Research Article

Antisalmonellal Activity and GC-MS Analysis of *Piliostigma thonningii* leaf extract

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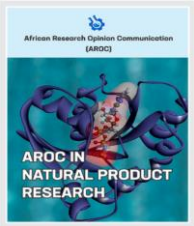

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Abstract

Background: Typhoid fever is a serious bacterial infection which causes bacteremia and inflammatory destruction of the intestine and some internal organs in the body. The widespread emergence of multi-drug resistant *Salmonella typhi* and *Salmonella paratyphi* has necessitated the search for other therapeutic options. The study was conducted to screen the antisalmonellal activity of *Piliostigma thonningii* leaf crude extract, fractions and isolated compounds. Methods: The plant leaves were extracted with 70% methanol, the crude extract was partitioned into fractions and was tested for antibacterial activity against *S. typhi*, *S. paratyphi* A, *S. paratyphi* B and *Salmonella paratyphi* C using agar well diffusion technique. Column and thin layer chromatographic methods were used for phyto-constituent separation of plant extract. The most effective antisalmonellal column chromatography isolated compound was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Results: The crude extract and the fractions except n-hexane fraction possess antibacterial activity on at least one of the Salmonella strains tested, however, the ethyl acetate fraction (PT1-03) exhibited the widest zone of inhibition on the test bacteria (14-16 mm) at the concentration of 100 mg/ml. The zones of growth inhibition increased with the increasing concentration of the fractions. The corresponding increase in concentration and growth inhibition zone was significant ($p<0.05$). The isolated compound obtained from the column chromatography also showed significant inhibition on the Salmonella strains (12-15 mm) at the concentration of 50 mg/ml. GC-MS analysis of the column chromatography isolates revealed Levomenthol and hexadecanoic acids as the major compounds. Conclusion: The study clearly indicates that *P. thonningii* possesses bioactive compounds that are active against some Salmonella species. Therefore, these phytochemicals can be formulated into drugs for the treatment of typhoid and paratyphoid fevers.

Keyword: Antisalmonellal; Chromatography; Growth inhibition; Phytochemicals; *P. thonningii*

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1.0 Introduction

Typhoid fever is one of the most serious infectious bacterial diseases in third world countries [1]. The *Salmonellae* that cause significant human disease are classified in most countries under the taxon *Salmonella enterica*, subsp. *enterica*. Enteric fever caused by *Salmonella enterica* serotype t*yphi* (*Salmonella typhi*) and *Salmonella enterica* serotype p*aratyphi* A, B or C are short Gram-negative rods with rounded ends closely related to *E. coli* [2-3]. Enteric fever remains a major disease burden in developing countries and is associated with poor sanitation and contaminated water and food; a faecal–oral transmissible disease [4]. In areas of endemicity and in large outbreaks, most cases occur in persons aged between 3 and 29 years. Humans are the only natural host and reservoir [5].

The emergence and spread of multidrug resistant *Salmonella serotypes* to many commonly used antibiotics (Ampicillin, Amoxicillin, Chloramphenicol, and trimethoprim sulfamethoxazole) is now a subject of international concern. The problem has become endemic in many developing countries, causing

enormous childhood morbidity and high cost of treatment [6]. There is therefore, the need for the continuous search of indigenous plants for the treatment of typhoid fever [7]. Phytomedicines are believed to have promising potential because they contain compounds that can eliminate the quick resistance development and of low toxicity compared to synthetic drugs [3].

Piliostigma thonningii of the Fabaceae Family (subfamily Caesalpiniaceae) commonly known as camel's foot (English), Abefe (Yoruba), Kalgo (Hausa) and Okpoatu (Igbo) is an erect perennial tree grown throughout the tropics and sub-tropics [8]. Traditionally, the plant is used in the treatment of cough, dysentery, sores, snake bites, malaria, stomach upsets and also used as a pain reliever [9]. A number of pharmacological investigations have revealed that *P. thonningii* has some bioactivities such as antimicrobial [10,11], antimalarial [12,13], anthelmintic [14] and antioxidant [14,15]. The present research therefore evaluates the antisalmonellal activity of the crude extract and fractions of *P. thonningii* and also characterize the bioactive sub-fraction using Gas Chromatography- Mass Spectrometry **(**GC-MS)

2.0 Materials and Methods

2.1. Collection and Identification of the plant

Matured leaves of *P. thonningii* were collected along Gidan kwano Road Minna, Niger State. The plant was identified at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria where voucher number 171 was deposited.

2.2 Plant Preparation

The leaves were carefully washed under running water and air-dried at room temperature and then milled into fine powder. About 300 grams of the powdered leaves was macerated with 1.5 liters of 70% methanol for 72 hours. The extract was filtered using a muslin cloth and subsequently evaporated using a rotatory evaporator. The semi-dried extract was weighed, placed in sterile sample bottles and stored in a refrigerator until required for use [16].

2.3. Solvent partitioning of crude extract

The methanol crude extract was undertaken for solvent-solvent partitioning by using the methods employed by Emran *et al*., [17]. The crude extract was successively partitioned by using solvents of increasing polarity in the following order; n-hexane, chloroform and ethyl-acetate in a separating funnel. The resulting fractions of the crude extract were evaporated to dryness using rotary evaporator at 40 ℃. All the concentrated fractions were weighed and stored in air tight containers till further analysis. About one gram (1 g) of each extract and fraction were dissolved in 5 ml of 50% dimethylsulphoxide (DMSO) to make 200 mg/ml stock solution from which was serially diluted to give concentrations of 100 mg/ml, 50 mg/ml and 25 mg/ml.

2.4 Vacuum Liquid Chromatography (VLC) and Column Chromatography of Ethyl-acetate Fraction of *P. thonningii*

The most active partitioned fraction (ethyl-acetate fraction of *P. thonningii*) was further exploited in an attempt to isolate the active principle which exhibited the antibacterial activity. The method described by Amin *et al*., [18] was adopted for the isolation procedure. Different sub-fractions (VLC1- VLC7) were obtained by using vacuum liquid chromatography apparatus. The best fraction obtained from the VLC which exhibited significant activity against the test organisms was subjected to column chromatography using the method described by Dauda and Mudi [19] with slight modification. About 250 grams of washed silica gel (60-120 mesh size) was packed into a glass column (3.8 cm by 53 cm) in slurry of n- hexane. The fraction was dissolved in methanol and then mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the column already packed with silica gel. Sequential elution was

carried out using stepwise gradient solvents of increasing polarity. The process was monitored using the thin layer chromatography. An aliquot of 20 ml of the eluates were continuously collected into test tubes from the beginning to the end of the elution, in each case the eluates having similar TLC profile were pooled together into six major sub-fractions (Et1-Et6) which were further subjected to antibacterial activity.

2.5 Gas chromatography–mass spectrometry (GC-MS) analysis

The GC–MS analysis of the ethyl-acetate fraction of the Leaf extract was carried out in a (QP 2010 Plus SHIMADZU) instrument at 70 eV. One microliter (1 uL) of the extract was injected into the GC–MS using a micro syringe and the scanning was performed for 20 min.

2.6 Antisalmonellal assay

2.6.1 Test organisms

Clinical isolates of *Salmonella typhi, Salmonella paratyphi* A*, Salmonella paratyphi* B and *Salmonella paratyphi C* were obtained from the Microbiological laboratory of Aminu Kano teaching Hospital, Kano for the susceptibility tests. The organisms were used after their identity were confirmed at the Department of Microbiology, Bayero University, Kano. The stock culture was maintained on Nutrient agar slant at 4 ℃ in the refrigerator.

2.6.2 Antisalmonellal Susceptibility Test

The sensitivity of the crude extract and fractions were determined using the agar well diffusion method as described by Nas and Ali [20]. The prepared bacterial suspension equivalent to 0.5 McFarland Standard (1.5 x 10^6 CFU) was inoculated into sterile Mueller- Hinton agar medium in a sterile Petri-dish. A sterile 6 mm diameter cork borer was used to bore wells into the agar medium. The wells were filled with approximately 0.1 ml of the extract solution at a concentration of 25, 50 and100 mg/ml respectively, care was taken to prevent spillage onto the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 hour to allow proper diffusion of the extract into the medium after which the plates were incubated at 37 ℃ for 24 hours, and thereafter the plates were observed for zones of inhibition and measured using a meter rule. The same procedure was adopted for the Column chromatography isolates. The Reference antibiotic Amoxicillin (50 mg/ml) served as control.

2.7 Statistical Analyses

The statistical analyses were carried out using statistical package for social sciences (SPSS-computer package). Data from the antibacterial activities of *P. thonningii* were expressed as mean ± standard error of three independent replicates and also subjected to one-way analysis of variance (ANOVA) at $p<0.05$ level of significance for comparison of the extract activities.

3.0 Results

3.1 Antisalmonellal activity of crude extract and fractions

The antisalmonellal activity of the crude extract and soluble fractions (n-hexane, chloroform, ethyl-acetate, aqueous methanol) are shown in Table1. The most active fraction was ethyl-acetate soluble fraction as it showed the largest zone of bacterial inhibition. The n-hexane soluble fraction showed no activity on the *Salmonella* species tested.

3.2 Antisalmonellal activity of VLC subfractions

The Antisalmonellal activity of all the seven subfractions obtained from Vacuum liquid chromatography of *P. thonningii* ethyl-acetate fraction is shown in Table 2. All the sub-fractions except sub-fraction seven (VLC7) showed zones of inhibition on at least one organism. Sub-fraction five (VLC 5) was the most active against all the *Salmonella* strains tested

Table 1: Zones of growth inhibition (mm) of the crude extract and fractions of the leaves of *P. thonningii* on the test bacteria

Crude/ Fractions	Conc. (mg/ml)	*S. typhi*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*
PT1	25	10.33± 0.33^{c}	0.00± 0.00^{c}	09± 0.00^{c}	0.00± 0.00^{c}
	50	12.00± 0.58^{b}	09.33± 0.33^{b}	11.33± 0.33^{b}	9.33± 0.33^{b}
	100	15.33± 0.33^{a}	12.00± 0.58^{a}	14.67± 0.33^{a}	11.33± 0.33^{a}
PT1-01	25	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}
	50	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}
	100	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}
PT1-02	25	0.00± 0.00^{b}	0.00± 0.00^{b}	0.00± 0.00^{c}	0.00± 0.00^{c}
	50	0.00± 0.00^{b}	0.00± 0.00^{b}	11.00± 0.58^{b}	11.67± 0.33^{b}
	100	11.67± 0.33^{a}	8.33± 0.33^{a}	13.33± 0.33^{a}	14.00± 0.00^{a}
PT1-03	25	11.33± 0.33^{b}	0.00± 0.00^{c}	12.67± 0.33^{c}	12.33± 0.33^{c}
	50	11.67± 0.33^{b}	12.00± 0.00^{b}	13.67± 0.33^{b}	13.00± 0.58^{b}
	100	14.33± 0.33^{a}	15.33± 0.33^{a}	16.33± 0.33^{a}	15.33± 0.33^{a}
PT1-04	25	0.00± 0.00^{c}	0.00± 0.00^{c}	0.00± 0.00^{c}	0.00± 0.00^{c}
	50	10.00± 0.33^{b}	9.33± 0.33^{b}	9.33± 0.33^{b}	9.33± 0.33^{b}
	100	11.00± 0.33^{a}	12.33± 0.33^{a}	11.67± 0.33^{a}	10.67± 0.33^{a}

Values are presented in means ± Standard error of three replicates. Values with the same superscript on the same column are not significantly different at p>0.05. Keys: PT1 - Methanol extract; PT1-01 - n-hexane soluble fraction; PT1-02 -Chloroform soluble fraction; PT1-03 -Ethyl-acetate soluble fraction; PT1-04 -Aqueous methanol soluble fraction

3.3 Antisalmonellal activity of Column Chromatography subfractions

Table 3 shows the results of antisalmonellal activity of subfractions obtained from Column chromatography. The *Salmonella* strains tested were susceptible to sub-fraction Et1 and Et2. However, Et1 indicated the strongest antibacterial activity on the test organism at low concentration of 50 mg/ml (12-15 mm).

3.4 GCMS of Et1 subfraction

The chromatograph showed 28 peaks (Figure 1). The major constituents identified in the isolated compound were Levomenthol (cyclohexanol) (37.49%), n-Hexadecanoic acid (9.79%), hexadecanoic acid (Methyl ester) (6.66%) (Table 4) and many other constituents were identified as low level.

Table 2: Zones of growth inhibition (mm) of VLC subfractions obtained from ethyl-acetate fraction of *P. thonningii*

Fractions	Conc. (mg/ml)	*S. typhi*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*
VLC1	50	11.33± 0.33	10.33± 0.33	10.67± 0.33	11.33± 0.33
	100	12.33± 0.33	11.00± 0.58	11.33± 0.33	11.67± 0.33
VLC 2	50	10.33± 0.33	8.33± 0.33	-	8± 0.33
	100	11.00± 0.58	10.67± 0.33	8.67± 0.67	8.33± 0.33
VLC 3	50	8.33± 0.33	-	8.33± 0.33	-
	100	8.33± 0.33	8.33± 0.33	8.67± 0.33	8± 0.00
VLC 4	50	8.00± 0.58	8.33± 0.33	-	-
	100	8.33± 0.33	8.67± 0.33	-	-
VLC 5	50	12.67± 0.33	8.33± 0.33	11.67± 0.33	10.67± 0.33
	100	13.33± 0.88	8.67± 0.33	12.33± 0.33	11.33± 0.33
VLC 6	50	-	-	-	-
	100	9± 0.58	-	-	-
VLC 7	50	-	-	-	-
	100	-	-	-	-

Key: - No zone of inhibition

Table 3: Zones of growth inhibition (mm) of isolates obtained from Column Chromatography isolates of *P. thonningii*

Fractions	Conc. (mg/ml)	*S. typhi*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*
Et1	25	13.33± 0.33	14.33± 0.58	12.33± 0.33	12.67± 0.33
	50	15.33± 0.88	15.33± 0.33	13.33± 0.88	15.33± 0.88
Et2	25	11.33± 0.33	-	11.33± 0.33	-
	50	11.00± 0.88	-	11.00± 0.58	11.33± 0.33
Et 3	25	-	-	-	-
	50	-	-	-	-
Et 4	25	-	-	-	-
	50	-	-	-	-
Et 5	25	-	-	-	-
	50	-	-	-	-
Et 6	25	-	-	-	-
	50	-	-	-	-
Control	50	16.33± 0.33	10.33± 0.33	16.33± 0.88	11.33± 0.33

Key: - No zone of inhibition

3.4 GCMS of Et1 subfraction

The chromatograph showed 28 peaks (Figure 1). The major constituents identified in the isolated compound were Levomenthol (cyclohexanol) (37.49%), n-Hexadecanoic acid (9.79%), hexadecanoic acid (Methyl ester) (6.66%)(Table 4) and many other constituents were identified as low level.

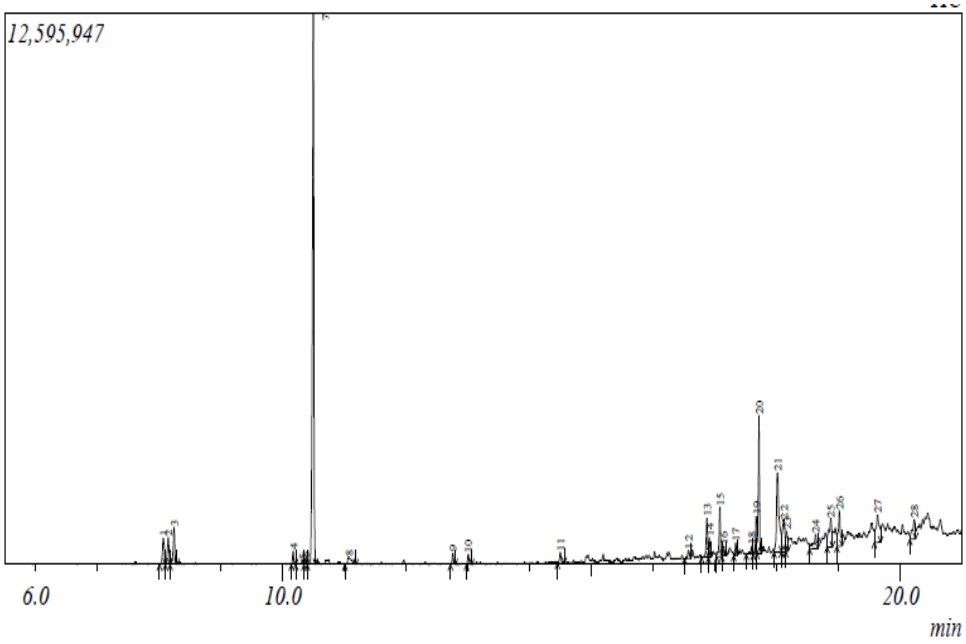

Figure 1: GC-MS chromatogram of Column isolate Et1

Table 4: The major chemical constituents of Column isolate Et1 by GC-MS

S/N	Peak	Retention time	Peak %	Derivatives	Molecular Formular
1	7	10.50	37.49	Levomenthol	$C_{10}H_{20}O$
2	20	17.70	6.66	Hexadecanoic acid	$C_{17}H_{34}O_2$
3	21	18.00	9.79	n-Hexadecanoic acid	$C_{16}H_{32}O_2$

4.0 Discussion

Plant extracts are considered to be valuable source of biological active compounds. In this study the antibacterial activity of the crude extracts and fractions were assessed against some *Salmonella* strains. The crude extract and all the other soluble fractions (n-hexane, chloroform, ethyl-acetate, aqueous methanol) had antibacterial activity against all the tested organisms except n-hexane soluble fraction. This could be that the bioactive compounds present in the Plant were more soluble in the polar solvents as compared to the non-polar solvent. Different extracts from same plant can show different constituents and antimicrobial activities on the same organism [19]. However, the most active fraction was ethyl-acetate soluble fraction as it showed the largest zone of bacterial inhibition as such was subjected to Vacuum liquid chromatography.

All the subfractions except sub-fraction seven (VLC7) showed zones of inhibition on at least one organism. Fraction five (VLC 5) was the most active against the bacteria strains tested, as a result of which, it was further purified using column chromatography. The *Salmonella* strains tested were susceptible to the column chromatography isolate Et1 and Et2. However, Et1 indicated the strongest antibacterial activity on the test organism at low concentration of 50 mg/ml (12-15 mm) when compared with the crude extract and subfractions. The activities of the isolates were significant as compared to that of the reference antibiotic Amoxicilin (control) used in the study. The present study has shown that antibacterial activity varies with the fractions. The observed difference could be attributed to the variation in the distribution of active principles according to their affinity for the solvent used in fractionation [21].

Few investigations on the antibacterial properties of *P. thonningii* have been reported. Ewansiha *et al.* [22] and Chukwunonye *et al.* [11] had also reported the potency of the leaf extract and fractions of *P. thonningii* on *S. typhi* and other bacteria. Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. Gas chromatography has gained widespread acceptance in numerous application areas, such as process control in chemical plants, quality control in the food industry, monitoring sample composition in the oil-industry, environmental and bio medical sciences [23]. The interpretation on mass spectrum was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Twenty-eight compounds were identified in the column isolate by GC-MS analysis. The compounds with the highest composition percentage were cyclohexanol (Levomenthol), n-hexadecanoic acid (palmitic acid) and hexadecanoic acid. The identified compounds possess many biological properties. Menthol possesses antibacterial and antioxidant activity [24]. n-Hexadecanoic acid and hexadecanoic acid have also been reported as major constituents in the GCMS analysis of some medicinal plants [23, 25-26], these compounds acts as an antimicrobial, antioxidant, hypocholesterolemic, nematicide, pesticide and lubricant activities [27-29]. The presence of the various bioactive compounds identified in this plant justifies its use as medicine for various ailments by traditional practitioners.

4.0 Conclusion

The antisalmonellal activity of *P. thonningii* leaf extract showed that the extracts and fractions were effective against the *Salmonella* strains with the column chromatography isolate exhibiting the highest activity when compared to the other fractions. The antibacterial activity of the plant may be due to the presence of the bioactive compounds identified in this study using gas chromatography–mass spectrometry. The ethyl-acetate fractions and the identified

column isolates may therefore be a readily available source of cheap and potent antibacterial agents to be used in the therapy of infections caused by these often multi resistant organisms. These findings provide a rationale for the use of the plant in traditional medicine.

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Conflicts of interest: The authors declare that they have no competing interests.

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