Asian Plant Research Journal

Volume 12, Issue 6, Page 12-26, 2024; Article no.APRJ.124856
ISSN: 2581-9992

Antisalmonella Activities of Essential Oil of *Psidium guajava* Leaves: An *In vitro* and *In vivo* Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/aprj/2024/v12i6277

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/124856

Original Research Article

Received: 16/08/2024
Accepted: 18/10/2024
Published: 23/10/2024

ABSTRACT

Background: Life-threatening typhoidal salmonella infection is becoming more difficult to treat due to rising resistance to commonly used antibiotics. This resistance is aided by the biofilm-forming ability of the bacteria. This study investigated the *in vitro* and *in vivo* antisalmonella activities of essential oil of *Psidium guajava* leaves.
Methods: Essential oil was extracted from the leaves of Psidium guajava with solvents. The sensitivity of Salmonella Typhi to the essential oil of Psidium guajava (EOPG) was assessed via the agar well diffusion method. Minimum inhibitory, bactericidal and biofilm-inhibitory concentrations (MIC, MBC and MBIC respectively) were measured using micro-broth dilution and crystal violet assay respectively. The effect of EOPG on the bacterial cell membrane was evaluated by measuring the efflux of potassium ions, inorganic phosphate and pyruvic acid. Bioautography and

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Cite as: *OMOTOSO, Michael Femi, Temitope Deborah OLANIYI, Abiodun Bukunmi ABORISADE, and Adewale ADETUTU. 2024. "Antisalmonella Activities of Essential Oil of Psidium Guajava Leaves: An In Vitro and In Vivo Study". Asian Plant Research Journal 12 (6):12-26. https://doi.org/10.9734/aprj/2024/v12i6277.*

GC-MS were employed to identify the constituents of the active fraction of the EOPG. Effects of EOPG on the level of bacteremia and bone marrow infection in male Wistar rats were determined by plating caudal blood on salmonella-shigella agar. Selected serum cytokines were measured using ELISA kits. Effect on hepatic DNA was measured by comet assay while histological evaluation of the liver of infected rats was done by Hematoxylin and Eosin staining.

Results: Essential oil of *Psidium guajava* leaves (EOPG) inhibited the growth of *S.* Typhi with a zone of inhibition of 13±0.2mm compared to 31±0.3mm recorded by ciprofloxacin. Minimum inhibitory, minimum bactericidal and minimum biofilm inhibitory concentrations were 25, >50 and 12.5 mg/mL respectively. Exposure to EOPG caused efflux of potassium ions, inorganic phosphate and pyruvic acid in *S*. Typhi. Oral administration of 50 mg/mL of EOPG reduced bacterial load in the blood and bone marrow of *S*. Typhi-infected rats significantly (P<0.05) when compared to untreated animals. Infection-induced reduction of serum IL-1β and rise in serum IFN-γ were ameliorated considerably in treated animals. The hepatic DNA of infected animals were protected from breakage in the group administered 50 mg/mL/day of EOPG compared with untreated, ciprofloxacin-treated as well as those administered 100 mg/mL/day of EOPG.

Conclusion: EOPG has both *in vitro* and *in vivo* anti-salmonella activities, this could be exploited in developing new drug leads for treatment of typhoid fever

Keywords: Essential oils; psidium guajava; salmonella typhi; typhoid fever.

ABBREVIATIONS

EOPG : Essential Oil of Psidium Guajava
MBC : Minimum Bactericidal Concentration
MBIC : Minimum Biofilm Inhibitory Concentration
MIC : Minimum Inhibitory Concentration

1. INTRODUCTION

Salmonellosis continues to pose a critical challenge to the global healthcare system with overwhelming statistics of incidence and mortality, especially in the sub-Saharan Africa [1,2]. Although non-typhoidal salmonella species cause self-limiting diarrheal disease, typhoid fever caused by *Salmonella* Typhi (*S.* Typhi) is a systemic, life-threatening infection of the reticule-endothelial system. The emergence of the multi-drug resistance strains of (*S.* Typhi) is making the treatment of typhoid fever more challenging. Some infected individuals also develop the carrier status due to the formation of the bacterial biofilm in harder-to-reach niches, especially in the gall bladder. These two important factors make searching for alternative anti-salmonella substances a top priority.

Essential oils (EOs), also known as volatile oils, are products of the secondary metabolism of aromatic plants. They are complex mixtures of primarily terpenoids and phenolic compounds. EOs have been used globally in food processing as flavour enhancers, preservatives, remedies, and skin and hair treatment [3]. EOs are currently being highlighted as potential resistance-modifying substances due to their chemical diversity which enables them to attack microorganism at multiple targets. Interaction between the components of EOs also leads to synergistic, additive, or sometimes even antagonistic effects on microorganisms. There have been many scientific reports of antibacterial activities of EOs of various plants against both gram-negative and gram-positive bacteria, including many *Salmonella spp* [4, 5]. Adetutu et al*.* [6] reported that the n-hexane fraction of *P. guajava* exhibited a bactericidal effect against the typhoidal pathogen. However, there is a dearth of information on the activity of essential oil on the deadly, typhoidal salmonella, *S.* Typhi.

Psidium guajava (common: guava), is native to Mexico and grows in all the tropical and subtropical areas of the world. A decoction of new shoots is taken as a febrifuge. An aqueous leaf extract is used to reduce blood glucose levels in diabetics. Leaves are applied on wounds, and ulcers, while they are chewed to relieve toothache and as antiseptic for treatment of diarrhoea (Teixeira et al*.*, 2003). Guava leaf extracts are also used in various herbal preparations for a variety of purposes; from herbal antibiotics and diarrhoea formulas to bowel health and weight loss formulas, anorexia, cholera, diarrhoea, digestive problems,

dysentery, gastric insufficiency, inflamed mucous membranes, laryngitis, mouth swelling, skin problems and sore throat (Holetz et al., 2002); [7]. Various extracts of *P, guajava* leaves have been reported to contain aromatics compounds such as flavonoids, coumarin and alkaloids with antioxidant anti-inflammatory and antibacterial activities [8]. Both gram-positive and gram-negative foodborne pathogens were susceptible to leaf extracts of *P. guajava* [9] Haven established the activities of leaf extract of *P. guajava* against *S.* Typhi [6] and, we sought to assess the activities of the essential oil fraction of the *n*-hexane extract of *P. guajava* leaves against the typhoidal pathogen.

2. MATERIALS AND METHODS

The study was conducted between June 2020 and October 2021 at the Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.

2.1 Extraction of Essential Oil of *Psidium guajava*

Leaves of *P. guajava* were harvested in the premises of Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria and authenticated at the herbarium unit of the Department of Pure and Applied Biology of the same institution.

The extraction of essential oils was done by solvent extraction process as described by Li et al*.* (2009) with slight modification. Fresh leaves of *P. guajava* were washed twice under running tap water to remove debris and sand. The leaves were air-dried for 10 days and ground into powder using an electric blender. About 50 g of powdered leaves of *P. guajava* were placed in the thimble and placed in the Soxhlet chamber 500 mL of n-Hexane was placed in a round bottom flask and assembled for extraction. After completion of the extraction process for 180 minutes at 70 °C. The solvent was removed at low pressure using water bath. Ethanol was added to the crude extract and allowed to cool at -4 °C for 1 hour. The mixture was then filtered with filter paper and the filtrate, allowed to concentrate in a water bath at 40 °C is the Essential oil of *P. guajava* (EOPG) while the residue is the concrete (Fathy et al., 2016).

2.2 *Salmonella* Typhi

Clinical isolates of *S.* Typhi were obtained from the Medical Microbiology unit of University of Ilorin Teaching Hospital, Ilorin, Nigeria and maintained on Salmonella Shigella Agar (SSA) throughout the study period.

2.3 *In vitro* Anti-Microbial Studies

2.3.1 Sensitivity of S. Typhi to EOPG (Agar Well Diffusion Method)

S. Typhi was cultured in freshly prepared, sterile Salmonella Shigella agar (SSA). A suspension of the pure culture (0.5 McFarland) was spread evenly over the face of a freshly prepared sterile Mueller-Hinton agar (MHA) plate. A well (6 mm) was bored at the centre of the agar and 50 µL of EOPG (100 mg/mL in DMSO) was applied to the well. The plate was incubated for 24 hours at 35 °C. A clear zone was observed (zone of inhibition) around the hole and the size was measured to know the extent of the antimicrobial activity of the essential oil. Ciprofloxacin and DMSO were used as positive and negative controls respectively [10]. The experiment was performed in triplicates.

2.3.2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The broth micro-dilution method described by Ramalivhana [11] was used to determine the minimum inhibitory concentration of the EOPG with slight modification. Miller-Hilton Broth (MHB) was prepared according to manufacturer's instructions. The essential oil was serially diluted to achieve a final concentration range of 50-0.39 mg/mL in MHB in a 96-well micro-titre plate. Exactly 0.5 McFarland standard suspensions of the bacteria were added to each well using a micropipette and incubated at 37 °C for 24 hours. Twenty microliter (20 µL) of 2,3,5-triphenyl tetrazolium chloride (TTC, 5 mg/mL) was added to each well and incubated for 4 hours. Each well was observed for colour change. *Salmonella* Typhi convert the light-yellow coloured TTC to deep red formazin. All experiments were performed in triplicate. The range of MIC for each extract was determined by observing the lowest concentration of the extract that inhibits the growth of the bacterium [12] (ie. Wells with no colour change). The MBC values were determined from the wells with no visible bacterial growth from the MIC assay. About 50µl of broth solution from each well with no growth was spread on freshly prepared sterile MHA plates, incubated at 37 °C overnight and observed for growth. The lowest concentration at which there is no visible bacterial growth was

taken as Minimum Bactericidal Concentration [13].

2.3.3 Determination of Anti-biofilm activity of EOPG

Anti-biofilm effect of the EOPG was assessed according to the methodology of González et al., [14] In other to stimulate the growth conditions of *S.* Typhi on cholesterol gall stone, microtitre plates were pre-coated with cholesterol by dispensing 5 mg/mL of cholesterol in ethanol: isopropanol (1:1) in each well of 96 well polystyrene micro-titre plate and allowed to dry at room temperature overnight. The same procedure as described in MIC was followed and the plates were incubated at 37 °C for 48 hours without agitation. After incubation, planktonic cells from each well were removed and washed thrice with sterile distilled water. Adhered cells were stained with 0.4% Crystal Violet (CV) solution for 3 minutes. The excess CV was discarded and the wells were washed twice with sterile distill water. The CV from the stained biofilms was dissolved in 33% glacial acetic acid for 10 minutes and the colour intensity was measured at OD_{570nm} using ELISA microtitre plate reader. The percentage biofilm inhibition was calculated using the following formula:

$$\text{Percentage (\%) inhibition} = \frac{\text{ODgrowth control} - \text{ODsample}}{\text{OD}growth\ control} \text{ X } 100$$

The lowest concentration of the essential oil that inhibits biofilm formation was taken as the Minimum biofilm inhibition concentration (MBIC)

2.3.4 Assay of Potassium and Phosphate Ions Efflux

The extracellular concentration of free potassium and phosphate ions upon exposure of *S.* Tyhi to EOPG was determined as described by Zhang et al*.* [15]. Fresh cultures of *S.* Typhi were incubated in sterile peptone water (0.1 g/100 mL) containing 0 and 25 mg/mL (MIC) of EOPG for control and test respectively for 0, 30, 60, 90, and 120 minutes. At each sampled interval, the extracellular potassium and phosphate concentrations were measured by a Kalium/Potassium kit and a phosphorus inorganic kit, respectively. Results were presented as the amount of extracellular free potassium and phosphate (mmol/L) in every interval of incubation.

2.3.5 Measurement of Pyruvic Acid Efflux

Efflux of pyruvic acid from *S.* Typhi incubated with EOPG was measured as described by Zou et al. [16]. Fresh cultures of *S.* Typhi were incubated in sterile peptone water (0.1 g/100 mL) containing 0 and 25 mg/mL (MIC) of EOPG for control and test respectively for 0, 3, 6 and 24 hours. At each sampled interval, aliquots were centrifuged at 6000 rpm for 15 min at 4 °C, and the supernatants were collected and stored in a refrigerator at 4 °C until measurement. The pyruvic acid content was measured using the 2,4-dinitrophenylhydrazine method. The supernatants (0.1 mL) and 2, 4-dinitrophenylhydrazine (1 mL) was added to test tubes containing 8 % trichloroacetic acid (0.3 mL) by rapid mixing. The mixture was placed in a hot bath at 37 °C for 10 min. Thereafter 0 mL of sodium hydroxide (0.4 mol/L) was added and mixed. The absorbance at 520 nm was recorded. The content of pyruvic acid was calculated on a pyruvic acid calibration curve.

2.3.6 Bioautography of the EOPG against S. Typhi

Aluminium-backed TLC Silica gel 60 F254 plates (Merck, Germany) cut to 10 by 5 cm was used for both analytical EOPG using hexane: ethyl acetate (100:0-0:100) as the mobile phase. The mobile phase was poured into the chromatographic tank to about 0.8 cm depth, covered and allowed for saturation. The samples were applied at the origin (1.0 cm from the base of the plate) and carefully lowered into the tank and allowed to develop. Two chromatograms were developed. The anti-salmonella activity of the bands (fractions) on one of the developed TLC plates of EOPG was assessed using the agar overlay bioautography technique as described by Dewanjee et al*.* [17]. The developed TLC plates were aseptically placed in Petri dishes, silica gel facing up, and freshly prepared, sterile MHA (cooled to 45 °C) overlaid on the plate (about 7 mm deep). After the agar solidified, the plates were inoculated with approximately $1.5x10^6$ CFU/mL of *S.* Typhi from an overnight culture and thereafter incubated for 24 hours at 37 °C. Bacterial growth was visualized by spraying with 2,3,5-triphenyl tetrazolium chloride (TTC, 5 mg/mL). Clear zones (no colouration) were taken as inhibition zones and the band(s) surrounded by the clear zones were designated as active anti-salmonella sub-fraction(s) of EOPG.

2.4 GC/MS Analysis of the Active Fraction of EOPG

The bands corresponding to the active fraction on the bioautogram were scraped into absolute

methanol and the silica gel was filtered out using a Whatman no 1 filter paper. The constituents of the active fraction of the EOPG were determined by Gas Chromatography/Mass Spectrometry (GC/MS). Volatile constituents were separated on a DB5-MS column (30 m length, 0.25 mm inner diameter, and 0.25 lm film. Injections were made in the split mode for 30 seconds, and the gas chromatograph was operated under the following conditions: injector 220 °C and column oven 40 °C for 3 minutes, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 minutes, and finally ramped at a rate of 40 °C/min to 220 °C and kept for 2 minutes, the carrier gas at 1 mL/min. The transfer line and ion–source temperatures were adjusted at 230 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range set at 40–500 m/z. The percentages of different components in the oil sample were determined by computerized peak area measurements relative to each other. The peaks were deconvoluted using AMDIS software (www.amdis.net) and identified by its retention indices (RI) relative to n-alkanes (C6–C20), mass spectrum matching to NIST.

2.5 *In vivo* assessment of Anti–salmonella Activity of EOPG

2.5.1 Grouping of experimental animal

Twenty-five male rats aged 8 weeks were used. They were acclimatized for 2 weeks prior to commencement of the experiment under standard laboratory conditions (12-hour light/dark cycle) and fed with standardized rat pellet and water. The animals were grouped as summarized in Table 1.

2.5.2 Infection and administration of EOPG to experimental animals with S. Typhi

The inoculum was prepared by picking a few colonies of *S.* Typhi with sterile wire loop from an overnight SSA culture into sterile Muller Hilton Broth solution and incubated at 37 °C for 18 hours. The suspension was then centrifuged at 4000 rpm for 10 minutes to remove the broth. The pelleted bacterial cells were then washed twice with sterile normal saline. The bacteria was re-suspended in normal saline and adjusted to 0.5 McFarland standard containing about 1.5 x 10^8 CFU/m.L Animals in Groups B to E were given 1 mL of the adjusted suspension orally.

To confirm infection, 50µL of blood was drawn from the tail of the infected animals after 48 hours into 1 mL of normal saline, plated on freshly prepared SSA and incubated at 37^{o}C for 24 hours. The emergence of colonies of *S.* Typhi on the SSA confirms the induction in the animals. Only animals with established infection were used for the experiment.

Treatment began 72 hours post-infection and was administered orally at 24-hour intervals for 7 consecutive days). About 50 µL of blood were drawn from the caudal vein of infected animals into 1mL of normal saline and plated on salmonella-shigella agar (SSA) on days 1, 3, 7 and 9 of treatments to monitor the bacterial load. The body weights of the animals were also monitored daily.

2.5.3 Collection of Animal Samples

After seven (7) days of treatment, the animals were fasted overnight and sacrificed by cervical dislocation. Blood was collected via cardiac puncture into a plain sample bottle for serum preparation. The liver, ileum and bone marrow were also quickly excised from the animals. The liver was portioned into three: a portion was rinsed in Phosphate-buffered saline (PBS 0.1M, PH 7.4) another other portion was fixed in 10% formalin (together with the distal ileum) for histological examination and the last portion was rinsed and stored in Hank Buffer Salt Solution (HBSS) kept on ice for assessment of DNA damage. Blood in plain bottles was allowed to clot and thereafter centrifuged at 4000 xg for 5 minutes. The serum (supernatant) was removed with a pipette and used for analysis of selected cytokines.

Table 1. Grouping of experimental animals

GROUPS	Infection with *S.* Typhi	ADMINISTRATION
Group A	No	Distill water
Group B	Yes	Distill water
Group C	Yes	Ciprofloxacin - 14.29mg/kg body weight/day
Group D	Yes	EOPG-100mg/kg body weight/day
Group E	Yes	EOPG-50mg/kg body weight/day

EOPG-essential oil of P. guajava

2.5.4 Enumeration of bacterial load in blood and bone marrow

Five microliter (5 µL) of caudal blood was collected in 1 ml of normal saline and 50 µL of the suspension was plated on sterile SSA. The bone marrow was aspirated with 1 mL sterile normal saline from the femur of the rats and 50 µL of the suspension was plated on SSA. The plates were inoculated at 37 °C overnight, and the actual bacterial content per mL was calculated.

2.5.5 Assessment of liver DNA damage

DNA damage in the liver was quantified using Single Cell Gel Electrophoresis (SCGE), also known as comet assay as described by Lu et al. [18]. Cellular suspensions from livers were obtained by mechanical shaking of 150 mg tissue in 10 mL phosphate-buffered saline (PBS, $Mg^{2+}$and Ca^{2+}-free) with 10% DMSO which was added as an antioxidant before treatment. Differential centrifugation was used to isolate cells from other tissue fragments. Slides were prepared in duplicate for each sample. Five microliters (5 µL) of the cell suspension was combined with 45 µL 1% molten low melting point (LMP) agarose (at 37 °C) and mixed gently by pipetting up and down. 30 µL of the mixture was immediately thinly spread onto a slide that had been pre-coated with 1% normal melting point agarose overnight. The slide was covered with a coverslip and was left on ice for 10 min to allow the agarose to solidify. The coverslip was gently removed and the third layer (75µL of 1% LMP agarose) was spread, covered and left on ice for 10 min. The slide was thereafter immersed in lysing solution (2.5 M NaCl; 100 mM disodium EDTA; 10 mM Tris base; 200 mM NaOH; pH=10; 1% sodium lauryl sarcosinate and 1% Triton X-100; final volme=1000 mL; 4 °C) at 4 °C in the dark overnight to lyse the cells, isolate the nuclei and eliminate the cytoplasm and membranes. The slides were gently removed from the lysing solution and gently immersed in alkaline electrophoresis buffer (Prepare 200 mM NaOH; 1 mM disodium EDTA; pH >13; final volume = 1,000 mL; 4 °C) for 1 h at 4 °C to allow DNA unwinding. Pre-chilled alkaline electrophoresis buffer in the electrophoresis slide tray, not exceeding 0.5 cm above the slides covered and electrophoresed (300 mA; 20 V; 1 V/cm) for 30 min at 4 °C. Excess electrophoresis solution was drained and the slides washed twice by immersion in neutralizing buffer (0.4 M Tris, pH 7.5) before dehydration and fixing in absolute ethanol for 10 min each. Nuclear DNA was stained with 25 µL ethidium bromide (20µg/mL) and observed using a fluorescence microscope (BX60 Olympus) connected to an image-analysis system (Komet 3:1 Kinetic Imaging Ltd.). The result was scored using Comet Score Software (comet 2.0) to analyze the degree of the DNA damage

2.5.6 Pro-inflammatory biomarkers

Serum levels of Interferon-γ (INT- γ), Tumor Necrosis Factor – α (TNF- α) and Interleukin-1β (INT-1β) were measured using ELISA kit for each cytokine (Cayman chemicals, Ann Arbor, MI, USA), according to the manufacturer's guidelines.

2.5.7 Histology of the Liver and Small intestine

Histological analysis was performed by hematoxylin and eosin staining procedure (Pearse, 1985) as described in Olaniyi et al. [10]. Photomicrographs were taken at x100 and x400 objective lenses were archived in the small intestine and the liver respectively.

2.6 Statistical Analysis

The data were expressed as Mean and Standard Error of Mean ± SEM (n=3 for *in vitro*, n = 4) for *in vivo*) animal samples and analyzed using one-way analysis of Variance (ANOVA) test using Graph Pad Prism version 5. The difference between the mean of different groups was analyzed using Duncan's multiple range test at $p< 0.05$

3. RESULTS

The Zone of inhibition of *S.* Typhi growth by EOPG and ciprofloxacin were 13±0.2 and 31±0.3mm respectively (Fig. 1a). The MIC of EOPG and ciprofloxacin are 25±0.00 and 0.313±0.00 mg/mL respectively (Fig. 1b), while the MBC of EOPG >50 mg/mL and 2.5 mg/mL respectively. The minimum biofilm inhibitory concentration of EOPG is 12.5mg/mL a ten-fold of the value for ciprofloxacin 1.25mg/mL (Table 2).

The extracellular concentration of potassium ions and inorganic phosphate increased rapidly in bacterial cells after 60 minutes of exposure to 25mg/mL of EOPG when compared to the control group. In the entire incubation process,

the extracellular level of pyruvic acid increased significantly (P<0.05) upon the exposure of S. Typhi to 25 mg/mL of EOPG The total pyruvic content of the control group remained steady at 1.7mM/L from 6 hours of the exposure to 24 hours of the exposure (Fig. 2a-c).

Thin layer Chromatogram of EOPG with n-hexane: ethyl acetate (9:1) as well as the bioautogram against *S.* Typhi are presented in Fig. 3. The bands marked with no visible growth shows the components of the EOPG that have anti-salmonella activity. These include bands with R_f values of 0.03, 0.09 and 0.18. The other bands show no anti-salmonella activity.

3.1 Compounds identified in active fraction of EOPG

Forty-five compounds were identified in the active fraction of EOPG that showed anti-salmonella activities. Some of the compounds are pyridines, aromatic compounds and amine derivatives (Table 3).

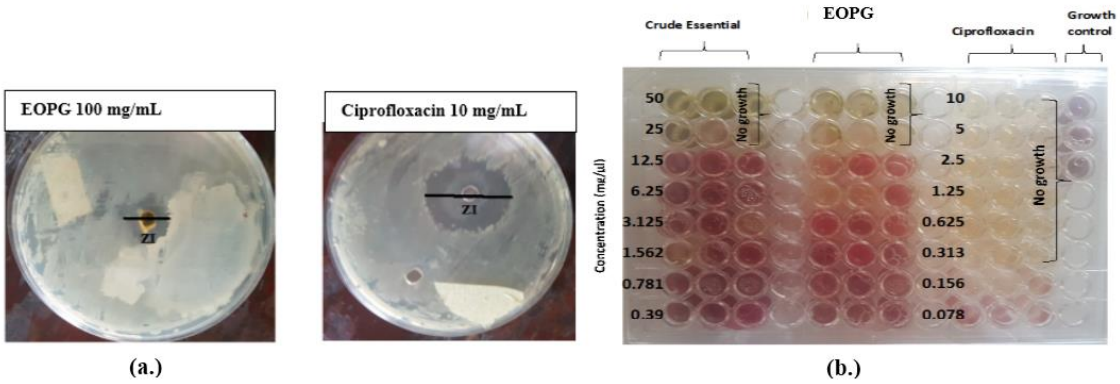

Fig. 1. *In vitro* activities of essential oil of *P. guajava* (EOPG) against *Salmonella* Typhi

Table 2. Biofilm inhibition potential of crude extract and essential oil of *P. guajava* in *S.* Typhi

EOPG (mg/mL)	% Biofilm inhibition	Ciprofloxacin (mg/mL)	% Biofilm inhibition
50.00	7.01	10.00	8.91
25.00	5.59	5.00	5.39
12.50	2.85	2.50	4.03
6.25	NI	1.25	0.78
3.13	NI	0.63	NI
1.56	NI	0.31	NI
0.78	NI	0.16	NI
0.39	NI	0.08	NI

NI – No Inhibition

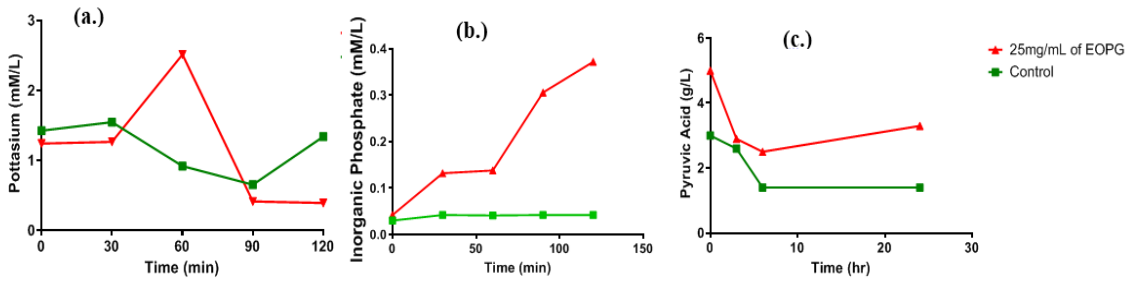

Fig. 2. Time course efflux of (a.) potassium ion (b.) inorganic phosphate and (c.) pyruvic acid from S. Typhi exposed to EOPG

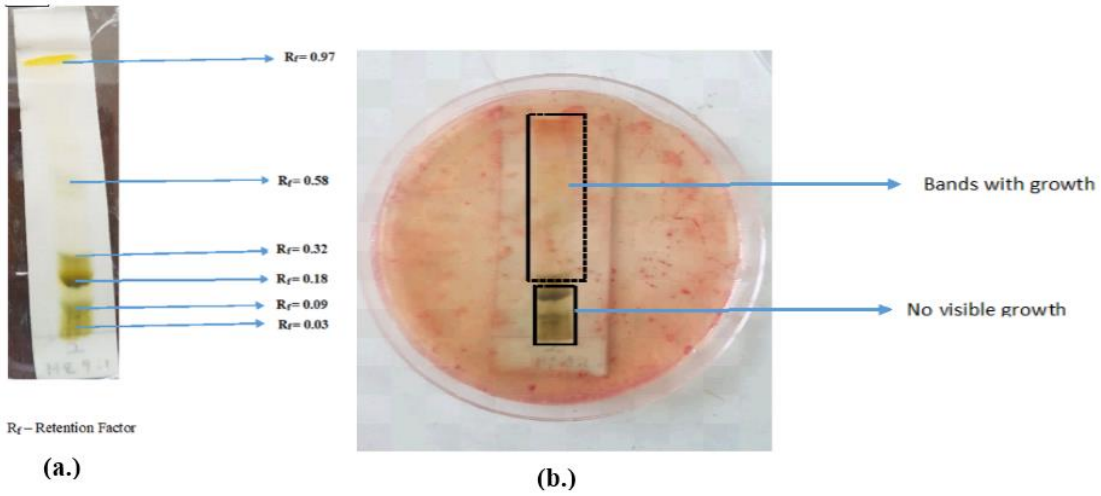

Fig. 3. (a.) Thin layer chromatogram and (b.) Bioautogram of EOPG against *S.* Typhi

Table 3. Compounds identified in active fraction of EOPG

S/no	Library ID	CAS#	Qual	Retention Time
1	Benzenesulfonic acid, 4-methyl-, -cyclopenten-1-ylidenehydrazide	098877-48-6	80	15.621
2	5-Iodo-6-hydroxybicyclo[2.2.1]heptane-2, 3-dicarboxylic acid, 2, 6-lactone	015573-45-2	78	9.203
3	Tricyclo [5.2.1.0 (2,6)] decan-3-one	100191-39-3	76	15.414
4	1,5:2,4-Dimethanopentalene-3,6-diol, octahydro-	066486-73-5	70	12.03
5	2H-Pyran, 2-(3-butynyloxy) tetrahydro-	040365-61-5	64	10.704
6	3-Pyradinamine, N-methyl-2-nitro-	032605-06-4	60	8.008
7	Hydrazine, N1-(4-dimethylaminobenzylideno)-N2-(5-nitrofurfurylideno)	096716-21-1	59	11.955
8	Bicyclo[2.2.2] oct-7-ene-2, 5-dione	017660-74-1	58	9.241
9	Pyridine-2-carboxylic acid 2, 2, 6, 6-tetramethyl-4-oxo-piperidin-1-yl ester	1000275-10-5	58	17.066
10	Tricyclo[3.3.1.1(3,7)] decanone, 4-(acetyloxy)-, (1.alpha.,3.beta.,4.beta.,5.alpha.,7.beta)	055821-13-1	58	12.937
11	Benzisoxazole-2-acetic acid, hydrozide	055244-10-5	56	9.578
12	1-Carboxymethyl-2(1H)-pyridone	056546-36-2	56	6.688
13	2, 6-Pyridinedicarboxaldehyde, 4-methoxy-, bis [methoxy-, bis[methyl(2-pyridyl)hydrazone]	118252-03-2	56	14.651
14	1, 3, 7, 9, 2, 8-Pyrazabole, 4, 5, 10, 11-tetrabromo-2, 2, 8, 8-tetraethyl-6, 12-dimethyl-	1000157-86-1	53	7.664
15	1, 2, 4-Oxadiazol-5(4H)-one, 3-(2, 3-dichlorophenyl)-4-(3-pyridul)-	288246-51-5	53	10.929
16	Pyridine, 2, 6-dichloro-3-nitro-	016013-85-7	53	12.262
17	Azetidine, 1-chloro-2-methyl-	038382-62-6	53	12.593
18	Trichloromethyl chlorodithioformat	091631-89-9	53	17.26
19	Ethanamine, 2-phenoxy-	001758-46-9	50	13.851
20	3-octyn-2-ol	041746-22-9	50	19.787
21	4, 25 - Secoobscurinervan-4-one, O-acetyl-22-ethyl-15, 16-dimethoxy-, (22. alpha.)-	054658-08-1	49	8.759
22	1, 2, 5-Oxadiazol-3-amine, 4-[5-(4-pyridinyl)-1, 2, 4-oxadiazol-3-yl]-	1000337-90-7	47	11.461

S/no	Library ID	CAS#	Qual	Retention Time
23	1-Ethanone, 1-[5-methyl-1-1-[4-(5-methyl-1,2,4-oxadiazol-3-yl)-1,2,5-oxadiazol-3-yl]-1H-1, 2,3-triazol-4-yl]-	1000338-01-5	47	13.425
24	Pyrimido[4,5-d] pyrimidin-4(1H)-one,2,3,5,6,7,8-hexahydro-1-phenyl-6-(2-phenyl)thioxo-	1000351-71-9	47	15.403
25	1-(4-Amino-furazan-3-yl)-5-methyl-1H-[1, 2, 3] triazole-4-carboxylic acid amide	1000300-80-6	46	5.462
26	Acetaldehyde, chloro-	000107-20-0	46	14.908
27	l-Alanine, N-acetyl-3-chloro-, methyl ester	018635-38-6	46	18.204
28	Bicyclo[2.2.1] heptan-2-one, 4,7,7-trimethyl-, semicarbazone	024230-79-3	46	18.104
29	3,5-Dinitro-4-(3,4-xylidino) benzoic acid	299965-36-9	46	11.949
30	Diethyl 2-[(1-oxo-3-(2-pyridinyl)tetrahydro-1H-pyrrolo[1, 2-c]imidazol-2(3H)-yl) (3-phenylpropanoyl)amino]succinate	1000146-10-4	45	17.016
31	Benzoic acid, 4-[3-(5-cyclopropyl-2H-pyrazol-3-yl)-5-mercapto-[1, 2, 4]triazol-4-yl]-	1000303-59-4	45	19.305
32	Diethyl phosphite	000762-04-9	45	7.314
33	Benzopyrazine, 1, 2, 3, 4-tetrahydro-2-oxo-3-difluoroacetylmethylene-	147917-07-5	43	6.795
34	Benzoic acid, 4-nitroso-, methyl ester	013170-28-0	43	11.886
35	Cyanomethyl 2-chloroethyl disulfide	1000226-70-1	43	13.119
36	N-Acrylonitrylaziridine	002407-61-6	43	16.941
37	2-[N-[1,4-Benzodioxan-2-methyl]] aminoethanethiosulfuric acid	060311-26-4	43	11.661
38	Furanzan -3-carbohydrazide, 4-amino-N2-(4-pyridylmethylene)-	1000264-22-7	43	13.938
39	Chromium, tricarbonyl (.eta.6-1, 4-dichlorobenzene)-	086409-62-3	42	15.852
40	2-Penten-4-yne, 2-Methyl-	001595-53-5	42	7.964
41	3-Pyridineacetaldehyde, [2-(3-pyridinyl)ethylidene] hydrozone	056114-45-5	42	16.384
42	N-(.alpha. -Methyl-4-nitrobenzylidene)-O-(phenylcarbomoyl) hydroxylamine	1000223-36-2	42	13.776
43	4-Methyl-2-mercaptopyridine-1-oxide	034341-26-9	42	18.085
44	(4Z)-5-Chloro-3, 4-dimethyl-2, 4-heptadiene	105949-75-5	40	12.118
45	Metaraminol	000054-49-9	40	7.727

3.2 *In vivo* Experiment

There was a significant ($P < 0.05$) weight gain in animals treated with ciprofloxacin and 50mg/body weight of EOPG. There was a little weight gain in the 100 mg/kg body weight treatment when compared to the negative control (Fig. 4).

Administration of treatments (EOPG and ciprofloxacin) led to the reduction of bacteremia count in infected rats while the bacteria load in the blood of negative control animals continued to increase over the period of the experiment. The bacteria load in the bone marrow was significantly ($p < 0.05$) reduced in animals treated with ciprofloxacin and EOPG (50mg/kg body weight and 100mg/kg body weight) as compared to normal ($p < 0.05$) (Fig. 5).

There was a decrease in the level of IFN-γ in treated groups compared to the values in negative control. The values of IFN-γ in *S.* Typhi-infected animals are significantly different compared to the normal control ($p < 0.05$). There was no significant difference in the level of TNF-α across the groups ($P<0.05$). There was an increase in the level of IL-1β of treated groups compared to the untreated groups. The values of IL-1β in the untreated groups are significantly different ($P<0.05$) compared to the treated groups (Table 4).

Animals treated with EOPG and ciprofloxacin showed a slight decrease in the percentage of DNA in tail as compared to the negative control ($p<0.05$) (Fig. 6).

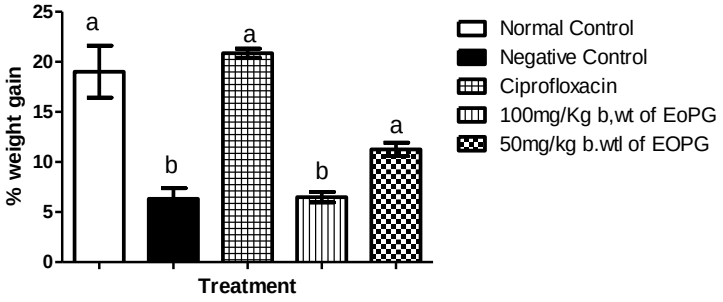

Fig. 4. Effect of essential oil of *Psidium guajava* leaves on percentage weight gain of *Salmonella* Typhi-infected rats.

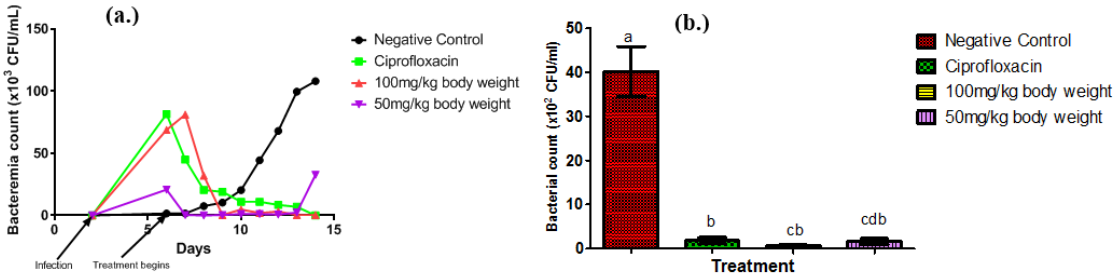

Fig. 5. Bacterial load in (a.) blood and (b.) bone marrow of experimental animals

Table 4. Selected serum pro-inflammatory markers in experimental animals

TREATMENT	IFN- γ (pg/ml)	TNF-α(pg/ml)	IL-1β(pg/ml)
Normal Control	40.19 ± 5.05^{a}	3.35 ± 0.14	64.29 ± 0.55^{a}
negative Control	104.64 ±13.99^{bc}	3.29 ± 0.08	63.99 ± 0.13^{a}
Ciprofloxacin	96.78 ± 6.30^{bd}	3.67 ± 0.23	77.66 ±2.21^{b}
100mg/kg body weight	88.97 ± 6.19^{be}	3.95 ±0.16	75.76 ± 0.50^{b}
50mg/kg body weight	90.26 ± 1.92^{bf}	3.90 ± 0.17	75.31 ± 0.69^{b}

Values are expressed in Mean±SEM (n=4) and considered significant at P value < 0.05. Different superscript alphabets represent significant differences across the row

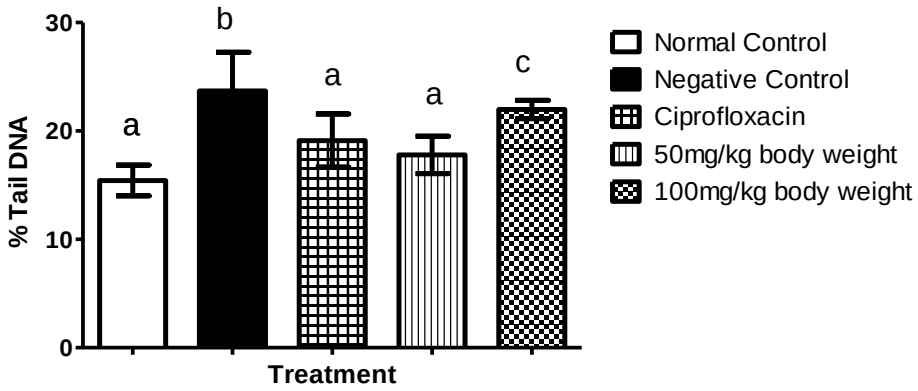

Fig. 6. Percentage of Tail DNA in the liver of experimental rats

Histological examination of the small Intestines of experimental rats (Fig. 7) revealed a normal architecture except for the negative control group and group treated with 50 mg/kg body weight. There was hypertrophy of the muscular layer, presence of perforations as well as vacuolations across intestinal villi and muscularis layer with some ulcerations (spot ulceration, deep ulcer, red colouration), fibrosis/haemorrhage stress is severely observable in the above-mentioned groups.

On examination of the liver, normal control, groups treated with ciprofloxacin and EOPG 50mg/kg body weight show normal central venules without a significant observable congestion, the morphology of the liver cells (hepatocytes) appear normal, the sinusoids appear normal and not infiltrated. Portal triad of normal control, ciprofloxacin, 100mg/kg body weight and 50mg/kg body weight showed an observable dilatations. Plates with significant observable morphocellular alterations is denoted by red thick arrows (Fig. 8).

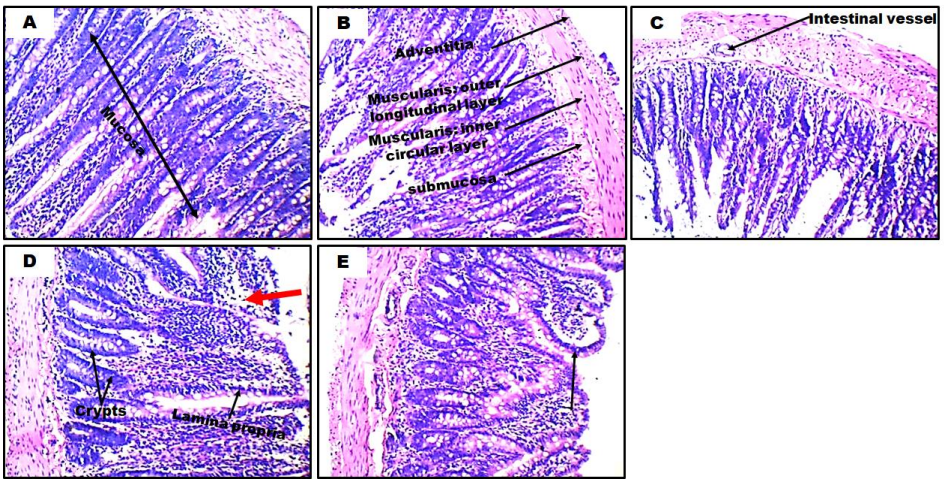

Fig. 7. Photomicrographs of panoramic views of intestine general micromorphological presentations in Adult Wistar rats across the study groups. The Muscularis mucosa, Smooth muscle layer, Submucosa, serosa, Gastric cells comprised of (Chief cells, Goblet cells, and Zymogenic cells), and Connective tissue layer are demonstrated across study groups. Hematoxylin and Eosin stain (X100). Fibrosis, ulceration, haemorrhage, depleting lamina propria and lymphatic nodes, degenerating gastric cells (red thick arrows).

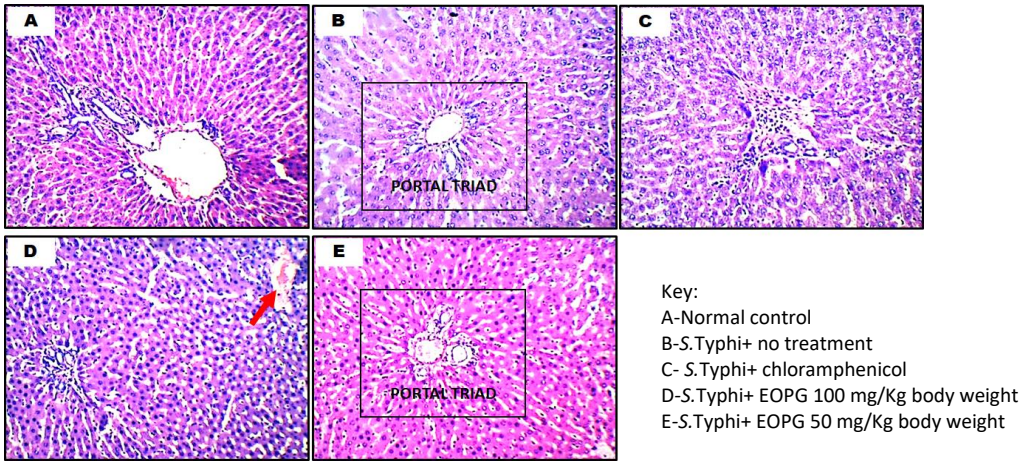

Fig. 8. Magnified views of a liver micromorphological section demonstrated by Haematoxylin and Eosin staining at high magnification (x400). The hepatocytes, sinusoids, and portal triad (hepatic vein, hepatic artery and bile duct) are all visible across the various groups. The red thick arrow denotes areas with observed significant alterations

4. DISCUSSION

Emergence of multidrug-resistant *S.* Typhi strain is a major global threat leading to failure of typhoid treatment cases. Reports of *S.* Typhi resistance to first-line antimicrobial drugs such as ampicillin, chloramphenicol, ciprofloxacin and trimethoprim-sulfamethoxazole are rendering these drugs less efficient in managing typhoid fever [19]. In recent times, essential oils have been reputed for strong antioxidant, antimicrobial, antiviral and antiparasitic activities against drug-resistant microbial strains [20]. In this study, essential oil of *P. guajava* leaves was investigated for activity against *S.* Typhi since the n-hexane soluble fraction of the leaves has demonstrated a reasonable anti-salmonella activity [6].

The essential oil of *P. guajava* leaves (EOPG) showed significant *in vitro* anti-salmonella activity with a clear zone of no bacterial growth around the agar well. Zone of inhibition (ZI) assays measure the ability of an antimicrobial substance to prevent the growth of a bacterium in a solid growth medium into which the substance has been applied [21]. Although the ZI is only 13±0.2 compared to 31±0.3 mm recorded for ciprofloxacin, it could be due to the low diffusion of the essential oil in the aqueous growth media since it is hydrophobic. The result agrees with the antibacterial activities of 11-18 mm (1.1-1.8 cm) exhibited by the same essential oil of *P. guajava* against several other gram-negative bacteria as reported by Baby and Mini [22].

Biofilm plays a major role in the development of chronic carriage of *S.* Typhi in some infected individuals. This community of sessile bacteria are most commonly present in the gallbladder. The ability of EOPG to limit the formation of biofilm *in vitro* could confer an added advantage to its anti-salmonella activity. essential oil

The bacterial membrane provides a permeability barrier to the movement of small molecules in and out of the cell to maintain cellular homeostasis. Therefore, minor changes to the structural integrity of cell membrane can detrimentally affect cell metabolism and lead to cell apoptosis [23]. The internal environment of cells is generally rich in K+, about 100 times higher than the extracellular levels [24], where it influences multiple physiological processes, including membrane potential, acid-base homeostasis, fluid and electrolyte balance and glucose metabolism [25]. Phosphates in the form of inorganic phosphates are important players in energy transfers, protein activation as well as carbon and amino acids metabolism [26]. Pyruvic acid is an extremely important metabolic intermediate. Pyruvic acid, apart from connecting glycolysis and the tricarboxylic acid cycle, plays an important role in infectivity and virulence of *S. Typhi*. Efflux of potassium ions, inorganic phosphate ion and pyruvic acid from the bacterial cells upon exposure to EOPG is an indication of an impaired membrane. These observations are in tandem with those of Bouyhaya et al*.* [27] who reported that essential oils of *Origanum compactum* induced leakages of cytosolic constituents of *Escherichia coli* and *Bacillus subtilis*. An important characteristic of essential oils components is their hydrophobicity, which enables them to partition into the lipids of the bacterial cell membrane, disturbing the cell structures, rendering them more permeable, and leading to lysis and leakage of intracellular compounds (Bajpai et al*.*, 2013).

Bioautography is a cost-effective and less laborious technique for linking fractionation and bioactivity of natural compounds. Bioactivity is assayed directly on a developed thin-layer chromatogram. In this study, bioautography revealed three fractions in which the activity of EOPG resides and GCMS identified 45 compounds from the active fractions. Some of these include bicyclo derivatives, oxadiazole, and aromatic derivatives which have been reported for the antibacterial properties (Jarrahpour et al*.,* 2014; Sabreen et al*.*, 2015; Jeshina, 2017)

It was observed in the *in vivo* study that administration of EOPG significantly reduced the bacterial load at the major culture sites of *S.* Typhi, the blood and bone marrow (). The reduction in bacterial load relieved the animals of some infection-induced anomalies such as retarded weight gain and alteration in serum levels of some pro-inflammatory cytokines. During salmonella infection, due to bacterial invasion of many host cells, there is increase in circulating cytokines such as IL-1, IL-6, TNF-alpha, IFN - α, β and γ) from activated T-cells, leading to clinical manifestation of the disease (Andrade and Andrade, 2003). A significant reduction in the serum level of IFN-γ, as observed in this study correlates with the reduction in bacteremia.

S. Typhi are known to produce endotoxins that damages the host DNA [28]. To assess these possible damages, comet assay was employed

in this study. The results of this study show that the EOPG significantly reduced the damage done to the host hepatic DNA by *S.* Typhi. However, this could be that the EOPG reduced the endotoxin effect by protecting the host DNA or due to the reduced load of the bacteria in the host system.

Salmonella typhi-rich bile flow into the small intestine causes enteritis and ulceration at Peyer's patches in the terminal ileum, caecum and ascending colon of the small intestine [29]. In the histology assessment of the small intestine carried out in this study, the presence of perforations as well as vacuolations across intestinal villi and muscularis layer with some ulcerations (spot ulceration, deep ulcer, red colouration) was observed in the untreated group but was mildly seen in the treated groups. *S.* Typhi may account for these injuries and it is therefore possible that the treatment with EOPG remedied these injuries. Typhoid fever is often associated with liver hepatitis and hepatomegaly has been reported in 55% of patients with typhoid fever [30], the liver histological studies shows normal central venules without a significant observable congestion in the treated groups while there was significant observable morpho-cellular alterations in the untreated which could be an indication of liver hepatitis.

5. CONCLUSION

This study established that the essential oil of the leaves of *P. guajava* (EOPG) has both *in vitro* and *in vivo* anti-salmonella activities. This justifies the traditional use of *P. guajava* in the treatment of typhoid as practised in traditional medicine. This study also analyzed some phytochemicals in the essential oil that could be responsible for its anti-salmonella activity. The presence of different compounds in the EOPG makes it possible to be used as an antimicrobial agent that may offer a low risk of microbial resistance development. The EOPG has been shown to possess anti-biofilm potential and could be used in dealing with carrier status development. The phytochemicals present in the oil can be considered for further investigation in drug development and its use in the treatment of typhoid.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/124856