

**INTERACTION OF CIPROFLOXACIN AND EXTRACT
OF *Hibiscus sabdariffa* LINN (ROSELLE) CALYSES IN
ANTIMICROBIAL ASSAYS BY 'AGAR-WELL CROSS-
REACTION TECHNIQUE'**

ABSTRACT

Aims: To evaluate the antibacterial activities of aqueous extract of *Hibiscus sabdariffa* Linn calyces and ciprofloxacin, separately and in combination, and introduce a simple method in the study of drug interactions.

Study design: Experimental design.

Place and duration of study: Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa, between March 2013 and June 2015.

Methodology: Antimicrobial assays using five bacterial isolates including *S. aureus*, *Pseudomonas aeruginosa*, and *S. aureus* NCTC 6571, by agar-well diffusion method were performed. Interaction assay of extract and ciprofloxacin by an 'Agar-well Cross-reaction Technique' was performed. Inferences for synergistic, additive, indifferent or antagonistic interaction were drawn from the percentage differences in the inhibition zone diameters (IZDs) produced.

Results: All test bacteria were susceptible to the extract, IZDs ranging from 10 mm to 32 mm. Four test bacteria were susceptible to ciprofloxacin, IZDs ranging from 15 mm to 35 mm. Out of the twenty five interactions against *S. aureus* O1, there were 6 (24%) synergistic, 6 (24%) additive, 3 (12%) indifferent, 5 (20%) antagonistic reactions, and 5 (20%) showed no activity. Out of the twenty five interactions against *P. aeruginosa* PA2, there were 2 (8.0%) synergistic, 2 (8.0%) additive, 1 (4%) indifferent, 9 (36%) antagonistic reactions, and 11 (44%) showed no activity. Summing up the fifty interactions for the two test isolates, 36 (72%) interactions were favorable or indifferent while 14 (28%) interactions were antagonistic. Results of Chi square test showed this to be statistically significant ($P = .05$).

Conclusion: *H. sabdariffa* calyces extract possesses antibacterial properties similar to ciprofloxacin, and inhibited ciprofloxacin-resistant isolates. Concomitant use of ciprofloxacin and *H. sabdariffa* extract may not produce antagonistic reactions. The 'Agar-well Cross-reaction Technique' proved to be sensitive and effective, eliminating many of the difficulties experienced with other methods of studying drug interactions.

Keywords: Interactions, agar-well, cross reaction, ciprofloxacin, *Hibiscus sabdariffa*, synergy

1. INTRODUCTION

Hibiscus sabdariffa L (Malvaceae), commonly called 'Roselle', is cultivated and used in all parts of the world for its nutritional and medicinal properties [1]. As food, *Hibiscus sabdariffa* is used in jam, preserves, colorant food and cocktails [2]. In Nigeria, an aqueous infusion of the calyces is commonly taken as a beverage drink called 'Zobo'. As herbal remedy, it is used for a variety of ailments including hypertension, cough and scurvy, cancer and diabetes. The coloring matter of the calyces is said to be lethal to *Mycobacterium tuberculosis*. *H. sabdariffa* has also been reported to be antiseptic, aphrodisiac, astringent, diuretic, emollient, purgative, sedative, stomachic, and tonic. It is also a folk remedy for abscesses, bilious conditions, cancer, cough, dysuria, and scurvy [3] and was also found to be anti-carcinogenic [4]. Previous workers have suggested that flavonoids, polysaccharides and organic acids may be mainly responsible for the pharmacological activities [5].

The *in vitro* antibacterial activities of *H. sabdariffa* extracts have been reported by various workers [6][7][8]. The *in vitro* activities of methanolic extract of *H. sabdariffa* calyces against *Staphylococcus aureus*, *Bacillus stearothermophilus*, *Micrococcus luteus*, *Serratia marcescens*, *Clostridium sporogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus* and *Pseudomonas fluorescens*, had been reported, with minimum inhibitory concentrations (MIC) ranging from 0.30 mg/ml to 1.30 mg/ml [8]. Other workers demonstrated the *in vitro* activities of aqueous methanolic extract of *H. sabdariffa* calyces against *E. coli* O157: H7 [9].

Given its dual use as food and phytomedicine, it is expected that *Hibiscus sabdariffa* is widely used by a significant fraction of the Nigerian population. Similarly, the use of antibiotics is on the increase globally

[10]. Fluoroquinolones, especially ciprofloxacin are frequently prescribed in Nigerian hospitals due to their broad spectrum activity and relatively low percentage of resistance offered by pathogens. Drug interactions have been known to occur *in vivo* between different medicinal plant extracts and conventional antibiotics. Results of previous research involving drug interactions *in vitro*, has indicated that the concomitant use of herbal remedy with a conventional antibiotic may result in a synergistic, additive or antagonistic interaction [11][12].

Several methods have been employed to investigate the interaction of antimicrobial agents. These include agar overlay method [12], checkerboard method, and time kill assays [13][14][15]. These methods present some technical difficulties which sometimes prevent a researcher from attaining the desired objectives. There is a need to modify existing methods of studying extract- antibiotic interactions or to devise simpler, less complicated methods.

The present study aims to evaluate the antibacterial effects of aqueous extract of *Hibiscus sabdariffa* calyces and ciprofloxacin, separately and in combination. Ciprofloxacin was selected as the test antibiotic because it has become a mainstream antibiotic in Nigerian hospitals and is commonly prescribed for many bacterial infections. Further, the author introduced a less complicated method of investigating drug interaction between solutions of antibiotics and plant extracts. 'The Agar-well Cross Reaction Technique' used in this study eliminates much of the tedium and difficulties experienced with existing methods.

2. Material and Methods

Dried *H. sabdariffa* calyces were obtained from a local market and transported to the Microbiology laboratory in Ondo State University of Science and Technology, Okitipupa, for analysis. The plant material was identified by Plant Taxonomist, Professor J. C. Okafor, (Emeritus) of Department of Botany, Faculty of Biological Sciences, University of Nigeria, Nsukka.

Ciprofloxacin injection (200mg/100ml) USP (UNIFLOX) was used in the study.

The test bacteria included *Staphylococcus aureus* (n=3), *Pseudomonas aeruginosa* (n=2), and a reference culture of *Staphylococcus aureus* NCTC 6571. The bacteria were isolated from swab samples from skin and nares of patients hospitalized at State Specialist Hospital, Okitipupa, Western Nigeria,

between March and April 2015. *S. aureus* was identified by a golden-yellow color on Mannitol Salt Agar, and profuse white colonies on Nutrient agar which on microscopy yielded Gram positive cocci in clusters, and gave coagulase positive and catalase positive reactions. *P. aeruginosa* was identified by observing the blue-green pigment on Nutrient agar, Gram negative rods, oxidase positive and catalase positive reactions.

H. sabdariffa extract was prepared by soaking a hundred grams (100 g) of dried *H. sabdariffa* calyces in a 1000 ml beaker containing 500 ml of boiling distilled water and stirred for five minutes. This was removed from the flame, kept on the bench to cool and then stored in a refrigerator at 4 °C for 24 h. Next, the liquid part of the mixture was decanted into another beaker to separate it from the calyces which form the residue. The liquid was distributed into glass Petri dishes which were placed in a hot air oven at temperatures of 45 °C to 65 °C to evaporate the water. The red colored extract which was left was stored in a bottle at 4 °C.

Serial dilution of extract and ciprofloxacin

A stock solution of 200 mg/ml of the extract was prepared by weighing out 2 g of extract in a balance. This was introduced into 10 ml of sterile distilled water in a Universal bottle and stirred with a glass rod until it dissolved. This resulted in a concentration of 200 mg/ml.

Two-fold dilution was carried out by transferring 5 ml of the stock solution into an equal amount of sterile distilled water in another Universal bottle. This gave a concentration of 100 mg/ml. Two-fold dilution was continued to obtain 50 mg/ml, 25 mg/ml and 12.5 mg/ml of the extract.

The stock solution of ciprofloxacin infusion (single dose) contained 200 mg/100 ml (2 mg/ml or 2000 µg/ml). Starting with the stock solution of concentration 2000 µg/ml, a two-fold dilution was performed by transferring 5 ml of the stock solution to 5 ml of sterile distilled water in a Universal bottle to give a solution of concentration 1000 µg/ml.

Next, a ten-fold dilution was performed by transferring 1ml of the 1000 µg/ml solution into a Universal bottle containing 9 ml of water to give 100 µg/ml. This was continued to give 10 µg/ml.

From 10 µg/ml solution, two-fold dilution was resumed by transferring 5 ml into a Universal bottle containing 5 ml of water to give 5 µg/ml. Two-fold dilution was continued to obtain 2.5 µg/ml, 1.25 µg/ml and 0.625 µg/ml.

Ciprofloxacin dilutions of 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 0.625 µg/ml were used for the antimicrobial assay.

Antimicrobial screening of extract and ciprofloxacin:

The test organisms were cultured for 24 hours at 37°C. Then two loopfuls of each test organism was transferred into 5 ml of sterile physiological saline in a test tube. The volume was adjusted until the turbidity of the cell suspension matched the 0.5 McFarland Standard, containing approximately 1.5×10^8 colony forming units per ml (CFU/ml).

Five concentrations of the extract (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml) and five dilutions of ciprofloxacin (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 0.625 µg/ml) were screened against each test bacterium using the agar-well diffusion method. Mueller-Hinton agar plates, in replicates, were first swabbed with a suspension of the standardized test organisms (0.5 MacFarland Standards). Next, a sterile cork borer with a diameter of 8 mm was used to bore wells into the Mueller-Hinton agar medium. By means of a sterile pipette, 0.1ml of each concentration of the extract was introduced into an appropriately labeled well. The plates were kept on the bench for 1 h at room temperature for proper diffusion to take place and subsequently incubated at 37°C for 24 h. After incubation, the diameters of the zone of inhibition were measured with a transparent ruler. The mean of the zone of inhibition for each set of replicate plates were recorded in millimeters [16][17].

***In vitro* evaluation of interaction of the extract and ciprofloxacin**

Interaction of the extract and ciprofloxacin was investigated using an 'Agar-well cross reaction technique'.

Two of the test bacteria susceptible to both the extract and ciprofloxacin were selected for the cross reaction assay. Sub-inhibitory concentrations of the extract for each organism were used.

Twenty-five millimeters (25 ml) of Mueller-Hinton agar in universal bottles were sterilized by autoclaving at 121 °C for 15 minutes, and cooling to about 50 °C. Then, 0.5 ml of five sub-inhibitory concentrations of the extract for each susceptible test organism was mixed with the molten agar in appropriately labeled bottles. The mixtures were then poured aseptically into labeled Petri dishes and allowed to solidify on the bench. The plates were inoculated with standardized suspensions of the test bacteria. Each plate was labeled with the test bacteria and concentration of the extract contained in the medium. Thereafter, five

wells were bored into each agar plate with a cork borer of 8 mm and the wells were labeled with the different concentrations of ciprofloxacin - 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 0.625 µg/ml. Then 0.1 ml of each concentration of ciprofloxacin was then introduced into appropriately labeled wells.

Statistical Analysis: The Chi square test was used to calculate the statistical significance between percentage antagonism and the other effects obtained, with confidence level of ($P = .05$)

3. Results and Discussion

Antimicrobial activity of *H. sabdariffa* extract and ciprofloxacin against the test bacteria

Table 1 shows the susceptibilities of the five bacterial isolates and reference culture *S. aureus* NCTC 6571 to *H. sabdariffa* extract and ciprofloxacin. *H. sabdariffa* extract produced inhibition zone diameters (IZDs) ranging from 0 mm to 32 mm, all the test bacteria being susceptible in varying degrees. Ciprofloxacin produced IZDs ranging from 0 mm to 35 mm. Four of the test bacteria (*S. aureus* O1, *S. aureus* P1, *P. aeruginosa* PA2 and *S. aureus* NCTC 6571) were susceptible to ciprofloxacin, while two test bacteria (*S. aureus* Q2 and *P. aeruginosa* PA1) were resistant to ciprofloxacin.

Table 1: Zones of inhibition (IZD in millimeters) produced by different concentrations of *H. sabdariffa* extract and ciprofloxacin against the test organisms

Test organism	Extract					Ciprofloxacin				
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	1.25 µg/ml	0.625 µg/ml
<i>S. aureus</i> O1	20	17	15	13	0	28	25	35	30	29
<i>S. aureus</i> P1	18	16	14	12	0	0	0	25	24	16
<i>S. aureus</i> Q2	32	26	22	18	16	0	0	0	0	0
<i>S. aureus</i> NCTC 6571	30	25	15	13	0	30	27	22	20	15
<i>P. aeruginosa</i> PA1	22	18	14	11	9	0	0	0	0	0
<i>P. aeruginosa</i> PA2	20	18	15	12	10	30	26	25	23	20

The results shown on Table 1 indicated that aqueous extract of *H. sabdariffa* possesses antibacterial properties, and this activity compared favorably with ciprofloxacin *in vitro*, and in addition inhibited two test bacteria resistant to ciprofloxacin. The antibacterial effects of *H. sabdariffa* extract demonstrated in this study are similar to what has been reported previously [12]. The susceptibility of antibiotic-resistant pathogens to plant extracts has been previously reported [18].

Interaction of ciprofloxacin with *H. sabdariffa* extract

Two isolates, *S. aureus* O1 and *P. aeruginosa* PA2, were selected for the interaction assay due to their susceptibilities to both the extract and ciprofloxacin. Sub-inhibitory concentrations of the extract for each the test bacteria were used, while the normal concentrations of ciprofloxacin were used. Table 2 shows the results of extract-ciprofloxacin interactions against *S. aureus* O1, the inhibition zones ranging from 0 mm to 45 mm, except the 6.25 mg/ml extract concentration which produced no inhibition zones with ciprofloxacin.

Table 2: Zones of inhibition (IZD in millimeters) produced by ciprofloxacin in interaction with *H. sabdariffa* extract against *S. aureus* O1

Extract concentration		Ciprofloxacin concentrations				
		10µg/ml	5µg/ml	2.5µg/ml	1.25µg/ml	0.625µg/ml
	12.5 mg/ml	21	25	28	30	30
	6.25 mg/ml	0	0	0	0	0
	3.13 mg/ml	20	25	45	43	39
	1.56 mg/ml	27	26	40	40	35
	0.78 mg/ml	24	28	40	40	40

The clear inhibition zones obtained in most of the interactions evidence the fact that some of the test bacterial isolates are susceptible to a combination of certain concentrations ciprofloxacin and *H. sabdariffa* extract, and that the two agents can interact *in vitro* to inhibit these test bacteria.

Table 3 shows the results of extract-ciprofloxacin interactions against *P. aeruginosa* PA2, the inhibition zones ranging from 0 mm to 30 mm.

Table 3: Zones of inhibition (IZD in millimeters) produced by ciprofloxacin in interaction with *H. sabdariffa* extract against *P. aeruginosa* PA2

Extract concentration		Ciprofloxacin concentrations				
		10µg/ml	5µg/ml	2.5µg/ml	1.25µg/ml	0.625µg/ml
	6.25 mg/ml	0	0	14	25	0
	3.13 mg/ml	0	0	19	13	0
	1.56 mg/ml	0	0	26	18	10
	0.78 mg/ml	20	14	25	30	28
	0.39 mg/ml	25	20	0	0	0

For the two test bacteria used in the interaction assays (Tables 2 and 3), the IZD values did not show a regular pattern, and in most of the interactions, lower concentrations of ciprofloxacin produced larger inhibition zones in interaction with the corresponding extract concentrations. The extract-ciprofloxacin combinations which produced large inhibition zones are worthy of note.

In Figure 1, two agar plates containing a single concentration of extract each (12.5 mg/ml and 6.25 mg/ml respectively), show clear zones of inhibition in interaction with various concentrations of ciprofloxacin.

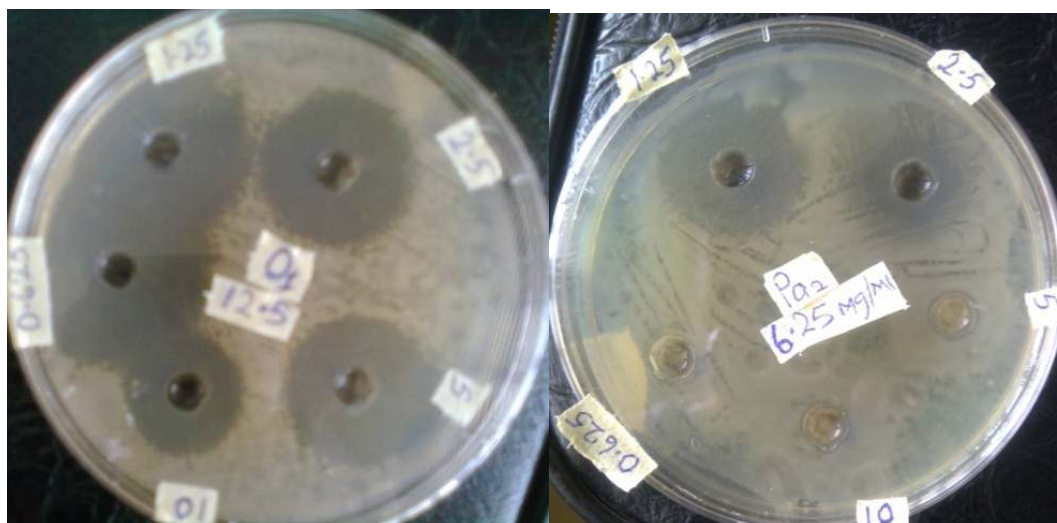


Figure 1- *Left*: Interaction assay. Zones of inhibition produced by five concentrations of ciprofloxacin against *S. aureus* O1 on an agar plate containing 12.5 mg/ml concentration of extract. *Right*: Interaction assay. Zones of inhibition produced by two concentrations of ciprofloxacin against *P. aeruginosa* PA2 on an agar plate containing 6.25 mg/ml concentration of extract.

Results of the interaction assay (interpretation and summary)

A summary of the interactions of extract and ciprofloxacin against the test organisms is presented in Tables 4 and 5. It has been suggested that for a synergistic effect, the diameter of the zone of inhibition in the test plate (plate containing the extract and ciprofloxacin in) should be greater than that in the control plate (plate screening ciprofloxacin alone) by at least 19%. A percentage increase in the inhibition zone diameter that is lower than 19% indicates additivity. Where the inhibition zone diameters in the test and the control are equal, the combined antibiotics have indifferent effect and if the zone diameter in the test is less than the one in the control, then there is antagonism [19].

Table 4: Summary of interaction of ciprofloxacin with *H. sabdariffa* extract against *S. aureus* O1. Values recorded in millimeters are diameters of zones of inhibition produced.

	Extract concentrations				
	12.5 mg/ml	6.25 mg/ml	3.13 mg/ml	1.56 mg/ml	0.78 mg/ml
Ciprofloxacin control (10 µg/ml)	28 mm	28 mm	28 mm	28 mm	28 mm
Ciprofloxacin/extract interaction	21 mm	0 mm	20 mm	27 mm	24 mm
Inference	Antagonistic	No activity	Antagonistic	Antagonistic	Antagonistic
Ciprofloxacin control (5 µg/ml)	25 mm	25 mm	25 mm	25 mm	25 mm

Ciprofloxacin/extract interaction	<u>25 mm</u>	<u>0 mm</u>	<u>25 mm</u>	<u>26 mm</u>	<u>28 mm</u>
Inference	<u>Indifferent</u>	<u>No activity</u>	<u>Indifferent</u>	<u>Additive</u>	<u>Additive</u>
Ciprofloxacin control (2.5 µg/ml)	<u>35 mm</u>	<u>35 mm</u>	<u>35 mm</u>	<u>35 mm</u>	<u>35 mm</u>
Ciprofloxacin/extract interaction	<u>28 mm</u>	<u>0 mm</u>	<u>45 mm</u>	<u>40 mm</u>	<u>40 mm</u>
Inference	<u>Antagonistic</u>	<u>No activity</u>	<u>Synergistic</u>	<u>Additive</u>	<u>Additive</u>
Ciprofloxacin control (1.25 µg/ml)	<u>30 mm</u>	<u>30 mm</u>	<u>30 mm</u>	<u>30 mm</u>	<u>30 mm</u>
Ciprofloxacin/extract interaction	<u>30 mm</u>	<u>0 mm</u>	<u>43 mm</u>	<u>40 mm</u>	<u>40 mm</u>
Inference	<u>Indifferent</u>	<u>No activity</u>	<u>Synergistic</u>	<u>Synergistic</u>	<u>Synergistic</u>
Ciprofloxacin control (0.625 µg/ml)	<u>29 mm</u>	<u>29 mm</u>	<u>29 mm</u>	<u>29 mm</u>	<u>29 mm</u>
Ciprofloxacin/extract interaction	<u>30 mm</u>	<u>0 mm</u>	<u>39 mm</u>	<u>35 mm</u>	<u>40 mm</u>
Inference	<u>Additive</u>	<u>No activity</u>	<u>Synergistic</u>	<u>Synergistic</u>	<u>Synergistic</u>

184

185 By inference, out of the twenty five interactions against *S. aureus* O1, 6 (24%) were synergistic, 6 (24%)
186 were additive, 3 (12%) were indifferent, 5 (20%) were antagonistic, while 5 (20%) showed no activity
187 (Table 4).

188

189 **Table 5: Summary of interaction of ciprofloxacin with *H. sabdariffa* extract against *P. aeruginosa***
190 **PA2. Values recorded in millimeters are diameters of zones of inhibition produced.**

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	Extract concentrations				
	<u>6.25 mg/ml</u>	<u>3.13 mg/ml</u>	<u>1.56 mg/ml</u>	<u>0.78 mg/ml</u>	<u>0.39mg/ml</u>
Ciprofloxacin control (10 µg/ml)	<u>30 mm</u>	<u>30 mm</u>	<u>30 mm</u>	<u>30 mm</u>	<u>30 mm</u>
Ciprofloxacin/extract interaction	<u>0 mm</u>	<u>0 mm</u>	<u>0 mm</u>	<u>20 mm</u>	<u>25 mm</u>
Inference	<u>No activity</u>	<u>No activity</u>	<u>No activity</u>	<u>Antagonistic</u>	<u>Antagonistic</u>
Ciprofloxacin control (5 µg/ml)	<u>26 mm</u>	<u>26 mm</u>	<u>26 mm</u>	<u>26 mm</u>	<u>26 mm</u>
Ciprofloxacin/extract interaction	<u>0 mm</u>	<u>0 mm</u>	<u>0 mm</u>	<u>14 mm</u>	<u>20 mm</u>
Inference	<u>No activity</u>	<u>No activity</u>	<u>No activity</u>	<u>Antagonistic</u>	<u>Antagonistic</u>
Ciprofloxacin control (2.5 µg/ml)	<u>25 mm</u>	<u>25 mm</u>	<u>25 mm</u>	<u>25 mm</u>	<u>25 mm</u>
Ciprofloxacin/extract interaction	<u>14 mm</u>	<u>19 mm</u>	<u>26 mm</u>	<u>25 mm</u>	<u>0 mm</u>
Inference	<u>Antagonistic</u>	<u>Antagonistic</u>	<u>Additive</u>	<u>Indifferent</u>	<u>No activity</u>
Ciprofloxacin control (1.25 µg/ml)	<u>23 mm</u>	<u>23 mm</u>	<u>23 mm</u>	<u>23 mm</u>	<u>23 mm</u>

µg/ml)

Ciprofloxacin/extract interaction	<u>25 mm</u>	<u>13 mm</u>	<u>18 mm</u>	<u>30 mm</u>	<u>0 mm</u>
Inference	Additive	Antagonistic	Antagonistic	Synergistic	No activity
Ciprofloxacin control(0.625 µg/ml)	20 mm	20 mm	20 mm	20 mm	20 mm

Ciprofloxacin/extract interaction	<u>0 mm</u>	<u>0 mm</u>	<u>10 mm</u>	<u>28 mm</u>	<u>0 mm</u>
Inference	No activity	No activity	Antagonistic	Synergistic	No activity

Out of the twenty five interactions against *P. aeruginosa* PA2, 2 (8.0%) were synergistic, 2 (8.0%) were additive, 1 (4%) was indifferent, 9 (36%) were antagonistic, and 11 (44%) showed no activity (Table 5). Summing up the fifty interactions for the two test isolates, 36 (72%) interactions were either favorable or indifferent while 14 (28%) interactions were antagonistic. Result of Chi square test showed this to be statistically significant ($P = .05$).

4. Conclusions

Results from this study revealed that aqueous extract of *H. sabdariffa* calyces possesses antibacterial properties similar to ciprofloxacin, and in addition inhibits ciprofloxacin-resistant isolates. At the same time, the interactions between ciprofloxacin and the *H. sabdariffa* extract showed a percentage of favorable interactions significantly higher than the antagonistic interactions *in vitro*. In addition, the 'Agar-well Cross-reaction Technique' devised for this study, has proved to be sensitive and effective, while eliminating many of the difficulties experienced with other methods of studying drug interactions.

Competing Interests

The author declares that no competing interests exist.

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