

Synergistic Antibacterial Activity of Ethanolic Extracts of *Mondiawhytei* roots and *Physalis peruviana* leaves against *S. aureus* and *E. coli*

ABSTRACT

Aims: This work investigated the potential synergistic antibacterial activity of ethanolic extracts of *Mondiawhytei* roots and *Physalis peruviana* leaves.

Study design: This study employed both qualitative and quantitative research designs.

Place and duration of study: All research works were performed at the Central Science Laboratories, Faculty of science, Kyambogo University between September 2021 and March 2022.

Methodology: Standard methods were followed for phytochemical screening of ethanolic extracts of *Mondiawhytei* roots, and *Physalis peruviana* leaves. The antibacterial activity and potential synergistic activity of ethanolic extracts were evaluated through agar well diffusion assay. The bacteria strains were cultured on Mueller-Hinton agar. Sterile cork borer (6 mm diameter) was used to create the wells on petri discs and filled aseptically with 10 μ L of the test extract (100 mg/mL) and Chloramphenicol (0.5 mg/ml) was used as the positive control. The interaction between the extracts and the combined extract was assessed by determining their fractional inhibitory concentration (FIC) index.

Results: Alkaloids, flavonoids, tannins, saponins, phenols were found to be present in both extracts. Cardiac glycosides, terpenoids, anthraquinones and steroids were present only in ethanolic extract of *Physalis peruviana* leaves. The antibacterial activities of *Mondiawhytei* root extract, *Physalis peruviana* leaves extract and combined extract were obtained with the following zone of inhibitions; 19.20 ± 0.72 mm, 19.77 ± 0.60 mm, 25.93 ± 0.83 mm for *S. aureus* and 15.50 ± 0.46 mm, 15.00 ± 0.26 mm, 17.87 ± 0.61 mm for *E. coli* respectively, as compared with the standard reference antibiotic with zone of inhibitions; 26.00 ± 0.00 mm for *S. aureus* and 24.00 ± 0.00 mm *E. coli*. The MICs were determined at 0.1-100 mg/ml, and the FIC index were obtained from the evaluated MIC values with the corresponding values of 0.5 (synergistic) for *S. aureus*

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and 1.0 (additive) for *E.coli*.

Conclusion:

The results obtained from the present study support the traditional claims of using the selected plants to treat bacterial infections. The extracts had low antibacterial activities on the selected human pathogens, Hence, if these extracts are used in unison, greater efficacy could be achieved; further studies should be done using combined extracts with the commercial antibiotics.

Keywords: **Phytochemical analysis, Synergistic activity, Antibacterial activity, *Mondiawhytei*, and *Physalis peruviana***

1. INTRODUCTION

Bacteria resistant to the available used antibiotics cause one of the greatest significant global health problems on both developed and developing countries due to factors such as inadequate sanitation, poor hygiene and overcrowded living conditions [1, 2]. With an escalating number of antibiotics resistance to high levels, there is a need to search for an alternative to antibiotics that may be easily available, and provides efficient and cheap alternatives to antibiotics.

Medicinal plants are rich in a wide variety of chemical compounds, which have been found *in vitro* to have antimicrobial, antioxidant, antidiarrheal, anticancer, and antimalarial agents [3-5]. The active ingredients present in plants which possess its medicinal properties act as a microbial control agents, especially on bacteria strains [6, 7]. A number of plants have been found to have antimicrobial properties as a result of biosynthesis of the secondary metabolites in the presence of their corresponding enzymes.

Physalis peruviana L. belongs to the family Solanaceae, its common names are cape goose berry (English), Ogwal Kongo (Acholi) and Aduduma (Ateso), is an herbaceous, perennial plant and a very popular plant in Uganda, largely consumed by both urban and rural dwellers [8, 9]. This plant is a native to Latin America but has been grown widely in tropical, subtropical, and also in temperate regions of the world. The plant is useful for income, food, and medicinal uses [10-12]. The fruits of *P. peruviana* are described as tomato-like in flavour (sweet and sour) which are hidden in an inflated calyx protecting it from insects, birds, diseases and harsh climatic conditions [13]. The ethnopharmacological properties of *Physalis peruviana* are antimalarial, antibacterial, anticancer, and also used in the treatment of asthma [14, 15].

Mondiawhytei (Hook.F.) Skeels belongs to the family Ascepiadaceae and is also known as Mondia, or White ginger (English), Lurono (Acholi), Emulondo (Ateso) [8, 9]. Mondia is a vigorous climber (3-6 m high) with attractive heart-shaped leaves and a vanilla aroma, the main threat to the species has been over exploitation by local communities for subsistence and commercial purposes. A root decoction of *M. whytei* is documented to induce labour in Uganda [16]. In Kenya, a survey showed that *M. whytei* is practically used for many problems, for example; ringworms, skin diseases, stomach worms, heart diseases and asthma [17]. It was reported in South Africa by Stafford et al. [18], that *M. whytei* act as a stimulant for appetite, as an aphrodisiac, and for treatment of fits in children. This study was done to evaluate the effect of ethanolic extracts of *M. whytei* roots and *P. peruviana* leaves, their combined extract and compare the synergistic activity with a standard antibiotic.

2. MATERIAL AND METHODS

2.1 Collection and identification of Plant materials

The whole plant of *M. whytei* were collected from Lamogo-ongu Forest Reserve in Lamwo district Headquarter along Padibe-Lokung road, Northern Uganda and the leaves of *P. peruviana* were collected in the month of September 2021

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Comment [U4]: Before you unfold your plants, provide some studies related to medicinal plants to have a smooth transition of ideas which is commonly used in your region.

Elaborate why just roots for *M. whytei* and leaves for *P. peruviana* L.

Comment [U5]: Add some readings on phytochemical (from the medicinal plants) that has antimicrobial properties to strengthen your claims.

Add related studies as well on synergistic activities of selected plants.

Comment [U6]: you may add images of the plants.

from Kkingo sub-county, Masaka district in central Uganda from the wild. The plant material was identified and authenticated by a taxonomist at Department of Botany, Makerere University, Uganda with a Voucher number (PO-002 and PO-003) and deposited at Makerere University Herbarium. The root sample of *M. whytei* and the leave sample of *P. peruviana* were dried at room temperature for four weeks. The samples were ground into powdered and kept in air tight bottled.

2.2 Preparation of Plant extracts

The dried powdered of each sample (200 g) were weighed and cold macerated with 80% ethanol with occasional shaking for 48 hrs. The extract was filtered with cotton wool followed by Whatman No.1 filter paper and concentrated to dryness at 40 °C using a rotary evaporator and kept at 4°C for further analyses.

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2.3 Phytochemical Analyses

Phytochemical screening of the resultant ethanolic extracts of *M. whytei* and *P. peruviana* were done following standard methods [19].

Test for alkaloids

To 3ml of the extract, 2ml of 1% Hydrochloric acid was added and steamed. Four drops of Wagner's reagent was then added and left to stand for 5 minutes. Reddish or brown precipitate indicated the presence of alkaloids.

Test for cardiac glycosides

Glacial acetic acid (2ml) was added to 5ml of the extract into a clean test tube followed by 3 drops of iron (iii) chloride solution and finally 1ml of concentrated sulphuric acid. The presence of glycosides is indicated by a brown ring at the interface.

Test for flavonoids

To 2ml of extracts in the test tube was added a few drops of 2% sodium hydroxide solution. An intense yellow colour which turned colourless on addition of a few drops of hydrochloric acid confirmed the presence of flavonoids.

Test for Terpenoids

To 2ml of extract, 1ml of chloroform was added followed by 2-3 drops of concentrated sulphuric acid. The immediate production of a reddish brown precipitate at the interface indicated the presence of Terpenoids.

Test for tannins

In a test tube containing 2ml of extract, 2ml distilled water was added to the resultant solution followed by 3ml of 10% alcoholic iron (iii) chloride solution. Formation of blue or greenish precipitate indicated the presence of tannin.

Test for Saponins

To 2ml of extract in a clean test tube, 6ml of distilled water was added and the mixture shaken vigorously until persistent foam obtained which confirmed the presence of Saponins.

Test for Phenols

To 5ml of extract, 3 drops of 5% iron (iii) chloride was added. Formation of a deep blue, green black colour indicated the presence of phenol.

Test for Anthraquinones

To 2ml extract in a test tube was added a few drops of concentrated hydrochloric acid. Formation of green precipitate showed the presence of anthraquinones.

Test for Steroids

A volume of 2ml Chloroform was added to 5ml of the extract (5ml) in a test tube followed by 2mL of conc. Sulphuric acid. A red colouration in the lower chloroform layer indicated the presence of steroids.

2.4 Bacteria

The bacterial strains used for the study included *Staphylococcus aureus* and *Escherichia coli* which were clinically isolated.

2.5 In vitro Antibacterial Assay

Evaluation of antibacterial activity of the ethanolic extracts of MW, PP and the combined extract of MWPP were completed by the agar well diffusion method. As Micicet *et al.*, [20], reported in detail, the nutrient medium (Müller– Hinton agar) was inoculated with bacterial suspensions (approx. 6 log CFU/mL) onto three sterile petri discs. Wells were then bored into the agar medium with flame sterilized cork borer (6 mm). The wells were filled with 100 µL concentration of ethanolic extracts of *M. whytei*; MW and *P. peruviana*; PP, their combined extract and antibiotic (chloramphenicol). The petri discs were allowed to stand for few minutes (approximately 30 minutes) before incubation at 37°C for 24 hours as a condition suitable for bacterial growth. After 24 h of incubation, the petri discs were examined for zone of inhibition. The diameter of the zone of inhibition formed by the ethanolic extracts of *M. whytei*; MW and *P. peruviana*; PP, their combined extract and Chloramphenicol were measured in triplicates and interpreted as follows: sensitive (diameter of inhibition zone above 26 mm), intermediary (inhibition zone 22–26 mm), and resistance (inhibition zone below 22 mm) as described by CLSI zone diameter interpretative standards [21]. As a negative control, DMSO was used, while positive controls was commercially available antibiotics; chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA).

Comment [U8]: Before you use coding, introduced first as *M. whyte*(MW) or *P. peruviana* (PP).

2.6 Minimum Inhibitory Concentration (MIC)

The MIC was evaluated for the two selected bacteria using the broth microdilution method labelled by Omara *et al.*[22]. The initial concentration was defined as 100%, while other concentrations were prepared using successive dilutions (0.1-100) µg/mL using Dimethyl sulfoxide (50 mg/mL) for all the samples and the concentration for the reference Antibiotic was 500 µg/mL. To determine their combinatorial effects, the extracts with concentration of 50:50 (v/v) were combined. The tubes were inoculated with 15 µL of each of the bacterial strains. The used solvent was inert for all tested bacteria and with no biocidal effect on bacterial. MIC represents the lowest concentration of antibacterial agents that, under defined *in vitro* conditions, prevents the appearance of visible growth of bacteria within a defined period of time.

2.7 In vitro synergistic activity

Measured weight (5 g) of each extract was dissolved in 20 ml of DMSO. For determination of potential synergistic activity, combination of ethanolic extracts (*M. whytei*; MW and *P. peruviana*; PP) was prepared by mixing 5 ml of each extract, and the same procedure was followed for detection antibacterial activity of individual extract [23, 24].

2.8 Fractional inhibitory concentration (FIC)

In vitro interactions between antibacterial agents were determined and quantified by calculating the fractional inhibitory concentration (FIC) index following a method described by Aberu *et al.* [25], using the following formula:

$$\text{FIC index} = \frac{\text{MIC of MW extract in combination}}{\text{MIC of MW extract alone}} + \frac{\text{MIC of PP extract in combination}}{\text{MIC of PP extract alone}}$$

Interpretations of the FIC index (FIC_I) are shown in Table 1 and Figure 1.

Table 1: Recommended fractional inhibitory concentration ($\sum FIC$) indices for assigning combination effects [26].

Combination effect	($\sum FIC$) range
Synergy	$\sum FIC \leq 0.5$
Additive	$0.5 < \sum FIC \leq 1.0$
Indifference	$1.0 < \sum FIC \leq 4.0$
Antagonism	$4.0 < \sum FIC$

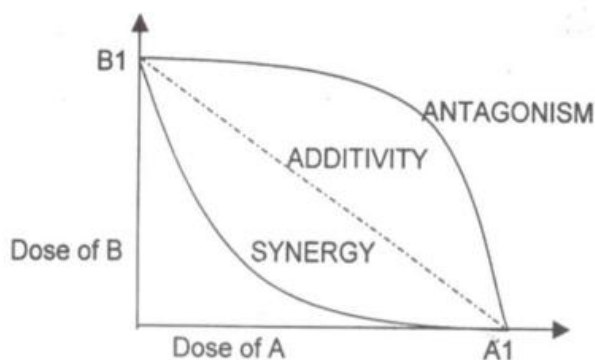


Fig 1: Isobologram illustrating Synergy, Additivity, and Antagonism [27].

A1 and B1 are the doses of the constituents A and B respectively, which produce an equal effect. The concave up isobole represents synergy. The concave down isobole represents antagonism.

The action of antibacterial agents is considered to be:

- Synergistic if their joint effect is stronger than the sum of effects of the individual agents;
- Additive if their joint effect is equal to the sum of the effects of the individual agents;
- Indifferent if their joint effect is equal to the effect of either individual agent;
- Antagonistic if their joint effect is weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent [28].

When more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC index was expressed as the average of the FIC values.

2.6 Data Analysis

Each experiment was done in triplicates and results are expressed as the mean \pm standard deviation (SD). The statistical analysis was performed by one way ANOVA test followed by t-test. The p -values were obtained statistically significant at ≤ 0.05 .

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

Preliminary phytochemical screening of ethanolic extracts of both *M. whytei* roots and *P. peruviana* leaves revealed that alkaloids, flavonoids, tannins, saponins and phenols were the major secondary metabolites. Meanwhile, cardiac glycosides, terpenoids, anthraquinones, and steroids were present only in ethanolic extract of *P. peruviana* leaves (Table 2).

Table 2: Phytochemicals identified in ethanolic extracts of *M. whytei* roots and *P. peruviana* leaves

Phytochemicals	Deductions	
	Ethanollic MW Extract	Ethanollic PP Extract
Alkaloids	+	+
Cardiac Glycosides	-	+
Flavonoids	+	+
Terpenoids	-	+
Tannins	+	+
Saponins	+	+
Phenols	+	+
Anthraquinones	-	+
Steroids	-	+

MW = *Mondiawhyte* roots, and **PP** = *Physalis peruviana*, (-) = Absent, (+) = Present

The findings are in agreement with the previous studies showing that *P. peruviana* contains secondary metabolites; alkaloids, flavonoids, tannins, saponins, phenols, cardiac glycosides, terpenoids, and steroids [29, 30], except for anthraquinones where we are reporting for its presence in *P. peruviana* leave extracts for the first time. Previous studies showed that many bioactive compounds have been isolated from the aerial parts of *P. peruviana*, such as phenolics, ticolidine, phytosterols [31], and various withanolides [32].

3.2 In vitro antibacterial activity

The ethanolic extracts of both *M. whyte* roots and *P. peruviana* leaves were found to be susceptible to the two selected microorganisms; *S. aureus* and *E. coli*. The combined ethanolic extract (MWPP) exhibited the highest (intermediate) zone of inhibition (25.93 ± 0.83) mm and (17.87 ± 0.61) mm against *S. aureus* and *E. coli*, respectively. But, for individual ethanolic extract, *P. peruviana* leaves extract exhibited the highest zone of inhibition (19.77 ± 0.60) mm and *M. whyte* roots extract exhibited the lowest zone of inhibition (19.20 ± 0.72) mm against a Gram positive microorganism (*S. aureus*) (Table 3). Meanwhile, *M. whyte* roots extract exhibited the highest zone of inhibition (15.50 ± 0.46) mm and *P. peruviana* leaves extract exhibited the lowest zone of inhibition (15.00 ± 0.26) mm against a Gram negative microorganism (*E. coli*) (Table 3).

Table 3: Antibacterial activity of the ethanolic extracts and the combined extract against two selected microorganisms

Extracts	Diameter of zone of inhibitions (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
Ethanolic MW	19.20 ± 0.72 (R)	15.50 ± 0.46 (R)
Ethanolic PP	19.77 ± 0.60 (R)	15.00 ± 0.26 (R)
MWPP	25.93 ± 0.83 (I)	17.87 ± 0.61 (R)
DSMO	0.00 ± 0.00	0.00 ± 0.00
Chloramphenicol	26.00 ± 0.00 (S)	24.00 ± 0.00 (I)

S = Sensitive, **I** = Intermediate, **R** = Resistant, **MW** = *Mondiawhyte* roots extract, **PP** = *Physalis peruviana* leaves extract, **MWPP** = combined extract of *Mondiawhyte* and *Physalis peruviana*, **DSMO** = Dimethyl sulfoxide

The results of the antibacterial activity obtained showed that the extracts were more active against Gram positive bacteria (*S. aureus*) but less active against Gram negative bacteria (*E. coli*), since previous studies showed that polar solvent extracts (ethanol, ethyl acetate, and acetone) posed higher biological activities [33, 34]. These could ideally be as a result of some secondary metabolites (like phenols) presence in the extracts which crossed or damaged the bacterial cell membrane, since Gram positive bacteria lacks outer membrane which aids easy penetration of the hydrophobic molecules, unlike Gram negative bacteria which bear cell wall acting as a barrier to stop the hydrophobic molecules to diffused into the cell [35, 36]. As reported by Kovacevi, [37]. Alkaloids from medicinal plants have the ability to intercalate with DNA by interrupting the enzymes activity. Anthraquinones and flavonoids have been reported to inhibit Nucleic acid synthesis and energy metabolism as well as cell wall interruptions and cell membrane synthesis [4, 38]. The values of zone of inhibition of the combined extract showed relatively similar values with that of the reference antibiotic (Chloramphenicol). In a previous study by Junio *et al.* [39], reported that some flavonoids and alkaloids from *Hydrastis canadensis* roots were synergistically tested against *S. aureus* with observed MICs of $75 \mu\text{g/mL}$. Since, the crude ethanolic extract of *P. peruviana* showed the highest sensitivity to zone of inhibition on *S. aureus*, and the crude extract

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from *M. whytei* showed highest sensitivity to zone of inhibition, they were considered for further tests and their Minimum Inhibitory Concentration (MIC) against each of the bacterial strains were investigated.

3.3 Minimum inhibitory concentration (mg/ml) and Fractional inhibitory concentration

As the bacterial activity of ethanolic extract of both *M. whyteiroots* and *P. peruviana* leaves on the two selected pathogens were assessed by agar well diffusion method, the required minimum concentration to inhibit bacterial growth were done by determining the MICs for each extracts, their combined extract and the antibiotic and the results are recorded in **Table 4**. And the impact of combining the extracts on the FIC index which produced synergistic interaction as shown in **Table 4**. The efficacy of each extracts on *S. aureus* and *E. coli* was identified as approximately equivalent to a value of 100 ± 0.00 $\mu\text{g/mL}$, but in the synergistic combination of the two extracts, the FIC was a maximum at 25 $\mu\text{g/mL}$.

Table 4: Minimum inhibitory concentration (mg/ml) and Fractional inhibitory concentration of the ethanolic extracts and the combined extract against two selected micro organisms

Extracts	<i>S. aureus</i>			<i>E. coli</i>		
	MIC ($\mu\text{g/mL}$)	FIC value	Interference	MIC ($\mu\text{g/mL}$)	FIC value	Interference
Ethanolic MW	100	NA	NA	100	NA	NA
Ethanolic PP	100	NA	NA	NIO	NA	NA
MWPP	25	0.5	Synergistic	100	1	Additive
Chloramphenicol	500	NA	NA	>1000	NA	NA

NIO= No inhibition observed; **NA** = Not applicable

Extracts synergy has been known and used for a long time such as in the traditional herbal formulation due to the diverse small molecules produced by plants. Although, most of these small molecules possess weak antibiotic activity, they are considered successful through their synergistic mechanisms [40, 41]. Even though the synergistic mechanisms in combined antibacterial agents are interesting, it may be due to a combination impacts instead of a single effect, decreased activeness of the bacterial isolates and increased activity of antibacterial components at the target sites [42-44], synergistic multi-target effects [45].

4. CONCLUSION

This study contributes to the knowledge of the presence of different bioactive compounds in *M. whyteiroots* and *P. peruviana* leaves extract possessing broad spectrum antibacterial efficacy. Although, the multitude of studies on the synergistic interferences of natural compounds and their combinations to treat different human pathogenic bacterial infections, few studies have focused on using this attractive approach in the treatment of the different bacterial infections. Both *M. whyteiroots* and *P. peruviana* leaves showed antibacterial activity against common human pathogens; *S. aureus* and *E. coli*, and the MWPP combined extract showed higher antibacterial activity. Overall, the antibacterial activity of the MWPP combined extract against the selected microorganisms could represent a promising new modes of treatment for disease-associated with infections caused by *S. aureus* and *E. coli*, especially patients with difficult curable infections, due to the increased antibacterial efficacy with negligible resistance to bacterial pathogens.

Comment [U11]: This is well written conclusion. Overall, this study is promising and the thoughts are well organized and re

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