the experimental conditions of this study, the ratio of activities ($K_{a's}$) of *G. kola* to Septrin was about 1:5; because $K_{Gk} = 4.254 \times 10^{-3}$ /ml/sec, and $K_{Sp} = 0.785 \times 10^{-3}$ /ml/sec. This ratio is similar to the ratio of MIC values for the two antibiotic agents obtained in similar studies with the same strain of *S. pyogenes* (Septrin 0.175 mm; *G. kola* 0.8 mm) by the present authors during earlier investigations (Ajibade and Adanlawo, in press). *G. kola* at 2 mg in the present studies caused a 50% inhibition of growth rate

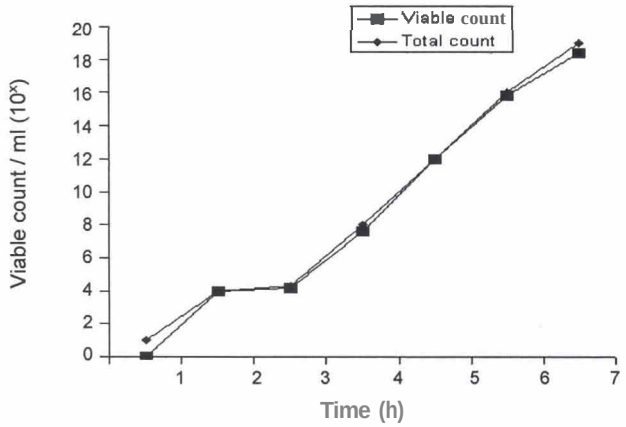

Fig. 3a. Quantitative relations between apparent ***Streptococcus pyogenes*** growth-rate constant (K_{app}) and concentrations of Septrin; the curves are plotted in accordance with the exprssion $K_o C/K_{app} = (K_a + K_b) \times C$ (see equation 3).

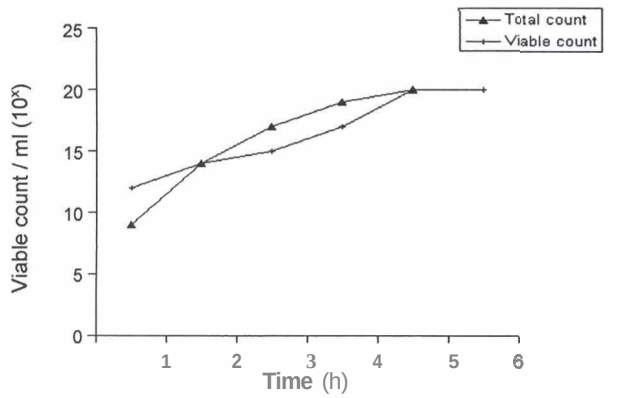

Fig. 3b. Quantitative relations between apparent ***Streptococcuspyogenes*** growth-rate constants (K_{app}) and concentrations of ***Garcinia kola;*** the curves are plotted in accordance with the exprssion $K_o C/K_{app} = (K_a + K_b) \times C$ (see equation 3).

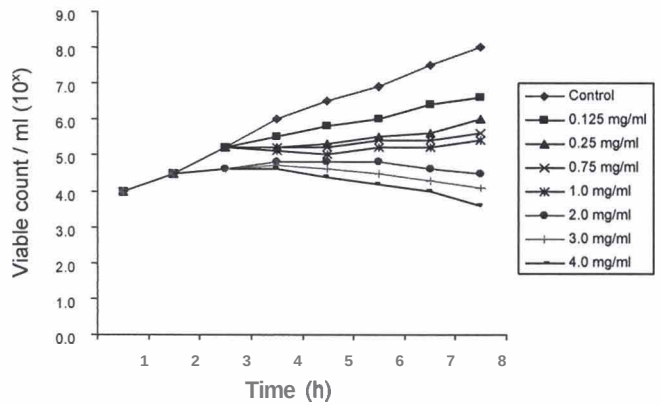

Fig. 4. Typical generation rate curves of ***Streptococcus pyogenes*** at 37 °C in the presence of various concentrations of Septrin.

(Fig. 4); one-half of the concentration (1 mg) does not cause 25% inhibition, but rather 33% inhibition. Since neither the activity of Septrin, nor that of G ***kola*** was linearly related to the concentration used, estimates of potency of combination based on assumptions of linear relationships between potency and concentration will be subject to error.

The second major difference between the effects of Septrin (Fig. 4) and G ***kola*** (Fig. 1 and Fig. 2) was that negative rate constants were obtained from experiments with viable counts at concentration as low as 2 mg. At these concentrations of Septrin, the total and viable counts obtained simultaneously did not coincide. In addition to the inhibition of cell division, killing was caused by Septrin. At the lower concentration of Septrin, viable and total counts did coincide, and no killing was observed. This means that Septrin was bacteriostatic at low concentration, but at higher concentrations killing was observed simultaneously with the inhibition. The threshold concentration seems to lie between 1 mg and 2 mg under the conditions used. The rate of killing was independent of the concentration of Septrin after the threshold concentration had passed. Although the rate constants were independent of the concentration of Septrin, they were dependent on the number of microorganisms.

The combined effects of Septrin and G ***kola*** on the growth of ***S. pyogenes*** is shown in Fig. 5. Due to the lag time in G ***kola*** action, the extract was added to the culture at different times of the logarithmic growth phase. G ***kola*** extract was added first, Septrin was added 1.5 h later. The results showed that G ***kola*** extract and Septrin inhibited the growth most at the respective combination ratio of 2:1, while the least inhibition was evident at single application. It can, therefore, be seen that the effect of the combination was significantly greater than when either sample was used alone. If the effects of the two samples were independently additive, one may predict the effects of the combination by combining the two expressions (equations 2) that were shown to hold for each sample alone, to obtain:

$$K_{app} = \frac{K_o (1 - K_{1(G\ kola)}}{1 + K_{1(G\ kola)}} \qquad (4)$$

$$K_{app} = \frac{K_o (1 - K_{2(Septrin)}}{1 + K_{2(Septrin)}} \qquad (5)$$

where:

$$K_1 = \frac{1}{K_{G\,k} K_o}$$

$$K_2 = \frac{1}{K_{Sp} K_o}$$

The calculated value of the generation rate constants obtained clearly show that the combination effects were much greater than those produced by simple additivity (Fig. 6). Since this combination of Septrin and G *kola* extracts led to an observed effect much greater than that predictable from simple additivity, it is justifiable to say that the combination has a synergistic effect.

The synergism was further demonstrated by the method of continuous variations (Fig. 6). In this study, generation rates

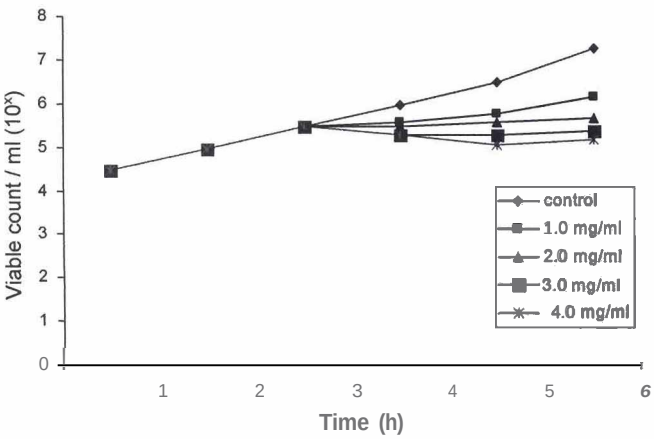

Fig. 5. Typical generation rate curves of *Streptococcus pyogenes* at 37 °C in the presence of combinations of different concentrations of Septrin and *Garcinia kola*.

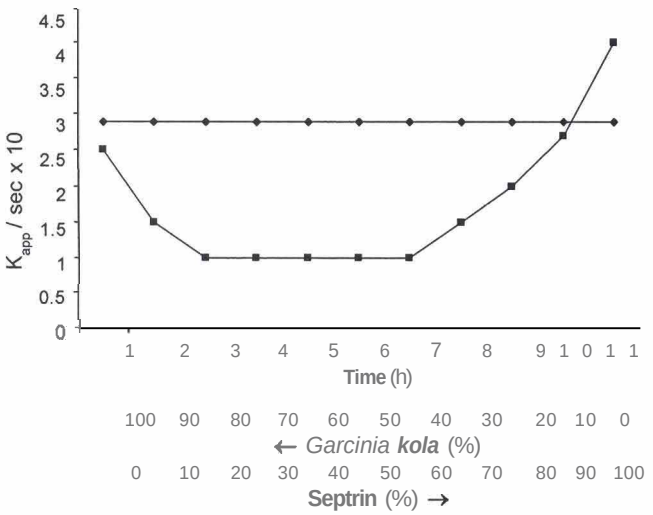

Fig. 6. Generation rate constants (K_{app}) of 1 mg/ml concentration of *Garcinia kola* and 0.015 mg/ml concentration of Septrin present in the culture (+), and when these were present in different percentage combinations (■) to show synergistic effect of the two.

were evaluated for a series of five cultures inhibited by mixtures of Septrin and G *kola, as* well as for each sample separately. The concentration of each sample chosen was a fraction of its concentration in the mixed culture so that the sum of the two sample fractions always equaled one. Equations 4 and 5 were used to calculate the curve showing the generation rates expected if the combinations were acting in an additive manner, The experimentally obtained generation rates were noted to be considerably lower than those predicted by this curve. Maximal synergy was observed when equipotent combinations of Septrin and G *kola* were used, but appreciable synergy was observed even when the potency ratio of Septrin to G *kola* was as low as 1 to 9, or as high as 9 to 1. Therefore, differences in the concentrations of two agents obtained in various body tissues should not have a large effect on the synergism.

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