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In vitro assessment of the synergism between extracts of *Cocos nucifera* husk and some standard antibiotics

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ABSTRACT

Objective: To evaluate the interactions between the crude extracts of *Cocos nucifera* (*C. nucifera*) and six front line antibiotics (ampicillin sodium salt, penicillin G sodium, amoxicillin, chloramphenicol, ciprofloxacin and tetracycline hydrochloride), against some bacterial pathogens linked with human infection.

Methods: The pulverized husk of *C. nucifera* was dissolved in 95% *n*-hexane and extracted using Soxhlet extraction method and sterile distilled water (aqueous). The antibacterial susceptibility of the crude extracts of *C. nucifera* was tested against environmental and clinical strains (6) obtained from the South African Bureau of Standards (SABS), *Vibrio* (6) and *Listeria* pathogens (6). The agar-well diffusion method was used for screening the extracts for their antibacterial activity. The minimum inhibitory concentration and minimum bactericidal concentration of the extracts were determined. Time-kill assay was used to evaluate bactericidal and/or bacteriostatic activity. The synergistic effect of the crude extracts and antibiotics was assessed and evaluated by adopting the checkerboard methods.

Results: With the time-kill assay, the highest bactericidal activity was observed on *Vibrio fluvialis* EL041 with a -5.6 ± 0.2 $\log_{10}$ CFU/mL decrease in cell density as a result of the combination of the extracts and chloramphenicol at two-fold minimum inhibitory concentrations. Synergisms using the time-kill assay constituted about 72%, while indifference constituted about 28%. The checkerboard method revealed synergistic interaction in 67% of the combinations, and indifference in 33%. There was no specificity in the observed synergy to a particular class of antibiotics.

Conclusions: This investigation suggests the crude extracts of *C. nucifera* to be a potential broad spectrum antimicrobial compound. Therefore, further study is needed to isolate the pure compounds from these crude extracts.

1. Introduction

Infectious diseases continuously denote a significant cause of illness and death among humans, mainly in third-world countries. Despite the production of new antibacterial drugs in the last years by pharmaceutical companies, multidrug resistance profile to these drugs by bacterial isolates has increased with public health implications [1]. Generally, bacteria have the inherent capacity to acquire and transmit resistance to drugs used as therapeutic agents genetically [2]. Antibacterial-resistant pathogens are on the rise. Currently, the incidence of multidrug resistance in pathogenic and opportunistic bacteria species has been increasingly acknowledged [3].

These multidrug-resistant pathogens have also been accompanied with severe clinical problems in immune-compromised persons. Among the various diarrhea causing serotypes of

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Escherichia coli (*E. coli*), enterohaemorrhagic *E. coli* O157:H7 have been implicated in a significant number of food-borne epidemics in different parts of the world [4]. According to the Center for Disease Control and Prevention [5], *Listeria* species have also been isolated from various environments and they are reported to cause about 25% of all the deaths resulting from foodborne outbreaks in the United States annually. Hence, the prominence of finding new effective and efficient antimicrobial agents cannot be overstressed. In balanced drug treatment, the simultaneous administration of two drugs is frequently vital and occasionally obligatory so as to attain the anticipated therapeutic objective or to treat co-existing infections.

Nonetheless, the drug interface may have different significant immune responses likewise the etiological microorganism. The prospective benefits of using synergistic antimicrobial remedy can be management of diverse infections, treatment of intense infections resulting from a known specific causative agent, augmentation of antibacterial activity, plummeting the time for long-term antimicrobial treatment and prevention of the occurrence of resistant disease causing microorganisms [6,7]. Drug combination between antimicrobial agents and bioactive plant extracts is a unique model and has been documented by several authors in recent times [8–10].

Drug combination treatment can be used to increase the spectrum of antimicrobial activity, to prevent the development of resistant strains, to reduce toxicity, and to achieve synergistic antimicrobial activity [11]. Infections resulting from strains that are resistant to main groups of antibiotics like the beta-lactams and aminoglycosides are treatable with vancomycin, chloramphenicol or other antibiotics [12]. However, resistance to these drugs is fast growing. Previously, resistance to the action of fluoroquinolone antibiotics was reported in certain bacteria by mutation but recent studies by Wang *et al.* [13] and Cheung *et al.* [14] have established the plasmid-mediated quinolone resistance (*qnrA* gene) in *E. coli* and members of the Enterobacteriaceae bestowing low level resistance to ciprofloxacin and other fluoroquinolones antibiotics.

Potential antibacterial actions of plant extracts have been documented which include inhibition of MDR-efflux pump [15] and β-lactamase activity [16], antibiotic resistance properties [17] and R-plasmid elimination [18]. On a similarly perspective, studies have shown that some plant extracts and phytochemical composites exhibited combination therapy with antibiotics against Gram-positive bacteria [19,20]. The discovery of novel antimicrobials that prevent and/or block resistance process can improve or eradicate the activities of these multidrug resistant pathogens [21].

This study was taken to assess the combination potentials of *Cocos nucifera* (*C. nucifera*) husk extract with some antibiotics. The research was aimed at enhancing the potentials of the antimicrobial properties of the plant with a view to discover new antimicrobial drugs effective against some pathogenic organisms.

2. Materials and methods

2.1. Plant materials

The plant samples were obtained from the environs of the research farm at Obafemi Awolowo University, Ile-Ife, Nigeria and identified by the curator of the herbarium at the Department of Botany, Obafemi Awolowo University, and a voucher specimen was deposited.

2.2. Preparation of the extracts

The husk of the coconut plant was sun-dried, milled into powdery form and sieved manually using filter with pore size of 2 mm × 2 mm to obtain the fine ground particles. Fifty grams of the dried powder husk of the plant were weighed and added to 200 mL of 95% *n*-hexane for 48 h with regular agitation. The supernatant collected was filtered using Whatman No. 1 filter paper into a clean sterile dried conical flask. The filtrate was concentrated *in vacuo* and lyophilized. Fifty grams of the pulverized husk were weighed and dissolved in 500 mL of sterile distilled water with regular agitation for the aqueous extract for 24 h. The aqueous extract was then centrifuged at 3 000 r/min for 5 min at 4 °C. The supernatant was then filtered through a Whatman No. 1 filter paper and the filtrate was lyophilized.

2.3. Preparation of test bacterial strains

The bacterial isolates used in this study included reference, environmental and clinical strains [*Streptococcus faecalis* (*S. faecalis*) ATCC 29212; *Escherichia coli* (*E. coli*) ATCC 8739; *Acinetobacter calcaoceticus anitratus* (*A. calcaoceticus anitratus*) CSIR; *Bacillus substilis* (*B. substilis*); *Shigella flexineri* (*S. flexineri*) and *Staphylococcus aureus* (*S. aureus*)] obtained from the South African Bureau of Standard (SABS), *Vibrio* [*Vibrio vulnificus* (*V. vulnificus*) EL047; *Vibrio metschnkovii* (*V. metschnkovii*) EL008; *Vibrio specie* (*V. specie*) EL009; *Vibrio fluvialis* (*V. fluvialis*) EL041; *Vibrio vulnificus* (*V. vulnificus*) EL039; *Vibrio fluvialis* (*V. fluvialis*) AL019] and *Listeria* pathogens [*Listeria ivanovii* (*L. ivanovii*) LEL1; *Listeria ivanovii* (*L. ivanovii*) LEL2; *Listeria grayi* (*L. grayi*) LAL3; *Listeria ivanovii* (*L. ivanovii*) LEL17; *Listeria ivanovii* (*L. ivanovii*) LAL10; *Listeria monocytogenes* (*L. monocytogenes*) LAL8]. The inocula of the test organisms were prepared using the colony suspension method [22]. Suspension of the test bacteria in sterile physiological saline buffer was prepared using district colonies picked from 18 to 24 h old cultures grown on nutrient agar to give an optical density of approximately 0.1 OD at 600 nm. A diluent of 1:100 of the suspension was then prepared to give approximately 10^5 CFU/mL cells density by transferring 0.1 mL of the bacterial suspension to 9.9 mL of sterile nutrient broth.

2.4. Antibiotics used

Amoxicillin (Duchefa), ampicillin sodium salt (Calbiochem), penicillin G sodium (Duchefa), ciprofloxacin (Fluka), chloramphenicol (Duchefa), and tetracycline hydrochloride (Duchefa) were the antibiotics used in the present study.

2.5. Antibacterial susceptibility testing

The susceptibility profile of the reference strains to crude extracts and standard antibiotics was evaluated in accordance with previous descriptions [23,24]. Standardization of the inoculum size at 10^5 CFU/mL cells of each test strain was

obtained using McFarland nephelometer standard. Solidified Mueller–Hinton agar plates were aseptically seeded with the reference bacterial strains and allowed to absorb at 37 °C for 3 h. With the aid of a sterile 6 mm cork borer wells were bored into the agar media and filled with the solution of the antibiotics and the extracts, taking care not to allow overflow of the suspension onto the surface of the Mueller–Hinton agar plates. Proper circulation of the extract and antibiotics into the media were allowed by placing the plates on the laboratory bench for 1 h and thereafter incubated at 37 °C for 24 h. Zones of inhibition were observed thereafter. Standard antibiotics (tetracycline and ampicillin) were used to determine the effects of the test bacterial isolates on the extracts at a concentration of 1 mg/mL and 10 µg/mL, respectively.

2.6. Determination of the minimum inhibitory concentrations (MIC)

The methods of Akinpelu and Kolawole [24] were used for the determination of the MIC of the crude *n*-hexane and aqueous extracts. Two milliliters aliquot of different concentrations of the solution prepared from two-fold dilutions of the extracts and antibiotics were added to 18 mL of sterilized molten Mueller–Hinton agar at 40 °C to attain final concentration regimes of 5.0–0.156 mg/mL and 0.01–0.50 mg/mL, respectively. The media were then poured into sterile Petri dishes and allowed to set under a laminar flow prior to streaking with 18-h old bacterial cultures. The plates were thereafter incubated at 37 °C for up to 72 h prior to examination for the presence or absence of growth. The MIC was determined at the least concentration of extracts that prohibited the detectable growth of the test bacteria.

2.7. Determination of minimum bactericidal concentration (MBC)

The MBC was determined from the MIC assays by sub-culturing 10 µL from each culture which did not show growth after 24 h of incubation and inoculating onto fresh Mueller Hinton agar plates. The plates were incubated for 48 h after which the numbers of colonies were counted. The MBC was defined as the lowest concentration that kills more than or equal to 99.9% of the inoculum compared with initial viable counts.

2.8. Extract-antibiotic combination assay

2.8.1. Time-kill assay

Time-kill assay was used to ascertain the effect of the combined action of the crude extracts and antibiotics [11]. The viable count of the test bacteria was initially determined. A 0.5 mL of known cell density (10^5 CFU/mL) from each test bacteria suspension was added 4.5 mL, using a concentration at 1/4 MIC and 2 times the MIC of crude extracts and antibiotics. The suspension was thoroughly mixed and held at room temperature (28–30 °C) and the killing rate was determined over a period of 24 h. A 0.5 mL of each suspension was withdrawn at the appropriate time interval and transferred to 4.5 mL nutrient broth recovery medium containing 3% Tween-80 to neutralize the effects of the antimicrobial compounds carryover from the test suspensions. The suspension was shaken properly then serially diluted up to the order of 10^5 in sterile buffered physiological saline. A 0.5 mL of the final dilution of the test bacteria was transferred into pre-sterile nutrient agar at 45 °C and plated out. The plates were allowed to set and incubated upside down at 37 °C for 24 h. Control experiment was set up without the inclusion of crude extracts and antibiotics. Viable counts were made in duplicate for each sample, where numbers of colonies were counted and expressed as Log_{10}. Synergy is defined as a 2 Log_{10} decrease in colony count at 24 h by the combination compared to the most active single agent, and the number of surviving organisms in the presence of the combination had to be $\geq$ 2 Log_{10} CFU/mL below the starting inoculum. Indifference is defined as $<$ 2 Log_{10} increase in colony count at 24 h by the combination compared by the most active single agent. Antagonism is defined as a $\geq$ 2 Log_{10} increase in colony count at 24 h by the combination compared with that by the most active single agent alone [11].

2.8.2. The checkerboard assay

The assay was carried out as previously described by Mandal *et al.* [25]. Standardized cultures were inoculated into plates by streaking in duplicates prior to incubation at 37 °C for 24 h. The MIC values were determined. The fractional inhibitory concentration (FIC) was thereafter derived from the least concentration of extract and antibiotic combination allowing no observable growth of the test bacteria on the plates [26]. Each agent was estimated for their FIC value using the standard formula:

$$\text{FIC (antibiotic)} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

$$\text{FIC (extract)} = \frac{\text{MIC of extract in combination}}{\text{MIC of extract alone}}$$

The interfaces amongst the extracts and the antibiotics were evaluated in expressions of the FIC guides calculated by using the formula:

$$\text{FIC index} = \sum \text{FIC} = \text{FIC (antibiotic)} + \text{FIC (plant extract)}$$

Combined action was categorized as synergistic, when the FIC guides were $<$ 1; additive when the FIC guides were equivalent to 1; indifferent when the FIC guides were $>$ 1 but $\leq$ 2 and antagonistic when the FIC guides were $>$ 2 [10,27]. When there was a change in the MIC value resulting from more than one combination of the antibiotic or extract, the FIC value was communicated as the average of the single FIC values [11].

2.9. Data analysis

One-way ANOVA was used for determination of the antimicrobial susceptibility by different solvents in the specific concentration. Values were expressed as mean ± SD by using SPSS version 16 software. The means of the two independent experiments (checkerboard and time-kill assay) were compared

using unpaired *t*-test. The *P*-values < 0.05 were considered statistically significant.

3. Results

The diameters of inhibition zones of the aqueous extract of *C. nucifera* husk ranged from 15 mm to 18 mm for the *Listeria* isolates, 13 mm to 17 mm for *Vibrio* isolates, 11 mm to 15 mm for the reference, environmental and clinical strains, while the diameters of inhibition zones of the *n*-hexane extract ranged from 14 mm to 19 mm against the *Listeria* isolates, 11 mm to 18 mm for the *Vibrio* isolates and 12 mm to 16 mm for the reference, environmental and clinical strains. The standard antibiotics, tetracycline and ampicillin showed zones of inhibition of 30–46 mm and 21–32 mm for the *Listeria* isolates, 18–30 mm and 19–25 mm for the *Vibrio* isolates and 27–32 mm and 14–28 mm for the reference, environmental and clinical strains respectively. Findings from these experiments showed that crude extracts of the husk of *C. nucifera* unveiled antibacterial actions against almost all the test Gram-positive and Gram-negative bacteria made up of the clinical, reference, environmental strains, as well as *Vibrio* and *Listeria* pathogens when screened at a concentration of 5 mg/mL (Table 1).

The *n*-hexane and aqueous extracts exhibited MICs which varied between 0.625 and 5.0 mg/mL (Table 2). Specifically, the MICs of the aqueous extract extended from 0.625 to 2.5 mg/mL for the *Listeria* isolates; 0.625 to 5.0 mg/mL for *Vibrio* isolates; and 0.625 to 5.0 mg/mL for reference, environmental and clinical isolates. The minimum bactericidal concentrations (MBCs)

Table 1
Diameter of inhibition zone of crude extracts of the husk fiber of *C. nucifera* (mm).

Bacterial isolate	Aqueous extract (5 mg/mL)	*n*-Hexane extract (5 mg/mL)	Tetracycline (1 mg/mL)	Ampicillin (10 µg/mL)
L. ivanovii LEL1	15.0 ± 0.0	18.0 ± 0.0	31.0 ± 0.4	21.0 ± 0.2
L. ivanovii LEL2	15.0 ± 0.1	16.0 ± 0.0	30.0 ± 0.2	26.0 ± 0.1
L. grayi LAL3	16.0 ± 0.0	19.0 ± 0.1	34.0 ± 0.5	25.0 ± 0.2
L. ivanovii LEL17	18.0 ± 0.2	18.0 ± 0.0	32.0 ± 0.1	25.0 ± 0.4
L. ivanovii LAL10	17.0 ± 0.1	16.0 ± 0.0	46.0 ± 0.2	32.0 ± 0.2
L. monocytogenes LAL8	16.0 ± 0.2	14.0 ± 0.1	34.0 ± 0.4	22.0 ± 0.1
V. vulnificus EL047	17.0 ± 0.2	18.0 ± 0.1	18.0 ± 0.2	25.0 ± 0.0
V. metschnkovii EL008	14.0 ± 0.0	12.0 ± 0.0	28.0 ± 0.2	19.0 ± 0.2
V. specie EL009	13.0 ± 0.2	17.0 ± 0.1	21.0 ± 0.4	20.0 ± 0.2
V. fluvialis EL041	14.0 ± 0.1	11.0¬± 0.0	22.0 ± 0.2	21.0 ± 0.1
V. vulnificus EL039	14.0 ± 0.0	11.0 ± 0.2	26.0 ± 0.1	24.0 ± 0.2
V. fluvialis AL019	16.0 ± 0.2	18.0 ± 0.0	30.0 ± 0.1	20.0 ± 0.4
E. coli ATCC 8739[r]	11.0 ± 0.2	12.0 ± 0.0	27.0 ± 0.0	28.0 ± 0.1
S. faecalis ATCC 29212[r]	15.0 ± 0.1	14.0 ± 0.2	27.0 ± 0.2	21.0 ± 0.1
A. calcoaceticus anitratus CSIR[r]	15.0 ± 0.2	14.0 ± 0.2	32.0 ± 0.4	14.0 ± 0.1
B. substilis[e]	11.0 ± 0.1	12.0 ± 0.0	22.0 ± 0.2	28.0 ± 0.1
S. flexineri[e]	14.0 ± 0.2	15.0 ± 0.0	30.0 ± 0.1	16.0 ± 0.2
S. aureus[c]	13.0 ± 0.2	16.0 ± 0.1	28.0 ± 0.0	19.0 ± 0.2

[r]: Reference strain; [e]: Environmental strain; [c]: Clinical strain.

Table 2
The MICs and MBCs of the *C. nucifera* aqueous and *n*-hexane husk extracts against susceptible bacterial isolates (mg/mL).

Bacterial isolates	Gram reaction	MIC		MBC	
		Aqueous extract	*n*-Hexane extract	Aqueous extract	*n*-Hexane extract
L. ivanovii LEL1	+	2.500	0.625	> 5.00	2.50
L. ivanovii LEL2	+	0.625	1.250	2.50	5.00
L. grayi LEL3	+	0.625	0.625	5.00	2.50
L. ivanovii LEL17	+	2.500	0.625	> 5.00	5.00
L. ivanovii LAL10	+	0.625	5.000	1.25	>5.09
L. monocytogenes LAL8	+	2.500	2.500	> 5.00	5.00
V. vulnificus EL047	–	0.625	0.625	2.50	2.50
V. metschnkovii EL008	–	0.625	1.250	5.00	2.50
V. specie EL009	–	5.000	2.500	> 5.00	5.00
V. fluvialis EL041	–	0.625	0.625	2.50	2.50
V. vulnificus EL039	–	0.625	0.625	2.50	5.00
V. fluvialis AL019	–	ND	1.250	ND	2.50
E. coli ATCC 8739[r]	–	0.625	5.000	2.50	>5.00
S. faecalis ATCC 29212[r]	+	0.625	0.312	5.00	1.25
A. calcaoceticus anitratus CSIR[r]	–	ND	2.500	ND	5.00
B. substilis[e]	+	ND	2.500	ND	5.00
S. flexineri[e]	–	ND	1.250	ND	2.50
S. aureus[c]	+	0.625	1.250	5.00	5.00

[r]: Reference strain; [e]: Environmental strain; [c]: Clinical strain; ND: Not determined.

were also determined for the entire susceptible organisms and it ranged from 1.25 to 5.0 mg/mL (Table 2). The MIC ranges for the standard antibiotics adopted were 0.01–0.50 mg/mL for penicillin G, 0.01–0.25 mg/mL for amoxicillin; 0.016 mg/mL for ciprofloxacin; 0.01–0.50 mg/mL for chloramphenicol; 0.01–0.25 mg/mL for ampicillin; 0.01–0.50 mg/mL for tetracycline (Table 3).

Synergy rates of 70% (extract + ampicillin; extract + amoxicillin), 90% (extract + chloramphenicol; extract + penicillin G), 80% (extract + ciprofloxacin) and 40% (extract + tetracycline) were detected on all the test bacterial isolates. Generally, insignificance constituted 28%, while combined action response formed 72% respectively of the respective combinations of antibiotics and extract against all test bacterial isolates using time-kill assay.

Table 3
The MICs of standard antibiotics exhibited against bacterial isolates (mg/mL).

Bacterial isolates	AMP	PEN G	AMX	CHL	CIP	TET
E. coli ATCC 8739	0.0312	0.0310	0.0160	0.1250	0.0160	0.0160
S. faecalis ATCC 29212	0.0312	0.0625	0.0620	0.0160	0.0160	0.0160
L. ivanovii LEL3	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160
V. vulnificus EL039	0.2500	0.0160	0.2500	0.0310	0.0160	0.0160
V. fluvialis EL041	0.2500	0.5000	0.2500	0.5000	0.0160	0.5000

AMP: Ampicillin; AMX: Amoxicillin; CHL: Chloramphenicol; PEN G: Penicillin G; TET: Tetracycline; CIP: Ciprofloxacin.

The effects and interactions of the time-kill on the antibiotics and the extracts combinations are presented in Table 4. The extracts revealed capacity to advance in the bactericidal effect from the antibiotics on both Gram-negative and Gram-positive microorganisms. A 5.6 Log_{10} decrease in cell density was considered as the utmost bactericidal activity and was produced by the combined action of chloramphenicol and plant extract against *V. fluvialis* EL041.

Table 5 reveals the combined action of the extract-antibiotic adopting the checkerboard method. Sixty-seven percent (67%) of the entire interactions were mutually stimulating, while insignificance interactions formed 33%. Comparison of the output for the checkerboard methods and time-kill showed the level of agreements observed between the respective methods ranged from 50% to 100%.

Table 4
Anti-bacterial activity of standard antibiotic plus extracts combinations using time-kill assay.

Bacterial isolates		*E. coli* ATCC 8739	*Streptococcus faecalis* ATCC 29212	*Listeria ivanovii* LEL3	*Vibrio vulnificus* EL039	*Vibrio fluvialis* EL041
EXT + AMP	1/4 MIC	−1.3 ± 0.1	−2.4 ± 0.2	−3.2 ± 0.1	−2.8 ± 0.2	−1.9 ± 0.1
	2 MIC	−2.4 ± 0.1	−2.6 ± 0.2	−4.4 ± 0.3	−1.6 ± 0.1	−2.3 ± 0.1
EXT + PEN G	1/4 MIC	−2.6 ± 0.1	−2.2 ± 0.1	−4.0 ± 0.2	−4.2 ± 0.2	−3.6 ± 0.2
	2 MIC	−1.4 ± 0.1	−2.0 ± 0.1	−3.2 ± 0.1	−3.4 ± 0.2	−2.4 ± 0.1
EXT + AMX	1/4 MIC	−2.4 ± 0.2	−1.2 ± 0.1	−3.6 ± 0.2	−2.2 ± 0.2	−1.8 ± 0.4
	2 MIC	−1.8 ± 0.2	−3.8 ± 0.1	−2.8 ± 0.1	−1.2 ± 0.1	−3.4 ± 0.1
EXT + CHL	1/4 MIC	−3.2 ± 0.4	−2.0 ± 0.1	−2.4 ± 0.1	−3.8 ± 0.4	−3.2 ± 0.2
	2 MIC	−1.4 ± 0.2	−3.4 ± 0.1	−2.0 ± 0.1	−4.0 ± 0.3	−5.6 ± 0.2
EXT + CIP	1/4 MIC	−4.4 ± 0.1	−3.8 ± 0.2	−4.6 ± 0.2	−2.1 ± 0.2	−3.0 ± 0.1
	2 MIC	−4.6 ± 0.4	−4.2 ± 0.2	−2.8 ± 0.1	−1.4 ± 0.2	−1.8 ± 0.2
EXT + TET	1/4 MIC	−1.6 ± 0.1	−1.8 ± 0.1	−2.0 ± 0.2	0.56 ± 0.1	−1.4 ± 0.1
	2 MIC	−2.4 ± 0.1	0.56 ± 0.1	−2.8 ± 0.2	−1.2 ± 0.2	−2.3 ± 0.2

Numbers of colonies were counted and expressed as Log_{10}. EXT: Extract; AMX: Amoxicillin; AMP: Ampicillin; PEN G: Penicillin G; CHL: Chloramphenicol; CIP: Ciprofloxacin; TET: Tetracycline.

Table 5
Anti-bacterial activity of standard antibiotic plus extracts combinations by checkerboard method.

Bacterial isolates	*E. coli* ATCC 8739	*S. faecalis* ATCC 29212	*L. ivanovii* LEL3	*V. vulnificus* EL039	*V. fluvialis* EL041
EXT + AMP	0.3 (S)	1.6 (I)	1.8 (I)	0.6 (S)	0.4 (S)
EXT + PEN G	1.9 (I)	0.8 (S)	0.9 (S)	1.2 (I)	0.8 (S)
EXT + AMX	0.6 (S)	0.8 (S)	1.4 (I)	0.7 (S)	1.4 (I)
EXT + CHL	0.4 (S)	1.4 (I)	0.4 (S)	0.6 (S)	1.2 (I)
EXT + CIP	0.4 (S)	0.8 (S)	0.4 (S)	0.6 (S)	1.1 (I)
EXT + TET	0.8 (S)	0.3 (S)	1.2 (I)	0.6 (S)	0.4 (S)

EXT: Extract; AMP: Ampicillin; PEN G: Penicillin G; AMOX: Amoxicillin; CHL: Chloramphenicol; CIP: Ciprofloxacin; TET: Tetracycline; I: Indifferent; S: Synergy.

4. Discussion

The use of medicinal plants to treat infectious diseases has been reported by several researchers. Antimicrobial combination therapy may be used frequently for diagnosis purpose such as to extend spectrum coverage, prevent the emergence of resistant mutants and gain synergy between antimicrobials [27]. Combinations of plant extracts with antibiotics are considered to be a fundamental therapy in the treatment of infections and diseases [28]. This experiment was carried out to assess and establish the combination potentials between antimicrobial drugs and plant extract against some pathogenic bacteria. The antimicrobial activity of plant extract with standard antibiotics against some bacteria pathogens were confirmed in the present study and it was observed that mutual stimulation was conceivable with all the antibiotics tested. No antagonism was observed and the degree of agreement detected amongst the antibiotics and all the plant extract using the time-kill assay and checkerboard method ranged between 50% and 100%. The checkerboard study was conducted in order to confirm the time-kill method while the time-kill studies revealed the capacity of the extract to build on the bactericidal action of the antibiotics on both Gram positive and Gram negative bacterial isolates.

Our time-kill studies were based on comparing the rate of kill of the combined action to that of the single agent. Mutual stimulation was observed at 1/4 MIC level of combination of extract + penicillin G; chloramphenicol and ciprofloxacin against the entire tested bacterial isolates. This indicates that the degree of the inhibition was constant at 24 h with the principles of synergistic effect. The effect of the inhibition resulting from the combination of extract + ciprofloxacin; ampicillin, penicillin G and chloramphenicol at 2 times the MIC persisted against all tested isolate except for *V. vulnificus* and *E. coli*.

The *in vitro* efficacy of extract + penicillin G; extract + chloramphenicol; extract + ciprofloxacin was superior to that of extract + tetracycline in producing synergy against all tested bacteria. A similar observation was reported by Otsuki and Nishino [28] and this has been attributed the penetrability of the external membrane to beta-lactam antibiotics that is elevated as a result of the interaction of quinolones with the external membrane as chelating agents. The process by which such combined action achieves such interaction is thought to be the expedition of access of beta-lactam antibiotics into the cells after incomplete interference of the cell wall as a consequence of the action of quinolones [29]. It is likely that the action of some substances extracted from plant on the ribosomal structure and bactericidal enzymes could result in the synergistic profile observed between inhibitors of cell wall, protein synthesis and plant extracts; conversely, the appreciation of synergistic process is central to development of new antimicrobial agents in the pharmaceutical pipeline to combat infectious diseases. Our findings further advocate that the medical effectiveness of this antimicrobial synergistic treatment should be therefore adopted in an animal model to inspect this occurrence. It would be an additional knowledge if medical investigations are carried out to cross examine the significance of our outcomes. As an alternative method the checkerboard method was conducted to observe variations in the MIC outputs [26]. With the aid of FIC indices, significant synergism was observed in all manners of combinations of the extract with all the antibiotics (except penicillin) against *E. coli* and *V. vulnificus* (Gram negative bacterial). The enhanced antimicrobial effect of the combination could be attributed to action of beta-lactams on the transpeptidation of the cell membrane structure coupled with the disconcertion of the cell membrane.

As seen in Table 3, the antimicrobial mechanisms of the drugs used in the present study varied; those that inhibit cell wall synthesis (penicillin) presented the best synergism of 90% followed by the protein synthesis inhibitor (chloramphenicol) and then the nucleic acid inhibitor using the time-kill and checkerboard assay method (ciprofloxacin). Synergistic potentials were promising for the combinations between the extract of *C. nucifera* and tetracycline in both assay methods. The presence of condensed tannin contained in the extract of *C. nucifera* husk was comprised of epicatechin-3-*O*-gallate, epigallocatechin, epicatechin, and flavonoids subunits catechin [30] which has antimicrobial and resistance modifying potentials. The mechanisms by which this naturally occurring tannins work have been reported to act by iron deprivation, hydrogen binding or specific interactions with vital proteins such as enzymes in microbial cells [31].

Zhao *et al.* [32] have revealed in their studies that some compounds derived from plant extract can increase the *in vitro* activity of cell wall synthesis by inhibiting some peptidoglycan structures. Kumar and Schweitzer [33] also attributed effect of antibiotic resistance in pathogenic organism to bacteria efflux pump system. For example Stermitz *et al.* [15] reported the compound 5'-methoxyhydnocarpin, isolated from *Berberis fremontii* against *Staphylococcus aureus* to be an inhibitor of efflux pump NorA, so it may be deduced that the husk of *C. nucifera* may contain broad spectrum efflux pump inhibitor compounds which could enhance its combination interaction with antibiotics against Gram negative and Gram positive bacterial isolates. Synergistic treatment is frequently acclaimed for first-hand management of infections caused by bacteria in intensive care units, where narrow spectrum antimicrobial is not likely to eliminate all latent pathogens, coupled with the emergence of resistance as a prospective threat [33].

It has been suggested that, plants extracts also produce compounds that inhibit multi-drug resistance (MDR) coupled with the release of inherent antimicrobial combinations, which improves the action of the antimicrobial compounds [15]. A study by Tegos *et al.* [34] revealed that the potential of some plant extracts against Gram negative and Gram positive microorganisms was expressively boosted by MDR inhibitors of efflux proteins, thus suggesting that plants extract can be prospective cradles of acceptable MDR inhibitors that can hypothetically increase the action of antibiotics against resistant bacterial strains.

The findings from the present study appear encouraging and may enhance its usage as an accepted product, revealing the prospect of this plant in the management of infectious diseases resulting from organisms implicated in food and wound infections. This corroborate the findings of Cheesbrough [35] who suggested that coconut oil contains antimicrobial agents which could make it suitable for medicinal purposes like the treatment of wound infection and urinary tract infection. The antimicrobial activities of potential plant extracts on strains of *Vibrio* and *Listeria* were deep-rooted and combined action was possible with all the antibacterial drugs used. All antibiotics revealed synergism with the plant extracts of *C. nucifera* against all tested bacteria, although with varying antimicrobial activity profiles.

Nevertheless, our research has suggested the potential usefulness of extract of *C. nucifera* plant and some front line antibiotics as a synergistic therapy for the cure of *L. ivanovii*,

V. vulnificus, *V. fluvialis*, and some reference bacterial strain infections. The recognition of synergism in this research establishes the capacity of this plant extract as a probable foundation of antibiotic resistance alternative compounds. Hence further analysis and identification of the possible compounds in the plant that could be responsible for the synergism observed as well as an *in vivo* study of the mechanism of action of the compounds in combination therapy are needed.

In summary, the combination interactions of the extracts of *C. nucifera* husk with six front line antibiotics were studied against a number of pathogenic organisms implicated in food and wound infections. The result from the present study indicates that the husk of *C. nucifera* possesses some antimicrobial properties with a greater potency when used concurrently with antibiotics against the tested pathogens, indicating the extract to be a promising plant to new choice of antimicrobial compounds for the treatment of infectious diseases. The data obtained in the present study support the use of antimicrobial combinations including beta-lactams, fluoroquinolones and glycopeptides with plant extracts in preliminary experimental therapy of serious infections possibly caused by strains of pathogenic organisms such as *Vibrio* and *Listeria* pathogens, though *in vivo* findings need to be validated by *in vitro* studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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