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In-vitro anti-salmonella activity of methanol and aqueous extracts and their associations of *Psidium guajava* and *Carica papaya* leaves

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Abstract

Background: Typhoid fever is a major public health problem and endemic especially in some developing countries. The present study was aimed at determining the anti-salmonella potentials of *Psidium guajava* (PG) and *Carica papaya* (CP) leaves extract association *in-vitro.*

Methods: The plant materials were extracted both in methanol and water separately. The methanol extract was concentrated using a rota-evaporator while the aqueous was dried in an oven. Phytochemical screening was done for the presence of alkaloids, phenols, flavonoids, steroids, triterpenoids, tannins, saponins, anthocyanins, and anthraquinons. The *Salmonella* strains were screened for susceptibility to *P. guajava* and *C. papaya* extracts using micro well dilution checkerboard test with colorimetric para-iodonitrotetrazolium chloride (INT) as revelator to obtain the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC).

Results: The results obtained showed the presence of alkaloids, phenols, flavonoids, steroids, triterpenoids, tannins, saponins, and anthraquinons secondary metabolites. The MIC of the methanol extract was 64 µg/mL for CP+PG and its MBC was 128 µg/mL. CP methanol extracts showed higher salmonella activities than PG methanol extracts with the most sensitive *Salmonella typhi* isolate S66 exhibiting the lowest MIC of 64 µg/mL. Synergic effects were observed with the association of CP + PG in 4 of the 10 salmonella studied strains/isolates. The FIC_{CP} was lower than FIC_{PG} in all the studied strains/isolates.

Conclusion: This study demonstrated that the CP methanol extract used in single or in association with PG could be used to develop phytomedicines against salmonella infections which are natural, readily available to all citizens, cheap, non-toxic with minimal side effects but contain active ingredients which can be used in the pre-formulation of phytodrug to fight against typhoid in our community.

Keywords: Edible plants; anti-salmonella activity; phytochemical; extract combination; MIC and MBC.

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Background

Typhoid fever is a major public health problem, especially in developing countries where they are endemic [1]. It is considered the second leading death cause in children under five years old (responsible for killing about 525 000 children every year) and is the fourth most common infectious disease globally [2]. *Salmonella enteritica* serotype typhi (*S. typhi*) causes typhoid fever and is responsible for an estimated 9,000,000 cases and 110,000 deaths globally per annum. Typhoid fever is a threat to many tropical countries showing a worldwide estimate of about 212 million cases with 129,000 deaths yearly with children and young adults being the vulnerable groups [3]. The high morbidity and mortality of typhoid fever in low and middle-income countries reinforce the need for an integrated control approach which may ultimately lead to its elimination in the 21st century [4]. The global estimate of invasive non-typhoidal salmonella is about 3.4 million cases and the most vulnerable groups (1.9 million cases and 380,000 deaths) are children and young adults in sub-Saharan Africa [5,6]. Outbreaks of extensively drug-resistant (XDR) *S. typhi* (resistant to first and second-line antibiotics as well as third-generation cephalosporins) strains in Pakistan have highlighted the dangers of improper and unnecessary use of antibiotics and the threats of having no treatment options left for patients with typhoid fever [7].

In developing countries like Cameroon typhoid is more severe due to poor hygiene, indiscriminate use of antibiotics, and a rapid rise in multidrug-resistant strains. We have about 800,000 infected cases and 200,000 deaths, giving a death rate of 5% [8,9]. Resistance to the first line drugs, chloramphenicol, ciprofloxacin, and amoxicillin in the course of salmonellosis management has been reported [10]. Timely and accurate diagnosis and treatment of typhoid fever in the community is needed to avert complications requiring hospitalization and death [11]. Typhoid fever is endemic in areas where water, sanitation, and hygiene (WASH) infrastructure is poor. Serious complications develop in approximately 10-15% of patients if left untreated and this is driven by inadequate diagnostic methods and the high burden of antibiotic-resistant strains [4]. The disease is treated with antibiotics without appropriate antibiotic treatment, 12–30% of people with typhoid fever die [12].

Risk factors for the development of drug resistance in *S. typhi* is nothing other than overuse misuse, and inappropriate antibiotic prescribing practices (especially in viral illness where antibiotic is of no use), a special factor in the case of typhoid fever is changing of different antibiotics unnecessarily during the treatment of typhoid fever by the treating physicians [13,14]. Multidrug-resistant typhoid fever (MDRTF) is defined as typhoid fever caused by *S. typhi* strains which are resistant to all the three first-line recommended drugs for treatment that is chloramphenicol, ampicillin, and co-trimoxazole (TMP-SMX) [14]. With this resistance, there is a need for the development of new antibiotic drugs [13].

Plants are recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities [15]. Hence a large majority of the population in tropical countries rely on traditional medicines for their primary health needs [16,17]. About 50% of current drugs are derived either directly or indirectly from plants [18]. Hence, plant-derived antimicrobials have received considerable attention in recent years [19]. The biologically active compounds present in plants are called phytochemicals has minimal side effects. Since the phytochemicals cure diseases without causing harm to human beings if not misused, these plants can also be considered “man’s-friendly medicines” [20]. However, some of these plant secondary metabolites are toxic and can cause health hazards due to indiscriminate use [21].

The search for new anti-typhic from plants around us is worth giving a try. *P. guajava* and *C. papaya* leaves are two traditionally used plants usually associated in the preparation of typhoid fever treatment. Moreover, several works have been done to support the individual anti-salmonella activity of *Psidium guava* or *Carica papaya* [22]. To the best of our knowledge, no reported study highlighted the scientific basis of association of the two medicinal plants for this therapy to justify their traditional usage and encourage the formulation of traditional improved medicines (pre-formulation of typhoid fever phytomedicine). This study was aimed at determining the anti-salmonella activities of the association of methanol and/or aqueous extracts of *P. guajava* and *C. papaya* leaves *in-vitro*.

Methods

Plant material and extraction

The plant material used for this work consisted of leaves of *Carica papaya* and *Psidium guajava* that were collected in December 2019 in Bambalang-Ndop, in the Division of Ngoketunjia, North West Region of Cameroon. These plants were identified at the National Herbarium in Yaounde-Cameroon under voucher specimens as 18647/SRF/CAM for *C. papaya* and 30044/SRF/CAM for *P. guajava*. The air-dried samples were powdered and 450 g from each of the two plants were separately macerated in 3.5 L of pure methanol (MeOH) for 48 hours at room temperature. The macerate was then filtered through a Whattman filter paper N°1 and concentrated under reduced pressure to afford the crude extract. The extraction procedure with distilled water was similar but the obtained extract solution was dried in the oven at 37°C. These extracts were then kept under 4°C in the fridge for further use.

Salmonella strains and culture media

The microorganisms used in this work were strains and isolates of *Salmonella typhi* and *Salmonella paratyphi.* They included *Salmonella typhi* (ATCC6539) from the American Type Culture Collection and clinical isolates of *Salmonella enteritica* (SE), *Salmonella typhimurum* (STM), and *Salmonella typhi* from Pasteur Center of Yaounde-Cameroon; and *Salmonella typhi* (S66, S68, S108, and S107), *Salmonella paratyphi* A (S07 and S104) from the Research Unit of Microbiology and Antimicrobial Substances of the University of Dschang. They were maintained on agar slant at 4ºC and sub-cultured on a fresh appropriate agar plates 18 hours prior to any antibacterial tests. Mueller Hinton Agar was used for the activation of bacteria while Mueller Hinton Broth (MHB) was used for MIC/MBC determinations.

Phytochemical screening of Psidium guajava and Caricapapaya leaves extracts

Major secondary metabolite classes in the various extracts namely; alkaloids, phenols, flavonoids, saponins, tannins, anthocyanins, anthraquinones, sterol, and triterpenes were revealed using classical phytochemical methods [23,24,25]. This was done simultaneously for methanol and aqueous extracts.

Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of methanol, as well as aqueous extracts from PG and CP against salmonella strains under study, were determined using micro well dilution test with colorimetric para-iodonitrotetrazolium bromine chloride (INT) as revelator [16,26,27]. Briefly, the test samples were firstly dissolved in DMSO/MHB. The solution obtained was then added to MHB, and serially diluted twofold (in a 96-wells microplates). One hundred microliters (100 µL) of inoculum (2×10^6 CFU/mL) prepared in MHB was then added. The final test concentration of plant extracts ranged from 2048 to 1 µg/mL. The plates were covered with a sterile plate sealer and incubated under agitation at 37ºC for 18 hrs. The final concentration of DMSO on the extract was lower than 1% (not affecting microbial growth). Wells containing MHB, 100 µL of inoculums, and DMSO served as a negative control. Ciprofloxacin was used as reference antibiotic. All tests were done in triplicate. The MICs of samples were detected after 18 hrs of incubation at 37ºC, following addition (40 µL) of 0.2 mg/mL INT to every two columns of each test sample and incubation at 37ºC for 30 min. Viable bacteria reduced the yellow dye to pink. MIC was defined as the lowest extract concentration that prevented this change and exhibited complete inhibition of salmonella growth.

Determination of Minimum Bactericidal Concentrations (MBCs)

After reading the different MICs using just two columns of the incubated plates, the third column without INT was used to determine the MBCs for each extract in triplicate assays. Briefly, 150 µL of newly prepared MHB were introduced into the wells of new plates, then 50 µL of the contents of each well of the third column without INT and where bacterial growth was inhibited were taken with a micropipette and introduced into the corresponding wells of the new plate. These plates were covered with a sterile plate sealer. Additional incubation was done at 37°C for 24 hours. After INT revelation, extract concentrations for which no bacterial growth was noted (no pink coloration) were considered bactericidal concentrations, and the smallest was noted as MBC. This test was equally done in triplicate. If the MBC/MIC ratio of an antimicrobial substance was less than or equal to four (≤ 4), it is classified as a bactericidal substance, while a ratio greater than four (> 4) it is said to be bacteriostatic [28].

Determination of anti-salmonella activity of the association of Psidium guajava and Carica papaya methanol extracts

The methanol extract with the highest antimicrobial activities in the previous study was selected for further study. These extracts were tested single and in combinations at various concentrations (ranging from 2048 to 2 µg/mL) using the micro well dilution checkerboard method [29]. Methanol extracts of CP and PG were tested against the 10 salmonella strains. Briefly, to each well of the 96 wells microplate, 100 µL of broth was added. Then 100 µL of CP was added and serial dilutions were done. The concentration of this first extract increases vertically then different concentrations (two-fold dilutions) of the second extract (PG) were separately prepared and 50 µL of each concentration set was added to the corresponding column of the CP. An amount of 50 µL of prepared inoculum at 4.10^6CFU/mL in broth was finally added to each well. One well was left as the positive control, and one well was added inoculum without extract as the negative control. Some columns were used for the single extract only (without combination). The plates were then labelled, sealed, and incubated for 18 hrs. The anti-salmonella effects of plant extract associations were sorted by revelation done as for the MIC determination using 40 µL of an aqueous INT solution added in each well of the microplates. This test was done in triplicate. The fractional inhibitory concentration index (FICI) for each drug combination per strains/isolates was calculated as the sum of the MIC of each drug when used in combination divided by the MIC of the drug when used alone. The fractional inhibitory concentrations (FICs) were calculated as follows [29]:

$$\mathrm{FICcp} = \frac{\text{MIC of the combined extract CP+PG}}{\text{MIC of the extract, CP alone}} \quad \text{and} \quad \mathrm{FICpg} = \frac{\text{MIC of the combined extract CP+PG}}{\text{MIC of the extract, PG alone}}$$

So the FIC Index was deduced by the formula:

FIC index= FICcp +FICpg

Synergy was established when FIC index range was]0-0.5], additive, when its range was]0.5- 1], indifference, when it was between]1- 4] and antagonism, when it was greater than 4.

Results

Phytochemical screening of crude extracts

The methanol and aqueous extracts of *C. papaya* and *P. guajava* all revealed the presence of tested secondary metabolites (Table 1). However, anthocyanins were not detected in our extracts. These classes of bioactive compounds varied slightly in quality across the plants and extraction solvents.

Anti-salmonella activity of the plant extracts

These plant extracts were tested for anti-salmonella activities against a panel of 10 Salmonella strains and isolates by the microdilution method. Summarized results (Table 2) showed that the CP extracts were more active compared to those of PG, and methanol extracts were more active compared to aqueous extracts in inhibiting Salmonella growth with MIC ranging from 64 to >2048 µg/mL. These extracts exhibited a bactericidal effect against most of the studied strains.

Effects of combined methanol extracts of CP and PG leaves on Salmonella growth

The anti-salmonella potential of methanol extracts of both plants in association was then studied *in vitro* using the same salmonella strains. According to the obtained results (Table 3), the various extracts association concentrations of the leaves of CP and PG presented 4 synergies, 3 additives effects, 3 indifferences, and no antagonistic effect on the 10 strains against which they were tested. CP had lower fractional inhibitory concentrations (FIC_{CP}) compared to those of PG (FIC_{PG}).

Discussion

Plants have been well-documented for their medicinal uses for thousands of years and traditional medicines are still a major part of habitual treatments of different illnesses in different parts of the world [30]. The specific molecular principles of medicinal plants in their natural states possess a variety of influences on human

physiological and biochemical systems as raised concern over their safety.

Plants were the sole source of active principles capable of curing man's ailments before the development of chemistry and synthesis of organic compounds in the 19th century [20]. From our results, it was observed that the crude extracts of *P. guajava* and *C. papaya* leaves contained active principles that inhibited the growth of test organisms. CP methanol is rich in flavonoids, alkaloids, sterols, and phenols while CP aqueous is very rich in saponins and PG methanol is very rich in alkaloids and phenols not leaving out flavonoids while PG aqueous is very rich in saponins. This result is in accordance with the publication which reported that the phytochemical screening of *P. guajava* and *C. papaya* leaves extracts demonstrated the presence of common photochemical in the leaf extracts which include alkaloids, flavonoids, saponins, terpenoids, steroids, tannins and glycosides as major active constituents [31]. The results obtained showed that the extracts of *C. papaya* leaves were more active against the test isolates of *S. typhi* and *S. paratyphi* (the causative agent of typhoid fever) than *P. guajava* extracts when used in isolation [31]. The *P. guajava* extracts were not very active against these *Salmonella* as equally reported [32]. The combined leaf extracts of *C. papaya* and *P. guajava* showed more synergistic and additive effects. Low MIC values (8 to 256 µg/mL for *C. papaya* in association and 256 to 512 µg/mL for *P. guajava* in association) were observed in most of the combined cases, indicating an increase in activity compared to MIC values up to >2048 µg/mL for a single extract. Synergistic effects and/or antagonistic and indifference effects of plant extracts have been reported [33].

Among the extracts of *C. papaya* tested, methanol extracts of the leaves showed higher activity against the growth of *S. typhi* and *S. papatyphi* isolates, while the aqueous extracts of *P. guajava* leaves showed higher antibacterial activity to *S. typhi* isolates compared to methanol extracts and these findings corroborate with other reports [6,34,35].

Reports showed that *P. guajava* contains alkaloids, phenols, and tannins and this might be the reason for its antimicrobial activity in both methanol and water extracts [34,36]. Following the phytochemical screening carried out within the framework of this study, several classes of secondary metabolites were highlighted in each of the extracts, which would justify the antibacterial activities observed like in the work on antimicrobial activities of some plants against selected microorganisms [37,38]. However, the activity of a plant extract does not depend solely on the presence of secondary metabolites but also on their quantity and possible interactions with other constituents. The plant extracts used in this work exhibited antibacterial activities that varied not only between extracts but also between bacterial strains and isolates. The antibacterial activities of plants are attributed to the presence of the different classes of secondary metabolites they contain [39,40,41]. According to the investigations of some researchers [42,43], the variability of the activities presented by the plants would also be attributed to differences in environmental conditions, seasons, and the modes and time of extraction of the plant. According to the classification scale of antibacterial activities of food plant extracts or parts of the plant, the plant extract is classified as highly active if it has a MIC<100 µg/mL, significantly active if 100 ≤ MIC≤ 512 µg/mL, moderately active if 512< MIC ≤ 2048 µg/mL, weakly active if MIC > 2048 µg/mL and not active if MIC > 10 mg/mL [44,45]. Moreover, based on an updated classification of antibacterial activity on both resistant and sensitive enterobacteria [46], the methanol extract of *C. papaya* had excellent activity (8 < MIC≤ 64 µg/mL) on one isolate (S68); very good activity (64 < MIC≤ 128 µg/mL) on two isolates; good activity (128 < MIC≤ 256 µg/mL) on three test microorganisms; average activity (256 < MIC≤ 512 µg/mL) on four bacteria. One can suggest that some of these test microorganisms could be resistant. On the other hand, *P. guajava* extracts were generally weak (512 < MIC≤ 1024 µg/mL) or not active (MIC> 1024 µg/mL).

Table 1. Qualitative phytochemical composition of the different plant extracts

Plants	Extraction Solvent	Alkaloids	Phenols	Flavonoids	Sterols	Triterpenes	Tannins	Saponins	Anthrocianin	Anthraquinones
C. papaya	Methanol	++	++	++	++	+	-	-	-	+
	Aqueous	+	+	+	-	+	-	++	-	-
P. guajava	Methanol	+++	+++	++	+	+	+	+	-	+
	Aqueous	+	++	+	-	+	+	+++	-	-

+: present; +++/++ : increase amount present; –: absent.

Table 2. Minimum inhibitory concentrations and minimum bactericidal concentrations of test substance

Test substances	Parameters	Strains									
		S104	**S107**	**S108**	**S68**	**S66**	**ST**	**SE**	**STM**	**S07**	**STS**
***C. papaya* methanol extract**	MIC	512	512	256	64	128	128	256	512	256	512
	MBC	512	2048	1024	512	128	1024	256	1024	1024	1024
	MBC/MIC	1	4	4	4	1	4	1	2	4	2
***C. papaya* aqueous extract**	MIC	512	512	512	>2048	>2048	>2048	1024	>2048	>2048	2048
	MBC	>2048	512	1024	>2048	>2048	512	>2048	>2048	>2048	>2048
	MBC/MIC	ND	1	2	ND	ND	ND	ND	ND	ND	ND
***P. guajava* aqueous extract**	MIC	1024	>2048	512	2048	1024	1024	>2048	1024	512	1024
	MBC	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048
	MCB/MIC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
***P. guajava* methanol extract**	MIC	1024	1024	1024	1024	1024	512	>2048	512	2048	2048
	MBC	2048	>2048	2048	>2048	2048	2048	>2048	2048	>2048	>2048
	MBC/MIC	2	ND	2	ND	2	4	ND	4	ND	ND
Ciprofloxacin	MIC	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
	MBC	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
	MBC/MIC	1	1	1	1	1	1	1	1	1	1

MBC/MIC ≤ 4: bactericidal; MBC/MIC >4: bacteriostatic; ND; not determined; MBC and MIC values are in µg/mL

Table 3. Methanol plant extracts association interaction patterns against salmonella growth

Bacteria strains	MIC (µg/mL)				FIC_{CP}	FIC_{PG}	FICI	Interaction pattern
	CP	**PG**	**CP in association**	**PG in association**				
S104	512	1024	256	512	0.50	0.50	1.00	Additive
S107	512	>2048	32	512	0.06	<0.25	<0.31	Synergy
S108	256	512	8	512	0.03	1.00	1.03	Indifference
S68	64	2048	16	512	0.25	0.25	0.50	Synergy
S66	128	1024	32	512	0.25	0.50	0.75	Additive
ST	128	512	16	512	0.12	1.00	1.12	Indifference
SE	256	>2048	32	512	0.12	<0.25	<0.37	Synergy
STM	512	1024	32	512	0.06	0.50	0.56	Additive
S07	256	512	64	512	0.25	1.00	1.25	Indifference
STS	512	512	64	256	0.13	0.50	0.63	Indifference

MIC: minimum inhibitory concentration; CP: *C. papaya*; PG: *P. guajava*; FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index (Fic_{cp}+Fic_{pg}); FICI values≤0.5 represent synergy/ >0.5 to 4 represent no interaction/ and >4 represent antagonism.

Conclusion

This study concludes that *C. papaya* and *P. guajava* leaves have antibacterial activity either used single or in association against *Salmonella typhi* and *Salmonella paratyphi.* The methanol leaves extracts of CP and PG have anti-salmonella potentials singly taken and in combination *in-vitro* as the important association outcomes were seen to be synergism and additive effects amongst the 2 plants much more than indifference. Further in vivo toxicological studies and mechanisms of actions of the plant products are needed to ascertain the use of these plants extracts to **formulate pre-phytodrugs** for typhoid fever treatment and/or new synthetic drug formulation.

Abbreviations

MIC: Minimal Inhibitory Concentration
MBC: Minimal Bactericidal Concentration
PG: *Psidium guajava*
CP: *Carica papaya*
FIC: Fractional inhibitory concentration
FICI: Fractional inhibitory concentration index
XDR: Extensively drug-resistant
MDRTF: Multidrug-resistant typhoid fever
MeOH: Methanol
INT: para-iodonitrotetrazolium chloride

Authors' Contribution

GNT conceived the study and designed the method. SLT monitored the experimental and laboratory work, analyzed data. TEN did the experimental and laboratory work, collected and transported data and other materials. GNT drafted and finalized the manuscript for publication. MTN and JRK edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest

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