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PHYTOCHEMICAL SCREENING AND ANTITYPHOID PROPERTIES OF ETHANOLIC LEAF EXTRACTS OF PARQUETINA NIGRESCENS

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ABSTRACT

The study carried out *in vitro* antibacterial activities of *Parquetina nigrescens* leaf extracts for the control of typhoid fever. Cold water extract, hot water extract and ethanolic extract of *Parquetina nigrescens* at various concentrations (50 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml, 250 mg/ml and 300mg/ml) were tested against *Salmonella typhi* at contact time 24, 48 and 72 hours, respectively. All the concentrations showed varying zones of inhibition with ethanolic extract having the highest zone 10.5±0.57 mm at 300 mg/ml and contact time of 24 hours while the least zone of inhibition was recorded for cold water extract (2.9±0.63) at 50 mg/ml and contact time 72 hours. Of the ten standard antibiotics examined, Ciprofloxacin had the highest zone of growth inhibition 12.6±0.20mm. Tarivid had the least zones of inhibition 0.4±0.05 mm while Septrin and Sparloxacin had no effect on *Salmonella typhi*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts on *S.typhi* varied, respectively with the ethanolic extract being the lowest values 12.5 mg/ml and 25 mg/ml. While the cold water extract had the highest values 50 mg/ml and 125 mg/ml, respectively. Phytochemical screening of the *P. nigrescens* extracts showed the presence of saponins, alkaloids, tannins, anthraquinone, terpenoids, flavonoids, ascorbic acid and cardiac glycosides.

Keywords: *Parquetina nigrescens*, ethanolic extract, antityphoid, phytochemical screening.

INTRODUCTION

Typhoid fever

Typhoid fever, known previously as "typhus abdominalis" (from the Greek, meaning smoke, fume, stupor) is an enteric fever manifested as a septicaemic illness, caused by *Salmonella enterica* serovar *typhi (S. typhi) (Eberth's bacillus).* Typhoid fever is a potentially fatal multisystem illness. The protean manifestations of typhoid fever make this disease a true diagnostic challenge. The classic presentation includes fever, malaise, diffuse abdominal pain, and constipation. Untreated, typhoid fever is a gruelling illness that may progress to delirium, obtundation, intestinal hemorrhage, bowel perforation, and death within one month of onset. Survivors may be left with long-term or permanent neuropsychiatic complications. *Salmonella typhi* has been a major human pathogen for thousands of years, thriving in conditions of poor sanitation, crowding, and social chaos (Paragrigorakis *et al*., 2007). The name *S.typhi* believed to cause disease and madness. In the advanced stages of typhoid fever, the patient's level of consciousness is truly clouded (Christie, 2000). Researchers have shown that Salmonella typhi has developed resistant to most of the antibiotics used to cure typhoid fever that is why attention is now shifted to the use of antimicrobial agents to cure the disease or to serve as a good source for new antibiotics.

MATERIALS AND METHODS

Plant collection and identification

Fresh *Parquetina nigrescens* leaves were collected from a compound in Akure metropolis. The leaves were authenticated using the herbarium specimens of the Department of Crop, Soil and Pest Management, Federal University of Technology Akure.

Source and confirmation of bacteria used

Pure isolate of *Salmonella typhi* obtained from the stock culture of the Microbiology Department, University Teaching Hospital, Ado-Ekiti, Nigeria into prepared nutrient agar slant. This bacterial strain was further characterized in the Postgraduate Microbiology Laboratory, Federal University of Technology, Akure.

Drying and extraction of the leaf samples

Parquetina nigrescens leaves were washed with sterile water. The leaves were air dried in room temperature (27±2°C) for 3 weeks. The dried leaves were then ground into fine powder by blending in a high-speed Philips model electric blender. They were separately kept in an airtight container to avoid the absorption of moisture. One hundred and fifty grams (150g) of the powdered sample was respectively soaked in one thousand five hundred millimeters (1500ml) of 70% ethanol, hot water and cold water as solvents to extract the bioactive components. Each container was labeled appropriately and left for 72hours. After which, it was sieved using muslin cloth and then filtered using 0.45µm membrane filter. The filtrates were vaporized to dryness using rotary evaporator (Resona, Germany). The ethanolic, hot and cold water extracts were each transferred into another clean container, packed and placed into a freezer overnight. Frozen extract of each plant was freeze dried in the lyophilizer. This extraction was repeated three times and average percentage yield was calculated. The extracts were preserved in sterile bottles at 4^0C before use (Oladunmoye, 2005 and Dada, 2008).

Phytochemical screening of *Parquetina nigrescens*

Phytochemical analysis was performed using standard procedures as described by Ayoola *et al.*, 2008 and Makanjuola *et al*., 2010 in order to determine the bioactive ingredients present in each plant extract. The phytochemicals determined in the three extracts are anthraquinones, terpenoids, flavonoids, saponins, tannins, alkaloids, cardiac glycosides and ascorbic acid.

Test for anthraquinones

A half grams (0.5g) of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for terpenoids (Salkowski test)

Two millimeter (2ml) of chloroform was added to 0.5g each of the extract. Concentrated H_2S0_4 (3ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

Test for flavonoids

Five millimeters (5ml) of dilute ammonia was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1ml) was added. A yellow colouration that disappears on standing indicates the presence of flavonoids.

Test for saponins

Five millimeters (5ml) of distilled water was added to 0.5 g of the extract in a test tube. The solution was shaken vigourously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigourously after which it was observed for the formation of an emulsion.

Test for tannins

A half grams (0.5g) of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

Test for alkaloids

A half grams (0.5g) of extract was diluted to 10 ml with acid alcohol, boiled and filtered. Two millimetres (2 ml) of dilute ammonia was added to 5ml of the filtrate. Five millimetres (5ml) of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

Test for cardiac glycosides (Keller-Killianitest)

A half grams (0.5g) of extract were diluted in 5 ml of distilled water, two millimetres (2ml) of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for phlobatanins

The production of a red precipitate from boiling aqueous solution of the extract in 1% aqueous hydrochloric acid confirmed the presence of phlobatanins.

Test for ascorbic acid

A tablespoon of cornstarch was mixed into 20 millimitres of water to make paste, 250 millimitres of water was added to the paste and boiled for 5 minutes. Ten drops of the starch solution was added to 75 millimitres of water (use an eyedropper) then iodine solution was added to produce a dark purple-blue colour. This serves as indicator. Five millimiteres of indicator solution was put in a 15 millimitres test tubes, a clean eyedropper was used to add 10 drops of 0.5g of the extract diluted in 5 ml of distilled water. The test tubes were held against a white background. The test tubes were lined up from lightest to darkest purple. The lighter the solution, the higher the vitamin C content. This is because vitamin C causes the purple indicator solution to lose its colour.

Preparation of crude extract of *Parquetina nigrescens*

The method of Akujobi *et al*., (2004) was adopted. The crude extract of ethanolic *Parquetina nigrescens* was reconstituted with 30% dimethylsulphoxide (DMSO) while hot and cold crude extracts were prepared in sterile distilled water to obtain concentrations of 300 mg/ml, 250mg/ml, 200 mg/ml, 150 mg/ml, 100 mg/ml, and 50 mg/ml, respectively.

Preparation of standard inocula of *Salmonella typhi* for *in vitro* assay

In preparing the standard inocula of *Salmonella typhi* for *in vitro* assay the method described by Andrews (2006) was employed. Three isolated overnight cultured colonies were transferred to a tube of sterile saline. The bacterial suspension was compared to the 0.5 McFarland standards against a sheet of white paper on which sharp black lines were drawn. The bacterial suspension was adjusted to be the proper density as the McFarland 0.5 by adding sterile saline or more bacterial growth. Then bacterial suspension was diluted to obtain 10^6 colony forming unit per millimetres (CFU/ml).

Antimicrobial sensitivity test

Method described by CLSI (2001) was used to determine the sensitivity of *Salmonella typhi* to the extracts of *Parquetina nigrescens*. With the aid of sterile pipette, half millimetres (0.5ml) of the bacterial

suspension was drawn out and was aseptically introduced into sterile petridishes. Sterilized Mueller Hinton Agar (MHA) that had been cooled to about 45^0C was aseptically poured into the petridishes containing 0.5 ml of *S. typhi*. Each petridish was gently swirled in a clockwise direction in order to ensure that the bacterium is homogeneously distributed with the MHA. The plates were allowed to stand for 40 minutes for the inoculated bacteria to be established in the medium. Four wells of about 6.0 mm diameters were aseptically bored on each agar plate using a sterile cork borer at allowance of 30mm between adjacent wells and between the edges of the petridish. A 0.1 ml of the different concentrations of the extracts was then introduced into each well in the plates using sterile syringe. A control well was in the centre with 0.1ml of the extracting solvent. The plates were labelled and allowed on the bench for 40 minutes for pre-diffusion of the extract to occur. The plates were incubated at 37^0C for 24 hours, 48 hours and 72 hours. The resulting zones of inhibition were measured using a calliper. The average of the three readings was taken to be the zone of inhibition of the bacterial isolate in question at that particular concentration and specific contact time.

Antibiotic assay

Disk Diffusion Method as described by Monica, (2006) was employed to compare the efficacy of the commercial antibiotics with the crude plant extracts on *Salmonella typhi*. Standard antibiotic disc with ten different antibiotics were used against *S. typhi*. With the aid of sterile pipette, half millimetres (0.5ml) of the bacterial suspension was drawn out and was aseptically introduced into sterile petridishes. Sterilized Mueller Hinton Agar (MHA) that had been cooled to about 45^0C was aseptically poured into the petridishes containing 0.5 ml of *S. typhi*. Each petridish was gently swirled in a clockwise direction in order to ensure that the bacterium is homogeneously distributed with the nutrient agar. The plates were allowed to stand for 40 minutes for the inoculated bacteria to be established in the medium. The filter paper strips of standard antibiotics (Septrin, Ciprofloxacin, Gentamycin, Ampicillin, Tarivid, Chloramphenicol, Perfloxacin, Amoxacillin, Augmentatin, and Streptomycin) were laid aseptically on the plate using a pair of forceps. The plates were incubated at 37^oC for 24 hour. After the incubation period, petridishes were observed for zones of inhibition around the disc to which the bacteria is sensitive. The diameter of the clear zone was measured in millimetres (mm) using a caliper and was recorded.

Determination of minimum inhibitory concentration (MIC) and determination of minimum bactericidal concentration (MBC)

A dilution method described by Monica (2006) was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the different crude extracts. Different concentrations of the crude extracts were prepared at 125 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.5 mg/ml. Mueller Hinton Broth (MHB) was prepared and five millimetres is drawn out with sterile pipette into test tubes with the aid of a sterile syringe, then 0.1 ml of inoculum of *Salmonella typhi* ($1.0x10^6$ cell/ml) was inoculated into each test tube and mixed thoroughly. With the aid of sterile syringe, one millimetre of the different concentration of plant extracts was withdrawn into each test tube containing the broth culture of *S. typhi.* These test tubes were then incubated at 37^0C for 24 hours. Growth in each tube was checked for by turbidity measurement using a spectrophotometer. Growth inhibition was indicated by low turbidity while growth was indicated by high turbidity. The concentration at which there was no growth as indicated by clear broth is taken as the minimum inhibitory (MIC) while the MBC was determined by first selecting tubes that showed no growth during MIC determination. A loopful from each tube was subcultured onto extract free agar plates, incubated for further 24 hours at 37^0C. The least concentration, at which no growth was observed, was noted as the MBC.

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and the Duncan's New Multiple Range test was used to separate the means. The level of significance was considered at P 0.05.

RESULTS AND DISCUSSIONS

The recovery of the plant extract expressed in percentage in (Table-1) showed that ethanol gave the highest recovery value of 30.6±1.38% followed by hot water with 21.0±0.57% and cold water with 16.7±0.11% respectively. The vary in the percentage yields of the extract using different solvents is in agreement with Kordali *et al.* (2003) who worked on the antifungal activities of leaves of three *Pistacia species* and reported that the percentage recovery was dependent on the solvent used. The results also corroborate those obtained by Campos *et al*., (2002) in which the percentage recovery of *Croton cajucana* varied with the different extraction solvents. The ethanol yielded the highest value of the extract. The reason might be due to the organic nature of ethanol as well as being polar permitting it to actively dissolve the chemical components of the leaves that have been shown to be organic in nature and slightly polar. Like solvent have been found to dissolve like solute compared with chloroform which solubilizes mainly flavanoids (Phenolic compounds from plant) (Mann *et al.*, 1997). Percentage recovery of the extracts from cold and hot water varied. Hot water produced higher yield. This is in line with David, (2001) and Oladunmoye, (2005) who reported that the solubility of non-volatile compounds has been shown to be temperature dependent. This could account for the higher values obtained for hot water.

The phytochemical screening of the plant extracts summarized in (Table-2) indicated that the phytochemical constituents of *Parquetina nigrescens* extracts varied with the extracting solvent. The amount of bioactive compounds present in ethanolic extract is more than that

of hot and cold water extracts while that of hot water extract is more than cold water extract as indicated by colour changes. Also hot water contains bioactive compounds higher than that of cold water. This result corroborate the report of Olowosu and Ibrahim, (2006) that the relative amount of phytochemical substances from plant extract depends on the solubility of the phytochemical in the solvent used for extraction.

The sensitivity pattern of *Salmonella typhi* to the antimicrobial activity of each extract at different concentrations is shown in Figures 1 to 6. The results agree essentially with several investigations and reports that *Parquetina nigrescens* contains antimicrobial substances (Ibekwe *et al*., 2001; Akujobi *et al.*, 2006; Flora *et al.*, 2008; Makanjuola *et al*., 2010 and Imaga *et al*., 2010). The result showed that cold and hot water extracts of *Parquetina nigrescens* were not as effective in antibacterial activity against *S. typhi* as ethanolic extract. This is not surprising since ethanolic extract has the highest amount of bioactive compounds and Flora *et al*., (2008) reported that ethanol is generally able to dissolve multivariable types of compounds; polar and non-polar, simple and complex chemical structures.

There was an increase in the zones of inhibition of the extracts as the concentration increased. It is noteworthy that the antibacterial activities of these plants extracts were dependent on the concentration of the extracts as reported by Ekwenye and Elegalam, (2005). Also, if the extract has high molecular weight, the rate of diffusion is always slow, reduced and also takes longer time, whereas an extract of low molecular weight diffuses faster and at a quicker rate (Parekh, *et al*., 2007). The decrease in the zones of inhibition of the extracts with increase in contact time can be attributed to the facts that crude extracts are liable to contamination and deterioration which reduces them in susceptibility unlike standard antibiotics which have stable antimicrobial potency over the period of 72 hours Lenta *et al*., (2007). This result could be attributed to fact that convectional antibiotics and other pharmaceutical products are usually prepared from synthetic materials by means of reproducible manufacturing techniques and procedures expressing purity and high fractionation which certainly will enhance antimicrobial effect and stability than crude extracts (Makanjuola, 2010).

The minimum inhibitory concentration is the least concentration of the extracts needed to inhibit the growth of *Salmonella typhi* while minimum bactericidal concentration is the least concentration of the extracts needed to kill *Salmonella typhi.* The observed MIC and MBC values of each extract on *S. typhi* are shown in Table-3. The MIC of 12.5 mg/ml was observed for ethanolic extract of *Parquetina nigrescens* on *S. typhi* which is the lowest value while 25.0 mg/ml was observed for hot water extract and 50 mg/ml which is the highest value was observed for cold water extract of *Parquetina nigrescens.* The least MBC value was observed for ethanolic extract of *Parquetina nigrescens* while the highest value was recorded for cold water of *Parquetina nigrescens.*

The result of antibiotic sensitivity pattern on *Salmonella typhi* is shown in Table-5. Ten commercial antibiotics (standard) were tested against *S. typhi*, eight (Ciplofloxacin, Ofloxacylin, Perfloxacin, Streptomycin, Augmentatin, Tarivid, Amoxycillin and Chloranphenicol) had antimicrobial effect in decreasing order. For example ciprofloxacin had the highest zone of growth inhibition against *S. typhi* and remarkably has become the antibiotics of choice in the treatment of typhoid fever. Unfortunately, resistance of S*almonella typhi* to all of these antibiotics is becoming more common globally. As such, appropriate treatment varies with geographic distribution of resistant strains (Flora *et al*., 2008). The antimicrobial properties exhibited by the extracts may be associated with the presence of tannins, sapoins, flavonoids, anthraquinone, terpenoids and alkaloids found in the plant extracts. A large number of flavonoids have been reported to possess antimicrobial properties (Olowusulu and Ibrahim, 2006; Akinjobi *et al*., 2006 and Makanjuola *et al*., 2010). Flora *et al*., (2008) attributed the antimicrobial activities of flavonoids to their ability to complex with extracellular and soluble proteins as well as their ability to complex with bacterial cell walls. They suggested that more lipophylic flavonoids exert antimicrobial activity by disrupting microbial cells membranes.

The result of comparative zones of inhibition of standard antibiotics with crude leaf extracts using Duncan multiple range test is shown in Table-6. The result shows that only Ciprofloxacin among standard antibiotics has the zone of inhibition that is higher than that of plant extracts at various concentrations. Ethanolic extract at 300 mg/ml has the zones of inhibition higher than other extracts at various concentrations and standard antibiotic discs except Ciprofloxacin.

Table-1. Percentage yield of plant extracts.

Name of the plant	Part used	70% ethanol	Hot water	Aqueous water
Parquetina nigrenscens	Leaf	30.6±1.38	21.0±0.57	16.7±0.11

Table-2. Phytochemical constituents of *Parquetina nigrescens.*

Test	Ethanolic extract	Hot water extract	Cold water extract
Anthraquinone	+++	+++	+++
Terpenoids	+++	++	+
Flavonoids	+++	+	-
Saponins	++	+	+
Alkaloids	+++	++	+
Tannins	++	-	-
Cardiac glycosides	+++	+	-
Phlobatanins	++	+	+
Ascorbic acid	++	+	++

Keys: - = beyond detectable limits; + = Present; ++ = moderately present;
+++ = Highly present.

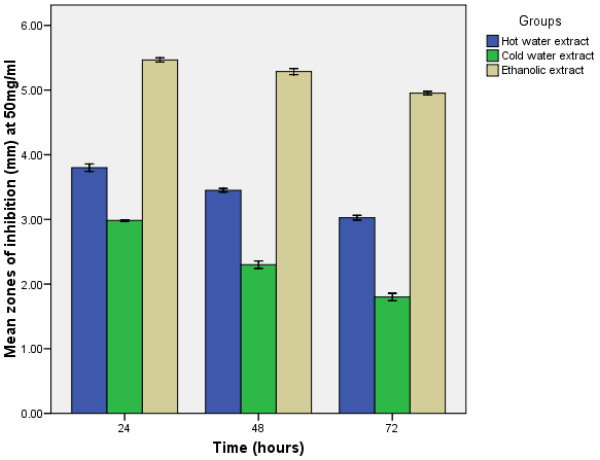

Figure-1. Inhibition zones (mm) of *Parquetina nigrescens* on *Salmonella typhi* at 50mg/ml.

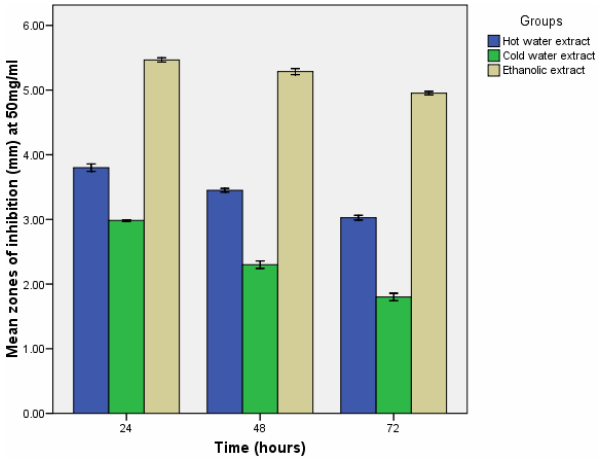

Figure-2. Inhibition zones (mm) of *Parquetina nigrescens* on *Salmonella typhi* at 100mg/ml.

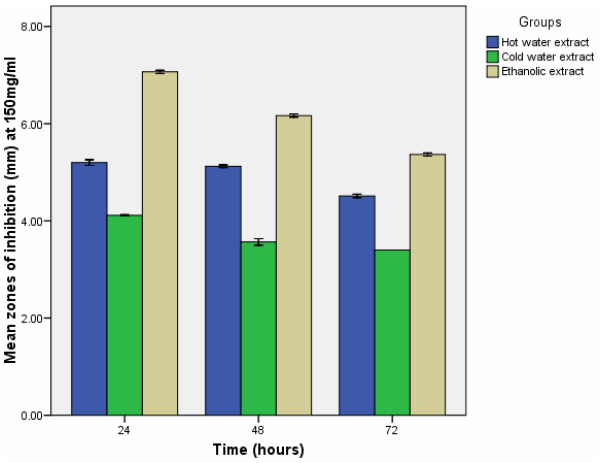

Figure-3. Inhibition zones (mm) of *Parquetina nigrescens* on *Salmonella typhi* at 150mg/ml.

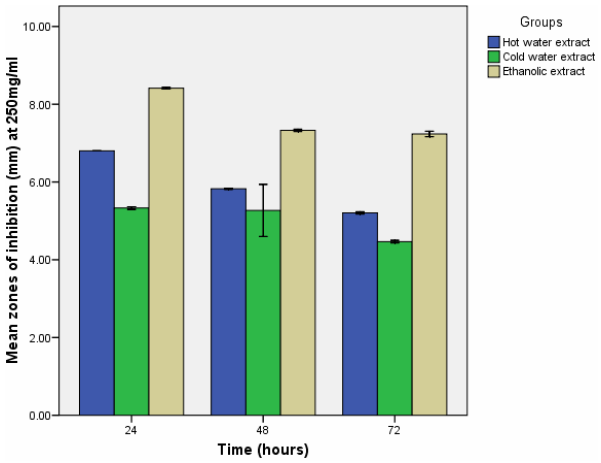

Figure-5. Inhibition zones (mm) of *Parquetina nigrescens* on *Salmonella typhi* at 250mg/ml.

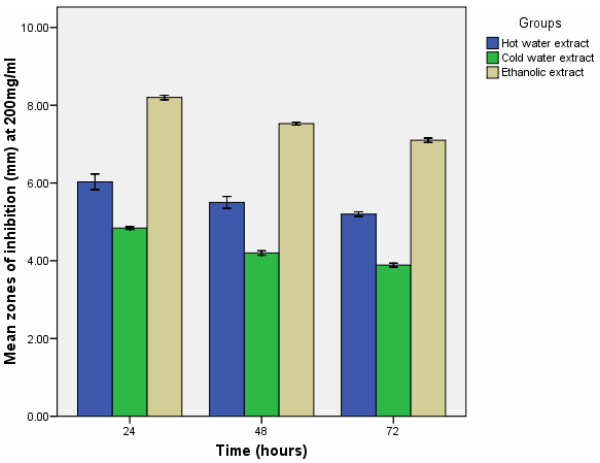

Figure-4. Inhibition zones (mm) of *Parquetina nigrescens* on *Salmonella typhi* at 200mg/ml.

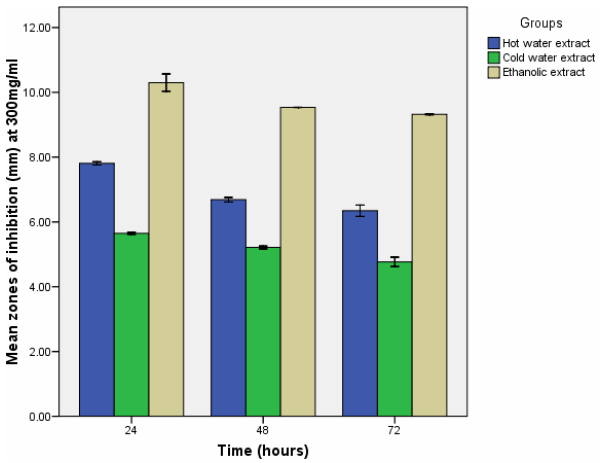

Figure-6. Inhibition zones (mm) of *Parquetina nigrescens* on *Salmonella typhi* at 300mg/ml.

Table-5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *prquetina nigrescens* extracts on *S. typhi*.

Extracts	MICs in mg/ml	MBCs in mg/ml
Cold water extract	50.0	125.0
Hot water extract	25.0	50.0
Ethanolic extract	12.5	25.0

Table-6. Zones of inhibition of standard antibiotics against *Salmonella typhi*.

Standard agents	**Zones of inhibition** (mm) **+ SD**		
	24 Hours	**48 Hours**	**72 Hours**
Septrin 30µg	Nil	Nil	Nil
Chloranphenicol 30µg	0.4±0.05	0.4±0.05	0.4±0.05
Sparloxacin 10µg	Nil	Nil	Nil
Ciprofloxacin 10µg	12.6±0.20	12.6±0.20	12.6±0.20
Amoxacillin 30µg	1.0±0.00	1.0±0.00	1.0±0.00
Augmentatin 30µg	2.0±0.00	2.0±0.00	2.0±0.00
Gentamycin 30µg	4.0±0.00	4.0±0.00	4.0±0.00
Perfloxacin 30µg	4.0±0.00	4.0±0.00	4.0±0.00
Tarivid 10µg	1.0±0.00	1.0±0.00	1.0±0.00
Streptomycin 30µg	3.6±0.05	3.6±0.05	3.6±0.05

Table-7. Comparative zones of inhibition of standard antibiotics with crude leaf extracts using Duncan multiple range test.

Antimicrobial agents	**Zones of inhibition** (mm)	**Duncan Multiple Range**
Septrin 30µg	0.0±0.00	a
Chloramphenicol 30µg	0.4±0.10	b
Sparloxacin 10µg	0.0±0.00	a
Ciprofloxacin 10µg	12.6±0.05	c
Amoxacillin 30µg	1.0±0.00	d
Augmentatin 30µg	2.0±0.00	e
Gentamycin 30µg	4.0±0.00	f
Perfloxacin 30µg	4.0±0.00	f
Tarivid 10µg	1.0±0.00	d
Streptomycin 30µg	3.6±0.05	g
Cold water extract (50mg/ml)	2.9±0.01	h
Cold water extract (100mg/ml)	3.4±0.02	i
Cold water extract(150mg/ml)	4.1±0.05	j
Cold water extract (200mg/ml)	4.8±0.01	k
Cold water extract (250mg/ml)	5.1±0.05	l
Cold water extract (300mg/ml)	5.6±0.00	m
Hot water extract (50mg/ml)	3.8±0.05	n
Hot water extract (100mg/ml)	4.2±0.04	j
Hot water extract (150mg/ml)	5.2±0.05	l
Hot water extract (200mg/ml)	6.0±0.01	q
Hot water extract (250mg/ml)	6.8±0.00	r
Hot water extract (300mg/ml)	7.8±0.00	s
Ethanolic extract (50mg/ml)	5.5±0.01	m
Ethanolic extract (100mg/ml)	6.5±0.00	u
Ethanolic extract (150mg/ml)	7.2±0.34	w
Ethanolic extract (200mg/ml)	8.2±0.05	x
Ethanolic extract (250mg/ml)	8.4±0.05	y
Ethanolic extract (300mg/ml)	10.5±0.05	z

Means within the column with the same alphabet are not significantly different

CONCLUSIONS

The information presented in this study on the antibacterial properties of the leaf extracts of *Parquetina nigrescens* might provide incentive for proper evaluation of the plant in ethnomedicine. The antimicrobial actions of ethanolic leaf extract of *Parquetina nigrescens* on *Salmonella typhi* are significant and therefore justify the use of the plant in traditional medicine. Therefore, it can be recommended as a good antimicrobial agent for treating typhoid fever.

However, there is need to conduct toxicity study on *Parquetina nigrescens*, its effect on enzymes, its action on the respiratory system, maximum duration of use and possibility of using other solvents for extraction. Further investigation into the antiviral, nematocidal, antiprotozoan and insecticidal activities of the *Parquetina nigrescens* will be useful in the field of human medicine. Pharmacologically, its anti-inflammatory, analgesic, antioxidant, hypoglacaemic, anti-diabetic and anti-carcinogenic properties can also be investigated.

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