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Combined Antibacterial Effect of *Croton macrostachyus, Calpurina aurea* and *Ocimum gratissimum* Against Selected Clinical and Standard Pathogenic Bacteria

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Abstract: *Background*: The use of medicinal plants as treatment options of human and animal diseases can be traced back in human history, and about ten percent of identified medicinal plants serve a pharmaceutical role because they have active chemical constituents such phenolic acids, flavonoids, tannins and lignin. Ethiopia is a place rich in medicinal plants, though most studies in the region have only considered the individual effects of their extracts while under-exploring their combined effects. *Objective*: The objective of this research was to assess the synergistic antibacterial activity of crude extracts of leaves of *Croton macrostachyus, Calpurnia aurea* and *Ocimum gratissimum* collected from Bahir Dar town against standard and clinical isolates of *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* by using methanol, acetone and chloroform as solvents. *Methods*: Extracts were prepared at a plant-to-solvent ratio of 50 g to 500 mL and then set to a concentration of 50 mg/mL by dissolving 100 mg of crude extract in 2 ml of 10% dimethyl sulfoxide in small cups, from which 100 μL was used for antibacterial assays using the disc diffusion method. Minimum inhibitory and bactericidal concentration assays were assessed with the broth microdilution and overnight bacterial culture preparation techniques, respectively. The fractional inhibitory concentration index was used for synergistic activity analysis. *Results*: Combinations of extracts showed relatively better effects against most test bacteria with inhibition zones reaching up to 23.00 ± 1.00 mm (*Salmonella typhi*) despite limited activity on both standard and clinical isolates of *Escherichia coli*. The lowermost minimum inhibitory and bactericidal concentrations were 3.125 mg/mL and 6.25 mg/mL, respectively, and a few synergistic and many additive effects were recorded for different forms of combinations on different bacterial isolates. *Conclusion*: The combined use of extracts is relatively promising, though further work is required to clearly set the safety margins of combinations used in vivo, as this is the first report on all settings used here. The findings of this study provide scientific evidence for communities, pharmaceutical industries, and other concerned bodies regarding alternative formulations of phytochemicals for the relief of different physiological deviations, with the combined use of plants showing better performance.

Keywords: Antibacterial Effect, Calpurina Aurea, Croton Macrostachyus, Medicinal Plant, Ocimum Gratissimum

1. Introduction

Human diseases are treated by using natural products from plants, animals and minerals [1]. In addition to their use in maintaining the stability and health of ecosystems, the use of plants as medicine has been progressively increasing since ancient times [2-4], as they started to be used as treatment options in early human history [5]. About ten percent or more of all identified plant species serve a pharmaceutical or cosmetic purpose [6, 7], although their distribution is highly limited to wild floral populations [8]. A medicinal plant is a plant variety with a direct or indirect

therapeutic role [3] due to compounds harvested from its seeds, roots, leaves, fruit, skin, flowers, or even the whole plant [9]. According to reports of the World Health Organization, over 60% of the population of developing countries exclusively depend on the consumption of herbal medications as a relief for their health complications [10]. Accordingly, scientists from different corners of the world are urged to explore the various biological activities of medicinal plants [6].

Medicinal plants possess various categories of phytoconstituents [11] with antibacterial, antimutagenic, anticarcinogenic, antithrombotic and/or vasodilatory activities [12]. The interaction of the constituents may produce an additive or dispersive effect on hard-to-treat illnesses such as cancer [13].

The use of herbal medicine is a solution for alarmingly increasing bacterial resistances, and it has been found to be a source of new medicinal agents that effective against organisms that cannot be treated by existing agents [14, 15]. The increase in the use of herbal medicine is also attributed to the increased toxicity and adverse effects of conventional and allopathic medicines [3]. Moreover, medicinal plants are demanded in Ethiopia due to culturally linked traditions and the trust of communities in traditional medicinal values [16]. Therefore, the cultivation and use of medicinal plants is not new to Ethiopia [17, 18]. 70% of human and 90% of livestock populations in Ethiopia depend on traditional medicine [13].

Plants such as *C. macrostachyus*, *C. aurea*, and *O. gratissimum* locally named "Bisana", "Zigita", and "Damacase" in Amharic, respectively, are used for the treatment of different ailments including skin infections, respiratory problems, and gastrointestinal complications. [16]. Plants are used intact or mixed with homemade substances such as "local beer" and "tella" [19], which are equivalent to ethanol, acetone, chloroform, and other chemicals used as active substances for scientific extraction [20, 21]. Sometimes, people use plants in combination if they are to treat extensive wounds. Accordingly, the use of appropriate extraction techniques and solvents is a prerequisite for the optimal harvest of a bioactive component from a chosen medicinal plant [13, 16, 22].

Ethiopia has over 6500 species of plants of medicinal importance. The majority of them have been assessed in different parts of the country, but their combined effects on target organisms have not been studied. Therefore, the objective of this research was to determine the combined antibacterial effects of *C. macrostachyus*, *C. aurea* and *O. gratissimum* against selected clinical and standard pathogenic bacteria, as well as the MIC and MBC values of all possible combinations. These findings will benefit the community using these plants by providing information about their combined effects. Researchers, health-conscious organizations, and drug processing and fabrication industries will also benefit from this knowledge because better and safer products can arise from it.

2. Materials and Methods

2.1. Collection Area of Plant Materials

Plant materials were collected from the shore of Lake Tana at Bahir Dar town, Northwestern Ethiopia, exit of Abbay River [23], an area hosting various types of vegetation with different levels pf medicinal importance [24].

2.2. Collection and Authentication of Plant Materials

Once leaves of *O. gratissimum*, *C. macrostachyus* and *C. aurea* were collected, they were identified by botanists at the University of Gondar, College of Natural and Computational Sciences, Department of Biology via visual comparisons with authenticated plant specimens and the use of taxonomic keys in the volumes of Flora of Ethiopia and Eritrea [25]. The voucher numbers of specimens were deposited at the National Herbarium (ETH) Northwest Ethiopia office, University of Gondar.

2.3. Preparation of Plant Extracts

The preparation of plant extracts was performed following the method presented in the article [26], i.e., leaves of plants were washed with distilled water, dried under shade and mechanically crushed by an electrical grinder. Fifty grams of each plant powder was added to 500 mL of an 80% solvent (methanol, acetone, and chloroform separately), mixed well using an orbital shaker, subjected to continuous shaking for four consecutive days, and filtered through Whatman No. 1 filter paper. The filtered extract was evaporated under a rotary evaporator (RE200B, UK Sterilin LTD., Newport, UK) of reduced pressure at 40°C before being pooled, dried in vacuum, and stored at 4°C until used in the next step [27].

2.4. Preparation of Test Organisms

Both clinical and standard isolates of *S. aureus* and *E. coli* and standard isolates of *Salmonella typhi* were obtained from the University of Gondar Teaching Referral Hospital. The bacteria were brought in vials and sub-cultured on nutrient agar to generate a fresh colony. Then, isolates were set to a 0.5 McFarland standard so that it was easy to seed them for the antibacterial test assays of crude extracts.

2.5. Detection of Antibacterial Activity of Plant Extracts

The agar well diffusion method [28] was used, i.e., the inhibition zones of crude extracts against the growth of test bacteria were assessed on a Mueller Hinton Agar medium prepared as per the manufacturer's instruction. Each Petri dish loaded with Mueller Hinton Agar was seeded with a bacterial suspension of a 0.5 McFarland standard or 1.5×10^8 colony-forming units (CFU/mL) and bored with a cork-borer of 4 mm in diameter at equal distances on its surface. Then, 100 µL of crude plant extracts was prepared as a solution by dissolving 50 mg of each in 1 ml of 10% DMSO. All activities were performed under a Vertical Laminar Air Flow Hood (Cat AC-356A, Abron Exports India, Ambala Cantt, India). An

equal volume of 10% DMSO, as a negative control, and a 10 µg of gentamycin disc, as a positive control, were also added to each Petri plate. Seeded Petri dishes were then incubated for 24 h at 37 °C and checked for any inhibition of bacterial growth around each extract. Extracts' inhibition zones were compared with inhibition zones of both positive and negative controls. All inhibition zones were measured in mm, with each test performed in triplicate and classified as effective if the mean inhibition zone was greater than or equal to 6 mm or not if the mean inhibition zone was less than 6 mm [29].

2.6. Determination of Minimum Inhibitory Concentration (MIC)

The broth microdilution method was used for MIC determination [30]. An extract concentration ranging from 50% to 1.56%, created by dissolving the extract in a broth medium in screw-capped test tubes, was prepared. Then, 100 µL of the bacterial suspension was added in the Vertical Laminar Air Flow Hood (Cat AC-356A, Abron Exports India). Tubes were incubated at 37 °C for 24 h, and then the growth of the test bacteria was checked by observing the turbidity of each tube compared with positive and negative control test tubes. The presence of turbidity confirmed growth of bacteria and, a clear view of the mixture showed the absence of growth. Each test tube with its specific extract concentration that showed no turbidity was recorded as the MIC of tested bacteria with the respective test extract.

2.7. Determination of Minimum Bactericidal Concentration (MBC)

Microdilution test units of the MIC assay with no turbidity were sub-cultured on fresh Mueller Hinton Agar plates and incubated overnight at 37 °C. Plate regions with certain concentration levels that showed no bacterial colonies at the end of the incubation period were recorded as minimum bactericidal concentration levels [31].

2.8. Determination of Combined Activity of Crude Extracts

Combined uses of different crude extracts were identified as synergistic by calculating the fractional inhibitory concentration index (FICI) using formula described by Stefanović, O. D [32].

$$FICI = (MICa \text{ in combination/ } MICa) + (MICb \text{ in combination/ } MICb)$$

where MICa is the MIC of plant extract one and MICb is the MIC of plant extract two, which can be interpreted as synergistic if an FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4, and antagonistic for an FICI > 4 according to the author [33].
1) Synergistic: if their joint effect is stronger than the sum of effects of the individual agents.
2) Additive: if their joint effect was equal to the sum of effects of the individual agents.
3) Indifferent: if their joint effect was equal to the effect of either individual agent.
4) Antagonistic: if their joint effect was weaker than the sum of the effects of the individual agents or weaker than the effect of either individual agent.

2.9. Data Analysis

SPSS version 20 was used to analyze all data. The inhibition zones of extracts are presented as mean ± standard deviation, and a one-way ANOVA was employed to define the difference in the effect of plant extracts on different test organisms at a significance level of (p-value) < 0.05. MIC and MBC values of extracts are expressed by bar graphs produced using Excel version 2010. The effects of combinations were defined with a simple algebraic calculation computing a fractional inhibitory concentration index.

3. Results and Discussion

3.1. The Antibacterial Assay

C. macrostachyus extracts in methanol and chloroform were highly active against *E. coli* ATCC35218, with inhibition zones of 17.00 ± 1.0 mm and 10.67 ± 0.56mm respectively, though these results disagreed with those of a previous study [34] in Debre Berhane. However, inhibition zones of 17.67 ± 1.53 mm and 15.00 ± 2.65 mm against standard and clinical isolates *of S. aureus,* respectively, with methanol as the solvent were lower than those of 25.00 ± 0.00 mm and 20.00 ± 0.00 mm reported earlier [35]. These differences could be attributed to variations in geography and the season of the collection time of plants, as both of these factors could contribute to differences in the phytochemical constituents and/or concentrations of plants.

C. aurea showed better effects against *S. aureus* ATCC43300 (14.67 ± 0.58 mm), *S. typhi* ATCC1333 (19.33 ± 3.21 mm), and *E. coli* ATCC35218 (16.67 ± 7.09 mm) compared with previously reported inhibition zones of 10mm, 11mm, and 14 mm, respectively, with methanol used as the extraction solvent [36]. However, crude extracts were found to be less potent (14.67 ± 0.58 mm) against *E. coli* ATCC35218 compared to the previously reported inhibition zone of 15.63 ± 0.12 mm [37]. A methanol extract of *O. gratissimum* was also found to perform well against standard isolates *S. aureus* and *E. coli*, with inhibition zones of 12 and 13.5 mm [38].

The effect of *C. aurea* was found to be better against clinical isolates of *S. aureus* and *E. coli* (17.33 ± 4.04 and 13.33 ± 1.15, respectively) using acetone compared with previously reported inhibition zones of 13.47 ± 2.01 and 12.4 ± 1.69, respectively, for the same bacteria with the same protocol [39]. Compared with reports of 25 ± 0.91 and 16 ± 0.26 against clinical isolates of *S. aureus* and *E. coli,* respectively, [40] from Nigeria, the respective inhibition zones of 18.00 ± 2.65 and 10.00 ± 1.00 reported in this study for the same bacteria were low. As in all other cases, the variations found in this study may have been associated with the agroecological location of plants, the season of harvest, and some inaccuracies in measurement during the actual laboratory work.

From crude methanol extracts, *C. macrostachyus* was found to have a significantly stronger effect against *S. typhi* ATCC1333 than *S. aureus* ATCC43300 ($p = 0.026$) and *E. coli* ($p = 0.013$) and clinical isolates of S. *aureus* ($p = 0.002$) and *E. coli* ($p = 0.007$). *O. gratissimum* was found to have a significantly lower effect against clinical isolates of *E. coli* than *S. aureus* ATCC43300 ($p = 0.03$) and against *E. coli* ATCC35218 than *S. typhi* ATCC1333 ($p = 0.014$). A significantly higher effect against *S. typhi* ATCC1333 than *S. aureus* (clinical) ($p = 0.019$) and *E. coli* (clinical) ($p = 0.00$) was also recorded with methanol. With the same solvent, *O. gratissimum* showed a significantly stronger effect against *S. typhi* ATCC1333 than clinical isolates of *E. coli* ($p = 0.001$), which agreed with the results [27].

The combined use of *C. macrostachyus* and *C. aurea* showed stronger effects than individual component extracts except for the stronger effect of *C. macrostachyus* on standard isolates of *S. aureus* ATCC43300 and that of *C. aurea* on *S. typhi* ATCC1333 using methanol. When using acetone, this combination was found to be relatively less effective against *S. typhi* ATCC1333. Moreover, using chloroform, the combination was less potent on clinical isolates of *S. aureus* compared with the effects of *C. aurea*. These results may have been caused by variations in the phytochemical extraction capacity of different solvents from the leaves of plants.

The combination of methanol extracts of *C. macrostachyus* and *O. gratissimum* was more active against most test bacteria than the individual components. When using acetone, the same combination was found to be less active against standard and clinical isolates of *S. aureus* than *O. gratissimum* (Tables 1 and 2).

***Table 1.** Antibacterial activity of individual plant extracts. The table indicates the mean inhibition zones of individual plant extracts against tested bacteria.*

Extract	Inhibition Zone on Test Organism (mm) (Mean ± Standard Deviation)				
	S. aureus ATCC43300	*S. aureus* (Clinical)	*E. coli* ATCC35218	*E. coli* (Clinical)	*S. typhi* ATCC1333
	Methanol				
C. macrostachyus	17.67 ± 1.53 [a]	15.00 ± 2.65 [a]	17.00 ± 1.00 [a]	16.33 ± 1.15 [a]	22.00 ± 3.00 [b]
C. aurea	19.33 ± 3.21 [a]	16.33 ± 2.52 [a]	14.67 ± 0.58 [a]	13.33 ± 1.53 [a]	16.67 ± 7.09 [a]
O. gratissimum	20.33 ± 3.21 [a]	18.00 ± 2.65 [a]	6.67 ± 5.86 [b]	10.00 ± 1.00 [c]	17.00 ± 6.25 [a]
Gentamycin (+ve control)	25.67 ± 1.53	25.33 ± 2.52	25.67 ± 1.53	24.67 ± 1.53	26.00 ± 2.00
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	acetone				
C. macrostachyus	16.67 ± 2.52 [a]	14.00 ± 1.00 [a]	10.67 ± 1.53 [b]	9.67 ± 1.15 [b]	19.33 ± 4.04 [a]
C. aurea	18.00 ± 3.61 [a]	17.33 ± 4.04 [a]	13.33 ± 2.08 [a]	13.33 ± 1.15 [a]	20.00 ± 2.65 [a]
O. gratissimum	19.33 ± 2.08 [a]	18.00 ± 1.73 [a]	12.67 ± 0.58 [b]	9.67 ± 1.53 [c]	17.67 ± 3.05 [a]
Gentamycin (+ve control)	26.67 ± 0.58	25.33 ± 1.53	25.33 ± 1.53	25.33 ± 1.53	25.33 ± 1.53
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	chloroform				
C. macrostachyus	16.67 ± 2.52 [a]	15.68 ± 0.58 [a]	10.67 ± 0.58 [b]	10.00 ± 1.00 [c]	19.67 ± 1.15 [d]
C. aurea	17.67 ± 2.31 [a]	17.33 ± 2.52 [a]	15.00 ± 1.00 [a]	12.33 ± 2.08 [b]	17.00 ± 1.00 [a]
O. gratissimum	15.67 ± 1.53 [a]	16.67 ± 1.53 [a]	13.33 ± 1.15 [a]	9.67 ± 2.08 [b]	18.00 ± 1.00 [a]
Gentamycin (+ve control)	24.00 ± 1.00	25.33 ± 0.58	25.33 ± 0.58	25.33 ± 0.58	25.33 ± 0.58
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

CmCa: *C. macrostachyus* and *C. aurea* combination; CmOg: *C. macrostachyus* and *O. gratissimum* combination; CmCaOg: *C. macrostachyus, C. aurea* and *O. gratissimum* combination; different superscripts in the same row indicate significantly different effects of extracts against respective bacteria; different superscripts in the same column indicate significantly different effects of extracts on the test bacteria.

***Table 2.** Antibacterial activity of combined extracts. The table shows the mean inhibition zones of combined effects against tested test bacteria.*

Extract	Inhibition Zone on Test Organism (mm) (Mean ± Standard Deviation)				
	S. aureus ATCC43300	*S. aureus* (Clinical)	*E. coli* ATCC35218	*E. coli* (Clinical)	*S. typhi* ATCC1333
	Methanol				
CmCa	19.00 ± 1.00 [a]	16.67 ± 3.055 [a]	18.33 ± 1.53 [a]	17.67 ± 0.58 [a]	21.33 ± 2.08 [a]
CmOg	22.67 ± 1.15 [a]	18.00 ± 2.00 [b]	16.00 ± 1.00 [c]	19.00 ± 1.00 [d]	22.33 ± 1.53 [a]
CaOg	19.33 ± 0.58 [a]	17.33 ± 4.16 [a]	17.67 ± 2.08 [a]	15.00 ± 1.00 [b]	23.00 ± 1.00 [a]
CmCaOg	20.67 ± 2.52 [a]	19.00 ± 2.65 [a]	17.67 ± 1.15 [b]	14.67 ± 0.58 [a]	23.00 ± 1.00 [a]
Gentamycin (+ve control)	25.67 ± 2.08	25.00 ± 1.00	25.00 ± 1.00	25.00 ± 1.00	25.00 ± 1.00
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	acetone				
CmCa	18.33 ± 2.08 [a]	17.33 ± 4.16 [a]	17.33 ± 0.58 [a]	18.00 ± 1.00 [a]	19.00 ± 1.00 [a]
CmOg	18.00 ± 1.00 [a]	17.33 ± 2.31 [a]	16.67 ± 1.53 [b]	14.33 ± 0.58 [c]	22.67 ± 0.58 [a]
CaOg	19.00 ± 3.61 [a]	19.00 ± 3.46 [a]	16.00 ± 1.72 [b]	14.33 ± 1.53 [a]	21.33 ± 1.15 [a]
CmCaOg	19.33 ± 2.52 [a]	19.67 ± 1.53 [b]	18.33 ± 2.08 [b]	17.00 ± 1.73 [a]	22.33 ± 1.15 [a]
Gentamycin (+ve control)	25.67 ± 0.58	25.67 ± 2.08	25.67 ± 2.08	25.67 ± 2.08	25.67 ± 2.08
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Extract	Inhibition Zone on Test Organism (mm) (Mean ± Standard Deviation)				
	S. aureus ATCC43300	S. aureus (Clinical)	E. coli ATCC35218	E. coli (Clinical)	S. typhi ATCC1333
	chloroform				
CmCa	20.00 ± 1.00 [a]	17.00 ± 1.00 [a]	20.67 ± 2.89 [ab]	18.33 ± 1.53 [ac]	20.67 ± 2.08 [a]
CmOg	23.00 ± 1.00 [a]	16.67 ± 3.15 [b]	20.00 ± 2.65 [c]	16.67 ± 0.58 [d]	24.00 ± 1.00 [a]
CaOg	19.67 ± 0.58 [a]	18.33 ± 3.05 [a]	16.67 ± 0.58 [a]	17.33 ± 1.53 [a]	20.67 ± 3.21 [a]
CmCaOg	21.33 ± 3.05 [a]	20.33 ± 2.89 [a]	18.33 ± 2.08 [a]	16.00 ± 1.00 [b]	21.00 ± 2.65 [a]
Gentamycin (+ve control)	25.67 ± 1.53	26.00 ± 1.00	26.00 ± 1.00	26.00 ± 1.00	26.00 ± 1.00
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

CmCa: *C. macrostachyus* and *C. aurea* combination; CmOg: *C. macrostachyus* and *O. gratissimum* combination; CmCaOg: *C. macrostachyus*, *C. aurea* and *O. gratissimum* combination; different superscripts in the same row indicate significantly different effects of extracts against respective bacteria; different superscripts in the same column indicate significantly different effects of extracts on the test bacteria.

The activity of the tested combinations of extracts was not found to be significantly different ($p > 0.05$) against test bacteria, as indicated by the same superscripts in the row of each extract shown in Table 2. Accordingly, the combined effect of the methanol extracts of *C. macrostachyus* and *C. aurea* was significantly higher against *S. typhi* ATCC1333 than that on clinical isolates of *S. aureus* ($p = 0.012$). With the same solvent, the effect of *C. macrostachyus* combined with *O. gratissimum* was significantly higher ($p = 0.008$) against *S. aureus* ATCC43300 than clinical isolates of *E. coli* and lower ($p = 0.002$) against *S. typhi* ATCC1333. The *C. aurea* and *O. gratissimum* extract combination was significantly less effective against clinical isolates of *E. coli* ($p = 0.046$) compared with all other test bacteria when using chloroform as the solvent. The combination of all three crude extracts was found to have significantly lower effects against clinical isolates of *E. coli* ($p = 0.025$) compared with all other bacterial isolates. The lowered activity of the combination compared with any of the individual components may have been due to phytochemical properties, as one may have biochemically neutralized the other(s) and hence diminished the effect.

Acetone extract combinations showed no significant differences among most tested bacteria except for the combination of *C. macrostachyus* and *O. gratissimum,* which showed significantly higher activity against *S. typhi* ATCC1333 compared with clinical isolates of *E. coli* ($p = 0.025$) and *E. coli* ATCC35218 ($p = 0.006$). The combination of *C. aurea* and *O. gratissimum* extracts showed significantly lower effects against clinical isolates of *E. coli* ($p = 0.046$) than both standard and clinical isolates of *S. aureus* and significantly higher effects against *S. typhi* ATCC1333 than clinical isolates of *E. coli* (0.006) and *E. coli* ATCC35218 (0.025). The combination of the three crude extracts was found to have significantly stronger effect against *S. typhi* ATCC1333 than *E. coli* ATCC35218 ($p = 0.025$) and clinical isolates of *E. coli* ($p = 0.006$) than that of each extract alone. No significant differences in the susceptibility of the test bacteria were recorded for the combination of *C. macrostachyus* and *C. aurea* with acetone. It is possible that such variations in the potency of the same combination on the same bacterial isolates may have been related to the variation in the chemical nature and the number of phytochemicals of antibacterial importance with different extraction solvents.

Relatively significant difference in susceptibility were observed in combinations of extracts using chloroform, e.g., the effect of the *C. macrostachyus* with *C. aurea* combination was significantly lower against *S. aureus* ATCC43300 than *S. typhi* ATCC1333 but higher against clinical isolates of *E. coli* ($p = 0.035$) in both cases. Significant variations were observed in the combination of *C. macrostachyus* with *O. gratissimum,* as the effect of the combination was significantly lower against clinical isolates *E. coli* compared with that against *E. coli* ATCC35218 ($p = 0.023$) and standard isolates of *S. aureus* and *S. typhi* ($p = 0.00$) in both bacterial isolates. The combination of *C. aurea* with *O. gratissimum* showed no statistically significant difference in activity against the test bacteria. However, the combination of three crude extracts had a significantly different effect against clinical isolates of *E. coli,* which was lower than that against standard isolates of *S. aureus* ($p = 0.024$) and *S. typhi* ($p = 0.031$). The differences among most bacteria in susceptibility to extracts may have been due to the different biological characteristics of the test bacteria; especially in cases of clinical versus standard isolates, bacteria can develop an increased resistance when clinically circulated in an environment.

3.2. MIC and MBC of Extracts Alone and in Combination on Test Bacteria

The MIC and MBC of the combination of *C. macrostachyus* and *C. aurea* were as low as 3.125 mg/mL and 6.25 mg/mL, respectively, for *S. typhi* ATCC1333 compared with *S. aureus* ATCC43300 and *E. coli* ATCC35218 and clinical *E. coli* (6.25 mg/mL and 12.5 mg/mL, respectively) and clinical *S. aureus* (12.5 mg/mL and 25 mg/mL, respectively). Very low MIC and MBC values of 6.25 mg/mL and 12.5 mg/mL, respectively, were also found for the combination of *C. macrostachyus* with *O. gratissimum* against *S. aureus* ATCC43300 with methanol and chloroform as solvents; in comparison, MIC and MBC values of 25 mg/mL are required in clinical isolates of *E. coli*. Combinations were both inhibitory and bactericidal at low concentrations compared with their component parts, indicating that their combined use was relatively advantageous (Figures 1–4).
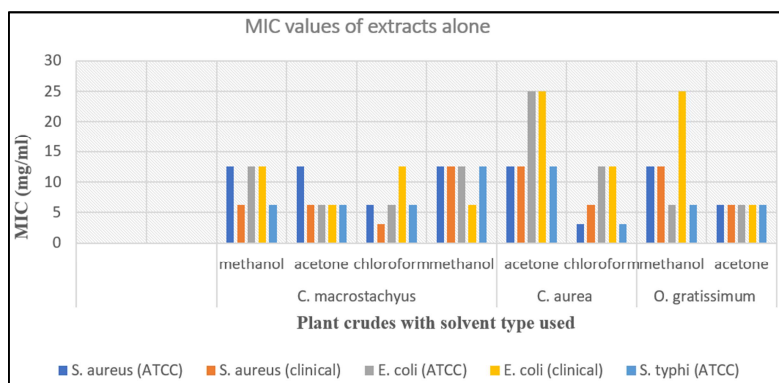
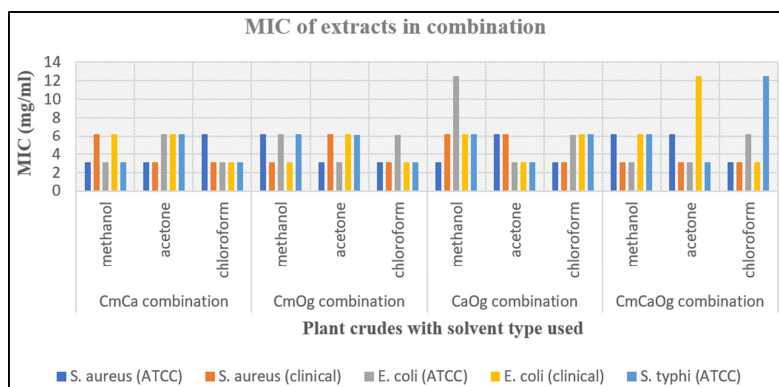**Figure 1.** *MIC values of extracts alone with different solvents on test bacteria.*

Figure 2. *MIC values of combined extracts with different solvents on test bacteria. CmCa: C. macrostachyus and C. aurea combination; CmOg: C. macrostachyus and O. gratissimum combination; CmCaOg: C. macrostachyus, C. aurea and O. gratissimum combination.*
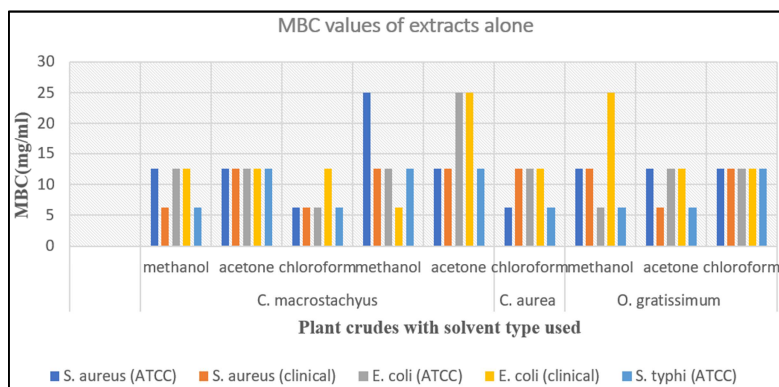

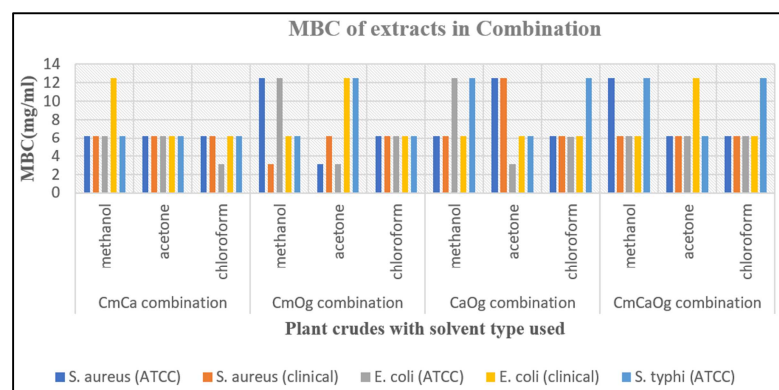**Figure 3.** *MBC values of extracts alone with different solvents on test bacteria.*

Figure 4. *MBC values of combined extracts with different solvents on test bacteria. CmCa: C. macrostachyus and C. aurea combination; CmOg: C. macrostachyus and O. gratissimum combination; CmCaOg: C. macrostachyus, C. aurea and O. gratissimum combination.*

The combination of *C. aurea* with *O. gratissimum* also produced MIC and MBC values as low as 3.125 mg/mL and 6.25 mg/mL, respectively, for both standard and clinical isolates of *S. aureus* with chloroform as the solvent and clinical isolates of *E. coli* with acetone as the solvent. The combination of three crude extracts showed an MIC of 3.125 mg/mL only in the case of *S. aureus* ATCC43300 with chloroform. The same MIC and MBC values were found for clinical isolates of *S. aureus* (12.5 mg/mL), both clinical and standard isolates of *E. coli* (6.25 mg/mL), and standard isolates of *S. typhi* (25 mg/mL). These results indicate that the extracts were inhibitory and killer at the same concentration.

3.3. Fractional Inhibitory Concentration Index (FICI) Determination

The ultimate goal of this work was to evaluate the synergetic effect of crude extracts in limited combinations such as *C. macrostachyus* with *C. aurea* against *S. aureus* ATCC43300 (FICI = 0.5) (Table 3), methanol extracts of the same combination against *E. coli* ATCC35218 (FICI = 0.5), the combination of methanol (FICI = 0.375) and chloroform (FICI = 0.5) extracts of *C. macrostachyus* with *O. gratissimum* against clinical *E. coli* in both cases, and the methanol extract combination of *C. aurea* with *O. gratissimum* against *S. aureus* ATCC43300 (FICI = 0.375). Most other combinations were additive and indifferent in effect against the test bacteria. However, the combination of all three crude extracts was found to be antagonistic against clinical isolates of *E. coli* (FICI = 4.5) with acetone as the extraction solvent. Such a lowered effect could be attributed to neutralizing actions of components when combined, as different phytochemicals from each may not share the same chemical nature. Therefore, one phytochemical may have neutralized others, thus lowering the overall effect.

Table 3. FICI values of different extract combinations. The table shows the different effects the combinations produced against test bacteria.

Extract	Solvent	Test Bacteria and FICI within Each Bacterium				
		S. aureus ATCC43300	*S. aureus* (Clinical)	*E. coli* ATCC35218	*E. coli* (Clinical)	*S. typhi* ATCC1333
CmCa	meth	0.75(Ad)	2(I)	0.5(S)	1(Ad)	1(Ad)
	acet	0.5(S)	0.75(Ad)	1.5(I)	1.25(I)	1.5(I)
	chlor	3(I)	1.5(I)	0.75(Ad)	0.75(Ad)	1.5(I)
CmOg	meth	1(Ad)	0.75(Ad)	1.5 (I)	0.375(S)	1(Ad)
	acet	0.75(Ad)	2 (I)	1(Ad)	2(1)	2(I)
	chlor	1(Ad)	1.5 (I)	1.5 (I)	0.5(S)	0.75(Ad)
CaOg	meth	0.375(s)	1(Ad)	3 (I)	1.25 (I)	1.5(I)
	acet	1.5 (I)	1.25 (I)	1(Ad)	0.625(Ad)	0.75(Ad)
	chlor	1.5 (I)	1(Ad)	1(Ad)	1(Ad)	2.5(I)
CmCaOg	meth	1.25 (I)	1.5 (I)	1(Ad)	1.75(I)	1.25(I)
	acet	2 (I)	1.25 (I)	1.5 (I)	4.5(AN)	1.25(I)
	chlor	2 (I)	2 (I)	2 (I)	0.75(Ad)	3.5(I)

CmCa: *C. macrostachyus* and *C. aurea* combination; CmOg: *C. macrostachyus* and *O. gratissimum* combination; CmCaOg: *C. macrostachyus, C. aurea* and *O. gratissimum* combination; S: synergy; Ad: additive; An: antagonistic; I: indifferent; meth: methanol; acet: acetone; chlor: chloroform.

4. Conclusions and Recommendations

This study revealed synergistic activity in some combinations of medicinal plant extracts, though many of the effects were shown to be additive. Compared with those of individual components, the effects of most combination options were amplified. These results can provide support to local communities attempting to heal different ailments in daily life. These findings may also be a source of data regarding the pharmacological properties of plants when combined and used, thus linking ethnopharmacological and traditional medicinal knowledge of plants as valuable sources of new, biologically active molecules possessing antibacterial properties. Ultimately, the results of this study are important for the development of a new generation of standardized and effective antibacterial preparations. Following this conclusion, the verification of the bacteriological causes of ailments against which local communities use the crude plant extracts discussed in this research, the in vivo confirmation of the extracts' best level of activity and bioavailability, and further research regarding best combination ratios of component parts need to conducted.

Abbreviations

ANOVA	Analysis of Variance
DMSO	Dimethyl Sulfoxide
FICI	Fractional Inhibition Concentration Index
MIC	Minimum Bacterial Inhibition Concentration
MBC	Minimum Bactericidal Concentration
TM	Traditional Medicine

Author Contributions

A. T. prepared the whole document including the figures and tables; N. B. and W. S. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

There are no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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