Biomedicine and Biotechnology, 2014, Vol. 2, No. 1, 20-28
Available online at http://pubs.sciepub.com/bb/2/1/4

DOI:10.12691/bb-2-1-4

Alkaloids and Phenolic Compound Activity of *Piper Nigrum* against Some Human Pathogenic Bacteria

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Received January 07, 2014; Revised January 20, 2014; Accepted February 10, 2014

Abstract This study focused on the production of antibacterial compound against human-pathogenic bacteria. Alkaloid and Phenols extract prepared with selected concentrations from Piper nigrum (fruits) to screening for antibacterial activity against four Gram-positive of Staphylococcus aureus and Streptococcus spp. strains with four Gram-negative of Escherichia coli strains bacteria. The antibacterial activities of the extracts (0.002, 0.004, 0.02, 0.04, 0.2, and 0.4%) were evaluated using agar diffusion methods. The completely randomized design (CRD) was used with three replications. The analysis of the alkaloid and phenol were made by fast liquid chromatography (FLC). The result showed antimicrobial activity against all tested bacteria with zone of inhibition ranged from (1-60mm). Alkaloids showed Maximum zone of inhibition against Gram negative bacteria Escherichia coli(PBR 322 standard strain) (26.4 mm) and minimum against Gram positive bacteria Staphylococcus aureus isolated from Urinary tract infection (10.5 mm). While Phenols showed maximum antibacterial activity towards Gram positive bacteria Staphylococcus aureus (isolated form Dermal infection) (22.4 mm) and least effect against Gram negative bacteria Escherichia coli (PBR 322 standard strain) (13.4 mm). Also the results showed maximum concentration activity was 0.4% which recorded for Alkaloids and Phenols (43.7 and 48.1 mm) respectively, while the minimum concentration activity was 0.002% recorded (0.6 and 1 mm) respectively. Beside FLC analysis results showed two alkaloid with ten phenolic compounds in P. nigrum crude extracts. The crude extract of P. nigrum demonstrated a significant antibacterial activity against the microorganisms investigated and could be used as antibacterial agents.

Keywords: *black pepper, antibacterial activity, alkaloid compounds, phenolic compounds*

Cite This Article: Ayyad Wajeh Al-Shahwany, "Alkaloids and Phenolic Compound Activity of *Piper Nigrum* against Some Human Pathogenic Bacteria." *Biomedicine and Biotechnology*, vol. 2, no. 1 (2014): 20-28. doi: 10.12691/bb-2-1-4.

1. Introduction

It is estimated that there are 250,000 to 500,000 species of plants on Earth. Relatively small percentages (1 to 10%) of these are used as foods by both humans and other animal species. It is possible that even more are used for medicinal purposes [1]. Hippocrates (in the late fifth century B.C.) mentioned 300 to 400 medicinal plants [2].

Black pepper (*Piper nigrum* L.) is a flowering vine of the Piperaceae family that cultivated for its fruit, which is usually dried and used as a spice and seasoning. *P. nigrum* native of south India and popularly known as "King of Spices". It is commonly used in curry recipes, as masalas and also included in the prescriptions of Ayurvedic and other traditional medicinal systems. Pepper is also used in folk medicine as aphrodisiac, carminative, stomachic, antiseptic diuretic and for the treatment of cough, rheumatoid arthritis, peripheral, acetic acid, neuropathy, melanoderma and leprosy due to the presence of volatile compounds, tannins, phenols, alkaloids, and other unknown substances [3].

According to (Friedman *et. al.,* 2008) [4] alkaloids play a significant role in plant physiology, agriculture, host-plant resistance, entomology, the diet and medicine. Among them piperine is the major chemical constituent responsible for the bitter taste of the black pepper. Phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl groups and range from simple phenolic molecules to highly polymerized compounds [5]. Polyphenols may be associated with various carbohydrates and organic acids [6]. These compounds exhibit a wide range of physiological properties, such as anti-inflammatory, antimicrobial and antioxidant effects [7,8]. Although a great amount of research has been performed to determine the antibacterial activity of medicinal plants, optimal extraction of bioactive compounds has not been well established for most plants. Unfortunately, the development of bacterial resistance to these antimicrobials quickly diminished this optimism. Therefore, the objectives of this study were to optimize the concentrations of *Pepper nigrum* alkaloids and phenols antimicrobial activity against some of human pathogenic bacteria.

2. Materials and Methods

2.1. Plant Material and Extraction

The *Piper nigrum* fruits were purchased from local market. After the fruit were air derided and powdered, it kept at 4°C until further investigations.

2.1.1. Preparation of Plant Extracts

a-Alkaloids

The extraction was prepared according to the method of (Harborne, 1984) [9] were considered. Quantities of 100 g dried materials were homogenized in electrical shaker with 350 mL of (4:1) ethanol: Distilled Water, then filtered through muslin. Then through a filter paper in Bouknner funnel, the filtrate was concentrated to quarter of original volume, then acidified by drops of 2% H2SO4 until the pH became between 1 and 2, then it was extracted with chlorophorm 3 times in the separating funnel; alkaloids were precipitated by the addition of drops of concentrated NH_4OH, pH became 9.0 and 10.0. Then extracted with chloroform-methanol (1:3) twice and with chloroform once, 2 layers appeared, lower layer was neglected because it containing weak alkaloids, while the upper layer, aqueous layer was dried by rotary evaporator; dried residue was extracted with methanol. Then kept in refrigerator until use.

b-Phenols

The extraction was made according to [10] and [9], 200 g of dried materials were divided into 2 equal quantities, one was mixed with 300 mL of Distilled Water. and another one was mixed with 300 mL of 1% HCl. Then samples were homogenized in electrical shaker for 5 min., and warmed by using centrifuge. The supernatants were mixed with equal volume of N-propanol and saturated with amount of NaCl in separating funnel, 2 layers were appeared: The lower one (aqueous layer) was extracted with amount of ethyl acetate and concentrated by using rotary evaporator. The upper layer was dried by rotary evaporator at 40°C. Dry material for both layers was dissolved with 5 mL of 96% ethanol, the 2 layers were dried using electrical oven at 40°C, and then kept in the refrigerator until use.

2.1.2. Concentrations Preparation

Stock solutions were prepared by mixing 2 g from the dried extract with 20 mL Ethylene glycol, and then it was sterilized with Millipore membrane filter (0.22 µm). Then different concentrations of (0.002, 0.004, 0.02, 0.04, 0.2, and 0.4%) mg. mL^{-1} were prepared by mixing known volume from the stock solution with Ethylene glycol using the following equation:

$C1V1 = C2V2$.

C1 = Concentration of stock solution.

V1 = Volume that obtained from stock solution.

C2 = Final concentration.

V2 = Final volume.

Ethylene glycol was the solvent which used as diluent solution.

2.2. Lant Extracts Antibacterial Activity Determination

2.2.1. Test Microorganisms

Test organisms were obtained from laboratory of Biotechnology Dept., College of Science, University of Baghdad; (Table 1). Four gram-negative *E. coli* strain, while four gram-positive bacteria were *Staphylococcus aureus, Streptococcus agalactiae* and *Streptococcus pyogenes*. For the purpose of antimicrobial evaluation, the microorganisms were cultured in Tryptone Soya Broth (TSB) (Oxford, England) at room temperature for 24 hr. and were adjusted to 10^7 $cfumL^{-1}$ with sterile saline.

Table 1. The microbial isolates used in the study

No.	Microorganism	Types of samples
1	*Streptococcus pyogenes*	Blood
2	*Staphylococcus aureus*	Blood
3	*Staphylococcus aureus*	Dermal infection
4	*Streptococcus agalactiae*	Urinary tract infection
5	*Escherichia coli*	Urinary tract infection
6	*Escherichia coli*	PBR 322 Standard strain
7	*Escherichia coli*	PBR 322 AMP Resistance TET resistance Contain plasmid standard strain
8	*Escherichia coli*	Patient stool

2.3. Activation and Maintenance of Isolates

Bacterial cultures were activated in test tubes containing 5 mL of nutrient broth, and sabaraud dextrose broth for the bacteria, and then incubated for 24 hours at 37°C; nutrient agar was used for bacterial strains storage at 4°C.

2.3.1. Plants Extract Anti-Bacterial Activity

The anti-bacterial activity of the extracted alkaloids and phenols compounds were tested against bacteria by using agar-well diffusion method as follows:

1. 25 mL of sterilized Mueller Hinton agar was poured into numbers of petri-dishes as required.
2. Bacterial broth of 18 hr. was prepared; a sterile cotton swab dipped into the broth, the entire agar surface of each plate was inoculated with this swab, first in the horizontal direction then in vertical direction to ensure even distribution of the organism over the agar surface.
3. Wells were prepared in plates with help of a sterile cork borer (8 mm in diameter).
4. 0.2 mL of different concentrations of the extracted oil was introduced into those wells; a control well was introduced by the diluent of oils (Ethylene glycol) instead of the oil. Replicates were made for each test.
5. Plates were incubated at 37°C for 24-48 hrs.
6. The anti-bacterial activity was recorded by measuring the diameters of clear zones of inhibition [11,12].

2.3.2. Analysis of Chemical Composition of the Plant Extracts by FLC

The analysis of the chemical composition was made by fast liquid chromatography (FLC). FLC consists from a mobile phase which is polar and consists of a mixture of solvents such as water and acetonitrile, while the stationary phase comprises of a column which is usually stainless steel and packed with silica particles, a sample of 50 µl was injected into the mobile phase using procedure outlined by Hartley and Buchan and it passes along the stationary phase, the time taken for a sample to pass through the system is recorded as its retention time and is one of the characteristic used to identify the compound, all the compounds were separated and identified using FLC

with separation conditions C-18, 3 cm particle size, 50 × 4.6 mm internal diameter of the column, detection U.V. set at 275 nm, flow rate 0.7 mL/min. and 30°C temperature, but the differences were in mobile phase which was 0.1 % acetic acid and acetonitrile using linear gradient from 0-100% B in 10 min. with phenolic compounds, However, it was deionized water: methanol 40:60 V/V in alkaloids case and 0.1% acetic acid in deionized water: acetonitrile (20-80V/V) in terpens. The area under a peak is used for calculating the concentration of a sample as the following formula:

$$\text{Conc. of sample}\left(\mu g.ml^{-1}\right) = \frac{\text{Area of the sample}}{\text{Area of the standard}} \times \text{Standard conc.} \times \text{Dilution factor}$$

Analysis of the chemical composition was made by injecting 20 µl of the extract of each sample in FLC for identification. The procedure that used outlined by Hartley and Buchan. The conditions of separation were listed in Table 2 and Table 3. The peaks were detected by UV detector. The analysis was carried out in the laboratories of Ministry of Science and Technology [13].

Table 2. Conditions of Fast Performance Liquid Chromatographic used for analysis of alkaloid compounds of the plants extracts

Parameter	Characteristic
Column dimensions	3µm particle size (50 × 4.6 mm ID)
Flow Rate	1.0 mL/min
Detector	UV spectrophotometer at 280 nm
Volume injection sample	20 µl
Type of Column	C-18
Mobile face	Were 0.01 M phosphate buffer pH 6.2: acetonitrile (75:25 V/V)
Temperature	30°C

Table 3. Conditions of Fast Liquid Chromatographic used for analysis of phenolic compounds of the plants extracts

Parameter	Characteristic
Column dimensions	3µm particle size (50 × 4.6 mm ID)
Flow Rate	1.4 mL/min
Detector	UV spectrophotometer at 330 nm
Volume injection sample	20 µl
Type of Column	C-18
Mobile face	Solvent A: 0.1% phosphoric acid in deionized water. Solvent B 50:50 V/V, 0.1% phosphoric acid in deionized water: acetonitrile HPLC grade, linear gradients 0% B-100% B
Temperature	30°C

2.4. Statistical Analysis

Complete Randomized Design (C.R.D.) was used as an experimental design. Data were analyzed by using statistical analysis system-SAS (2001) to study the effect of different factors on the diameters of inhibition zones. Least significant difference (LSD) was used to compare the significant difference between means at $P \leq 0.05$.

3. Results and Discussion

It is suggested that plant extracts exhibiting diameters of zones of inhibition ≥ 10 mm were considered active [14,15]. There for the alkaloid extracts showed significant zone of inhibition against Gram-positive and Gram-negative bacteria (Table 3). The maximum average for inhibition zone was 26.4 mm for E. *coli* (PBR 322 standard strain), while the minimum average was 11 mm for *S. aureus* which isolated from dermal infection (Table 4). Also for alkaloid extract concentrations the maximum average for inhibition zone 43.7 mm was for 0.4%, but the minimum was 0.6 mm for 0.002%. However interaction observation result from the Table 3 indicates that the maximum inhibition zone was 60 mm for *Escherichia coli* (PBR 322 standard strain) which treatment with 0.4% concentration, Further the minimum inhibition zone was 1 mm for the *Escherichia coli* (AMP resistance TET resistance Contain plasmid standard strain) treated with alkaloids 0.002% concentration.

Similar results on antibacterial activity were reported on Table 4 which indicates that there are significant variations of the inhibition zone for all tested bacteria with Phenols compounds. The average for maximum and minimum inhibition zone were 22.4 and 13.4 mm for *S. aureus* isolated from Dermal infection and *Escherichia coli* PBR 322 standard strain, receptively. Also the highest and lowest average inhibitory zone for concentrations were (48.1 and 1 mm) exhibited by 0.4 and 0.002%, respectively. Although the interaction result showed the maximum inhibition zone was 53 mm for both *Staphylococcus aureus* isolated from Urinary tract infection and *Escherichia coli* isolated from the same Source (Urinary tract infection) with 0.4% concentrations, while the minimum was 1 mm for three strain bacteria treated with phenols 0.002% concentration (Table 4).

The antibacterial activity results often pointed that 0.4% was the most concentration active against all tested bacteria, Especially *S. pyogenes* (gram positive) and three *E. coli* strain (gram negative) (Figure 1, Figure 2, Figure 3 and Figure 4). Beside the minimum inhibition concentration was 0.002%.

Table 4. Antibacterial activity of the Alkaloids plant extracts against some of pathogenic bacteria

	Concentrations of alkaloids (inhibition zone cm)							Average A
	Bacteria and source of isolate	0.002	0.004	0.02	0.04	0.2	0.4	
1	*Staphylococcus aureus* Blood + ve	0.00	1	9	15	25	45	15.9
2	*Staphylococcus aureus* Dermal infection + ve	0.00	0.00	2.5	10	19	35	11
3	*Staphylococcus aureus* Urinary tract infection + ve	0.00	0.00	3	11	19	30	10.5
4	*Streptococcus pyogenes* Blood + ve	2	5	19	23	25	51	20.9
5	*Escherichia coli* Urinary tract infection - ve	0.00	1	11	20	28	50	18.4
6	*Escherichia coli* PBR 322 standard strain - ve	2	4	21	30	41	60	26.4
7	*Escherichia coli* stool patient -ve	0.00	1	5	11	21	31	11.5
8	*Escherichia coli* PBR 322 AMP resistance TET resistance Contain plasmid standard strain - ve	1	2.5	20	26	30	47	21
	Average B	0.6	1.8	11.3	18.3	26	43.7	
	L.S.D P ≤ 0.05	A x B = 0.38		B = 0.09		A = 0.15		

Table 5. Antibacterial activity of the Phenolic plant extracts against some of pathogenic bacteria

	Concentrations of Phenols (inhibition zone cm) ns of Phenols (inhibition zone cm)							Average A
	Bacteria and source of isolate	0.002	0.004	0.02	0.04	0.2	0.4	
1	*Staphylococcus aureus* Blood + ve	0.0	1	5	20	22	52	16.7
2	*Staphylococcus aureus* Dermal infection + ve	3	8	15	30	30	48	22.4
3	*Staphylococcus aureus* Urinary tract infection + ve	0.0	2	16	22	32	53	20.9
4	*Streptococcus pyogenes* Blood + ve	0.0	1.5	10	17	22	52	17
5	*Escherichia coli* Urinary tract infection - ve	3	6	12	20	30	53	20.7
6	*Escherichia coli* PBR 322 standard strain - ve	0.0	1	10	10	20	39	13.4
7	*Escherichia coli* stool patient - ve	1	2.3	9	10	19	41	13.7
8	*Escherichia coli* PBR 322 AMP resistance TET resistance Contain plasmid standard strain - ve	1	3	5	12	20	47	14.7
	Average B	1	3.1	9.6	17.6	24.4	48.1	
	L.S.D P ≤ 0.05	A = 0.36		B = 0.50		A x B = 2.12		

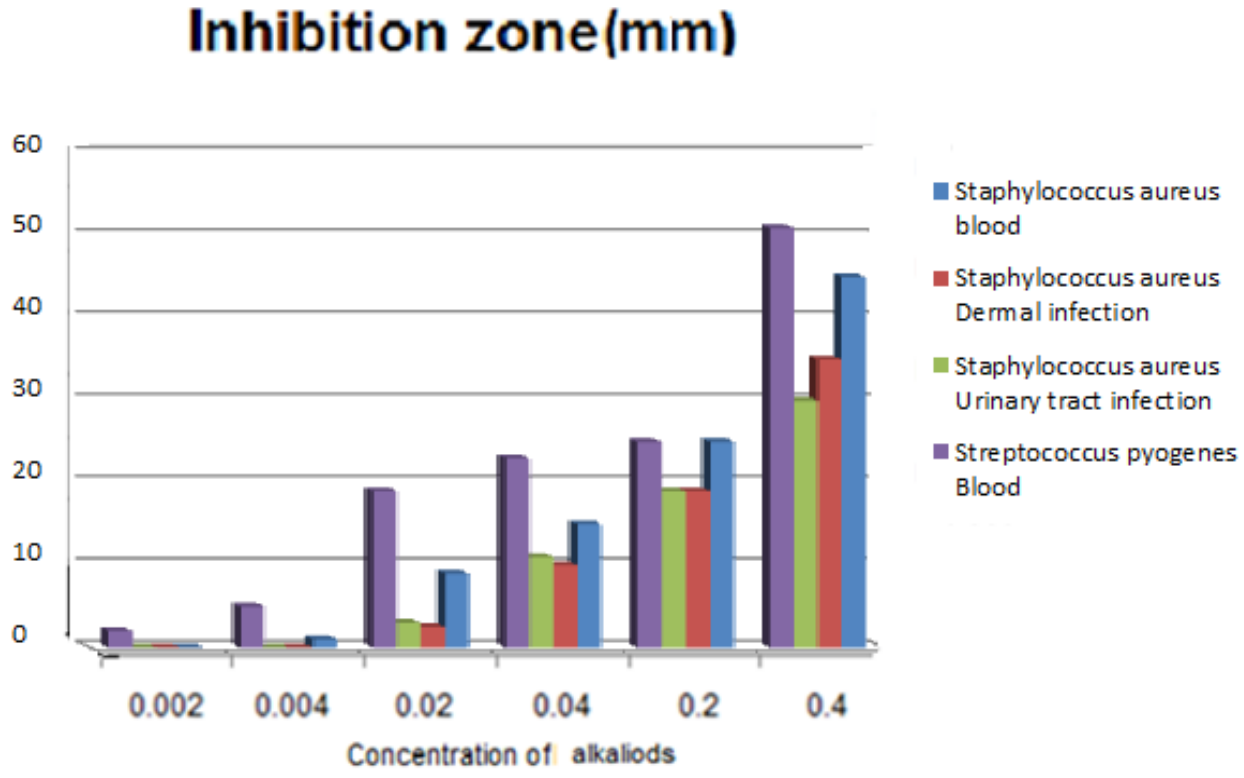

Figure 1. Antimicrobial activity of the alkaloids plant extracts, tested against different clinical microorganisms

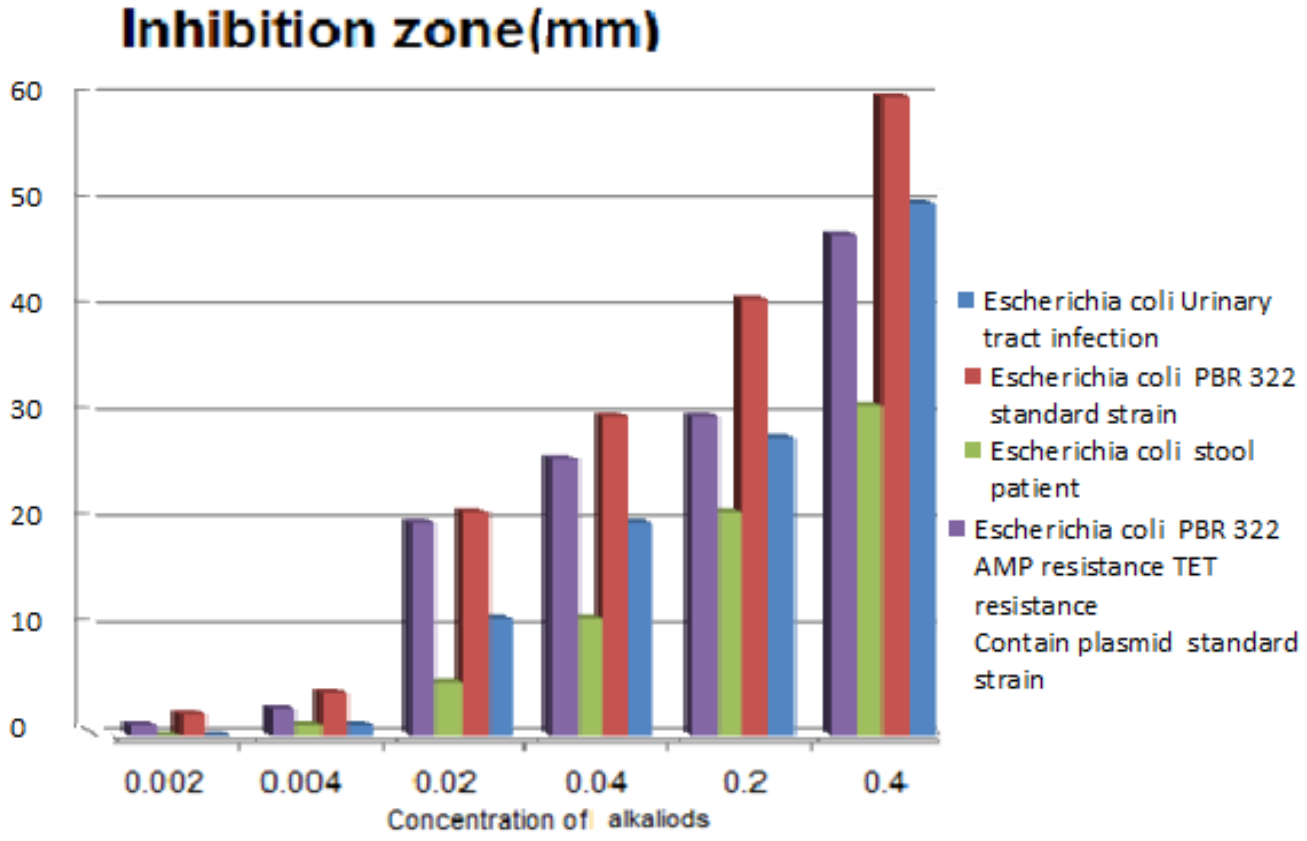

Figure 2. Antimicrobial activity of the alkaloids plant extracts, tested against different clinical isolated microorganisms

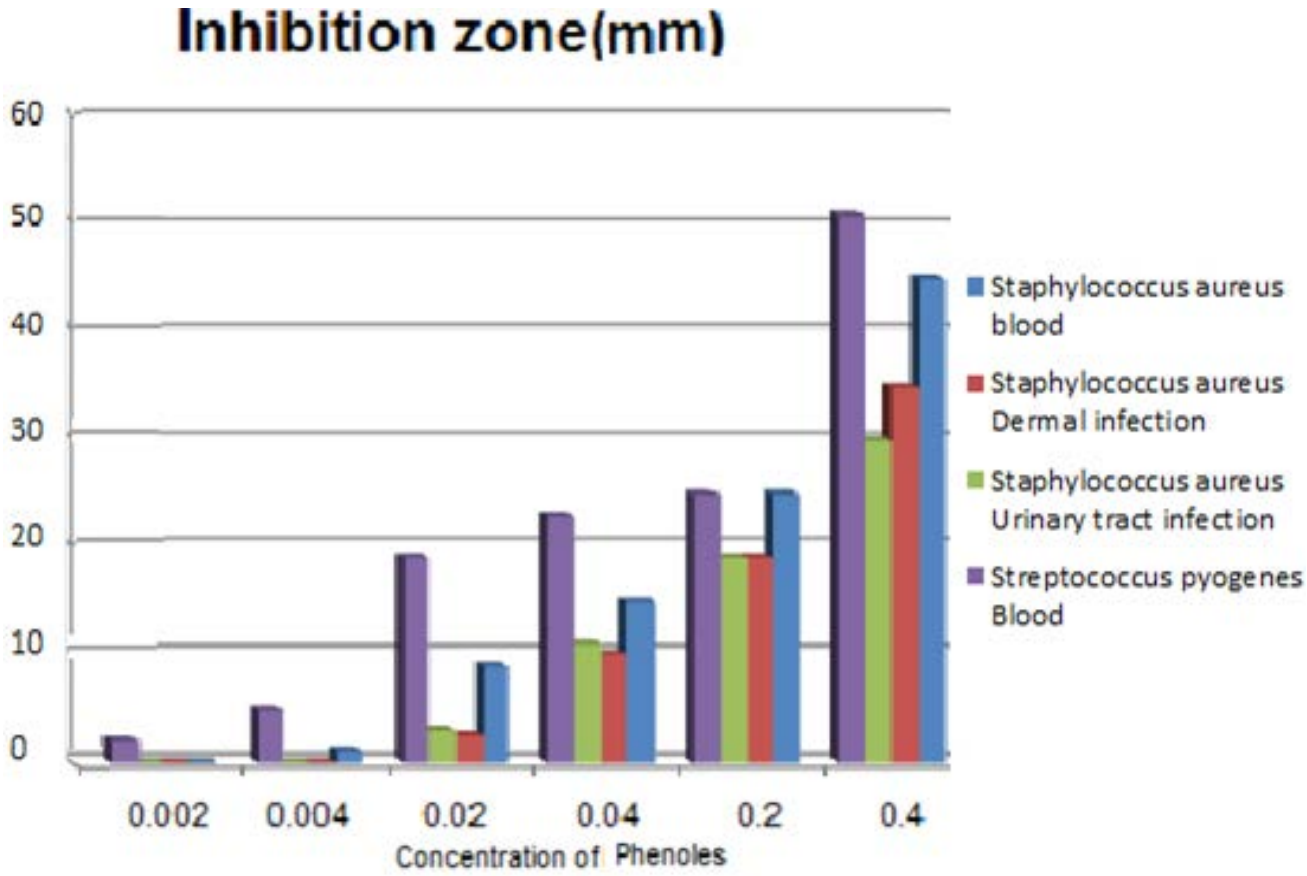

Figure 3. Antimicrobial activity of the phenolic plant extracts, tested against different clinical isolated microorganisms

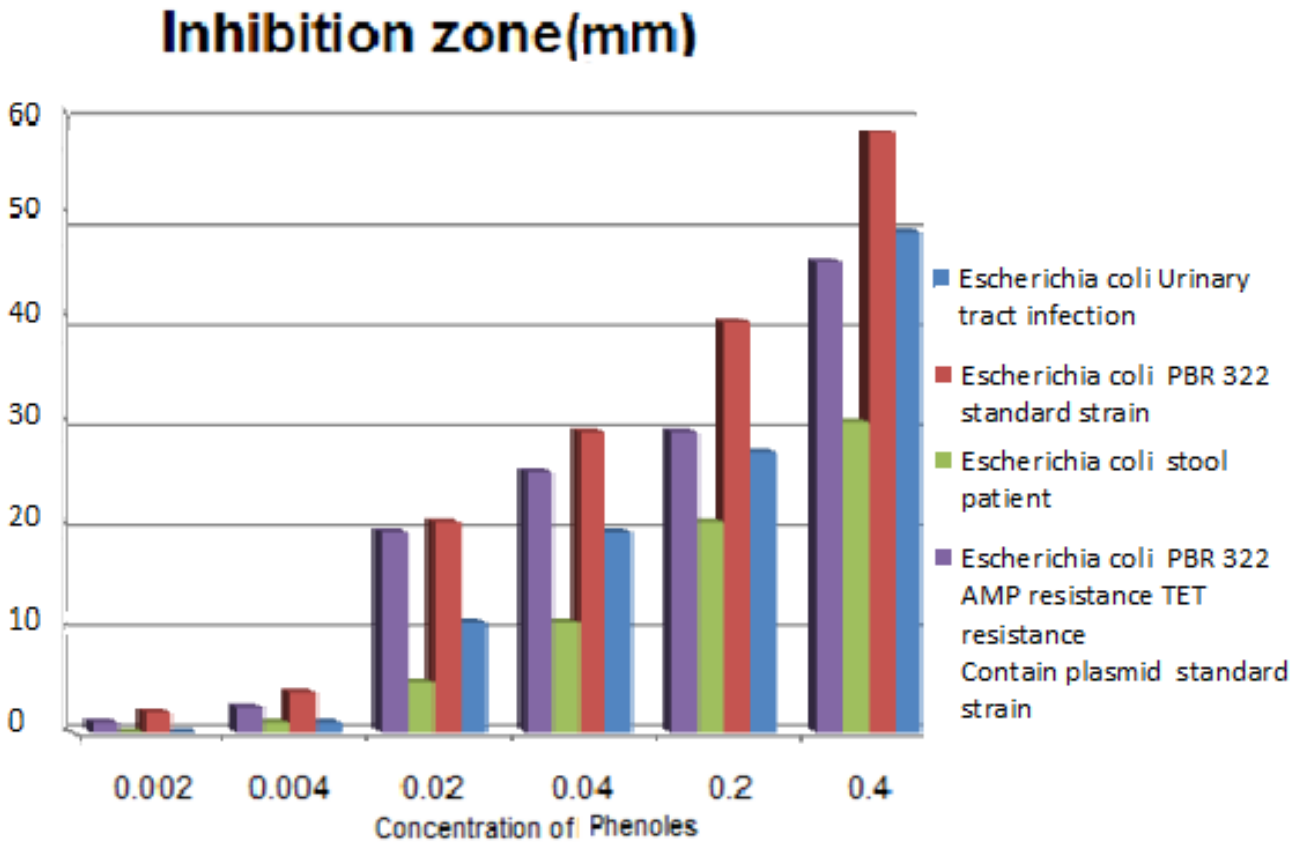

Figure 4. Antimicrobial activity of the phenolic plant extracts, tested against different clinical isolated microorganisms

3.1. Fast Liquid Chromatography (FLC) Analysis for Active Compounds in Plants Extracts

Results have been shown important and significant differences between the concentrations of each secondary metabolic compound among the extracted cruds.

3.2. Alkaloids Compound

Alkaloids present in the extracted plants were also identified by FLC, as elaborated in Table 6 and the peaks in Figure 5 and Figure 6. The total concentration of alkaloids in the extracted *P. nigrum* was 156.8 µgmL^{-1}. Capsaicin (137.2 µgmL^{-1}) was the major alkaloid, while 2-dihdrocapsaicin (19.6 µgmL^{-1}) was the minors in the *P. nigrum.*

Table 6. Types and concentration of alkaloids in plant extracts

alkaloids compounds	(µg/mL)
Capsaicin	**137.2**
2-dihdrocapsaicin	**19.6**
Total concentration (µg/mL)	**156.8**

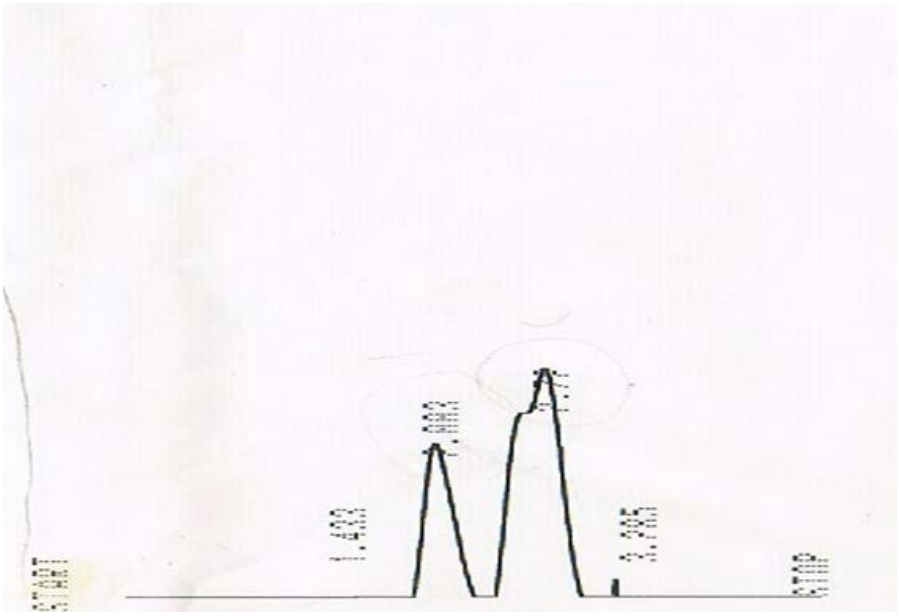

Figure 5. FLC profile of alkaloids standards of *Piper nigrum* (1) Capsaicin, (2) 2-dihdrocapsaicin

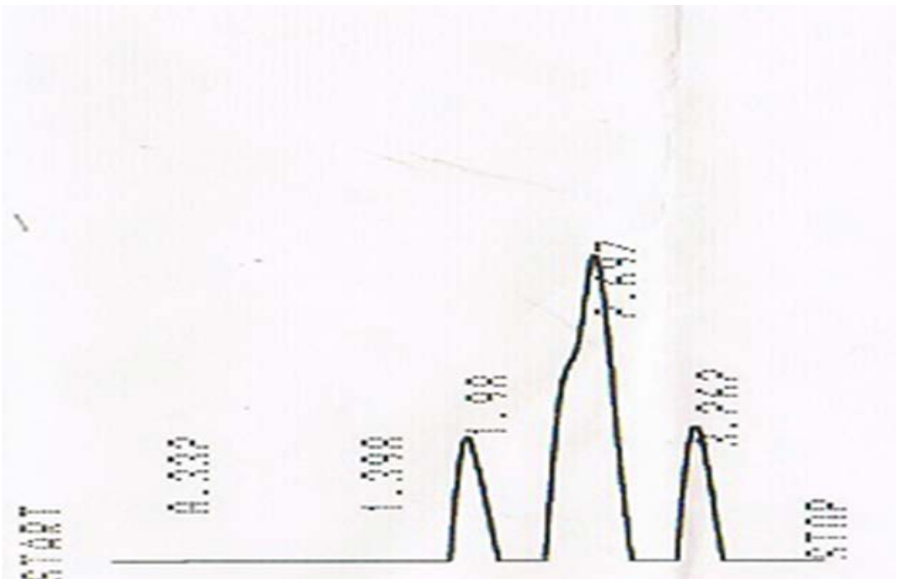

Figure 6. FLC profile of *P. nigrum* alkaloids (1) Capsaicin, (2) 2-dihdrocapsaicin

Table 7. Types and concentration of phenols in plant extracts

Phenolic compoundsin *P. nigrum*	(µg/mL)
Gallic acid	**32.42**
Trans-p-feruloyl-â-D-glucopyranoside	**3.68**
Trens-p-sinapyl-â-D-glucopyranoside	**147.4**
Quercetin3-O-R-L-rhamnopyranoside-7-O-â-D-glucopyranosyl	**62.6**
Quercetin3-O-R-L-rhamnopyranoside	**4.492**
Luteolin 6-C-â-D-glucopyranoside-8-C-R-L-arabinopyranoside	**8.02**
Luteolin 7-O-[2-(â-D-apiofuranosyl)-â-D-glucopyranoside-8-C-R-L-arabinopyranoside	**5.048**
Luteolin 7-O-[2-(â-D-apiofuranosyl)-4-(â-D-glucopyranosyl)	**10.84**
Kaempferol	**11.46**
Coumarins	**12.92**
Total concentration (µg/mL)	**274.5**

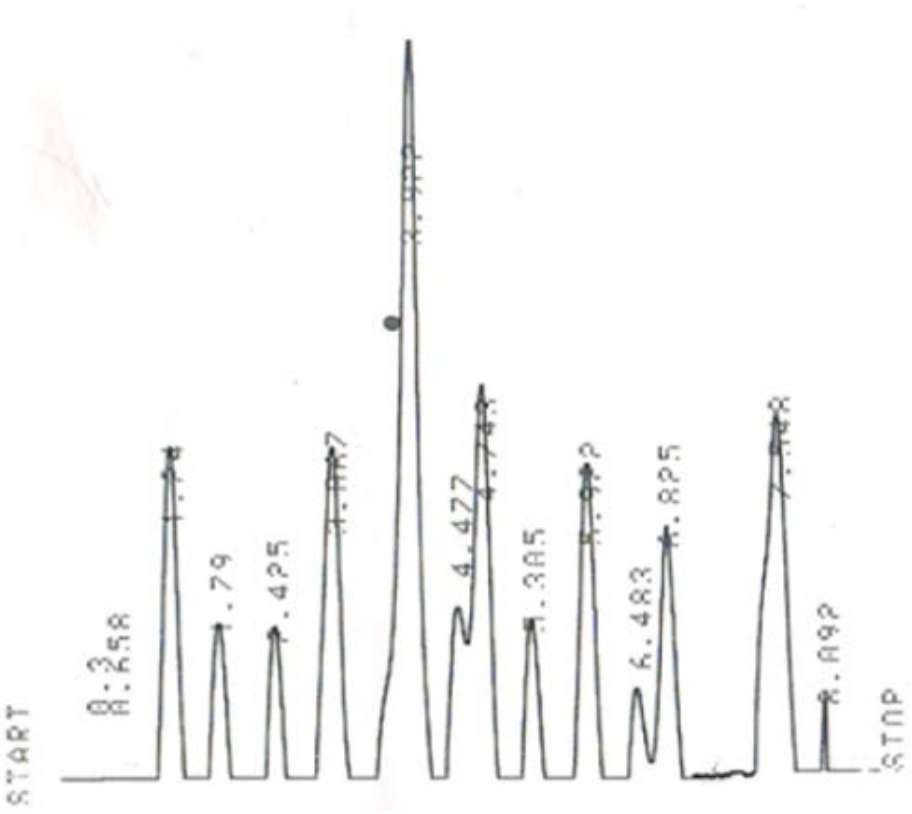

Figure 7. FLC profile of phenols standards of *Peper Nigrum* (1) Gallic acid, (2) Trans-p-feruloyl-â-D-glucopyranoside, (3) Trens–p-sinapyl-â-D-glucopyranoside, (4) Quercetin3-O-R-L-rhamnopyranoside-7-O-â-D-glucopyranosyl, (5) Quercetin3-O-R-L-rhamnopyranoside, (6) Luteolin 6-C-â-D-glucopyranoside-8-C-R-L-arabinopyranoside, (7) Luteolin 7-O-[2-(â-D-apiofuranosyl)-4-(â-D-glucopyranosyl), (8) Luteolin 7-O-[2-(â-D-apiofuranosyl)-â-D-glucopyranoside-8-C-R-L-arabinopyranoside, (9) Kaempferol, (10) Coumarins

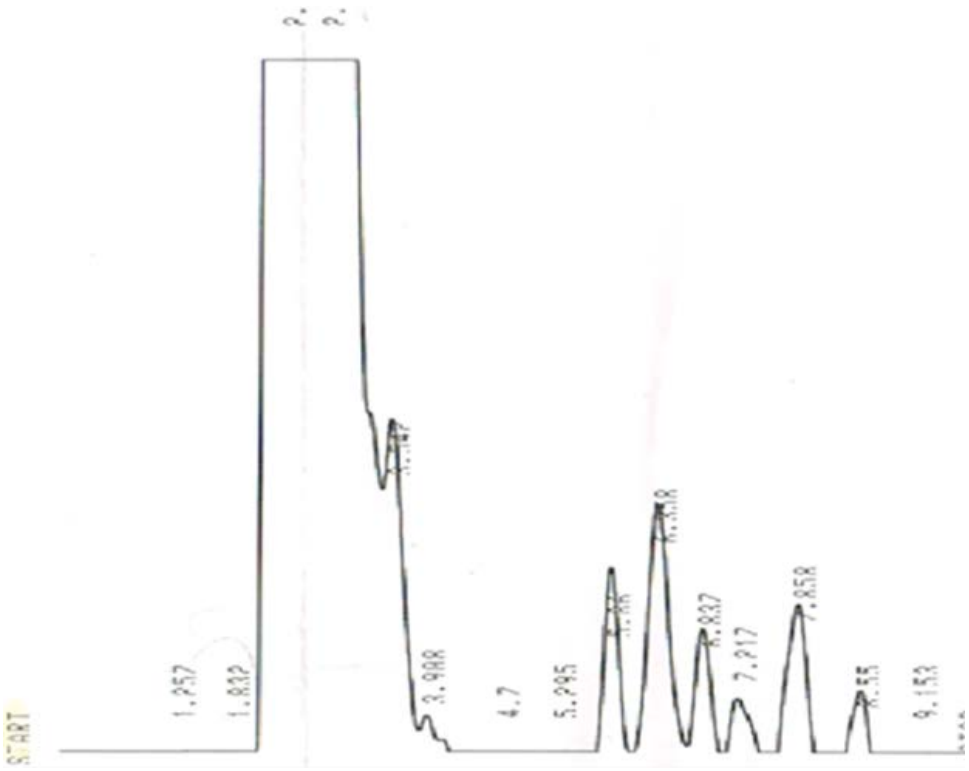

Figure 8. FLC profile of phenols of *P. nigrum* (1) Gallic acid, (2) Trans-p-feruloyl-â-D-glucopyranoside, (3) Trens–p-sinapyl-â-D-glucopyranoside, (4) Quercetin 3-O-R-L-rhamnopyranoside-7-O-â-D-glucopyranosyl, (5) Quercetin 3-O-R-L-rhamnopyranoside, (6) Luteolin 6-C-â-D-glucopyranoside-8-C-R-L-arabinopyranoside, (7) Luteolin 7-O-[2-(â-D-apiofuranosyl)-4-(â-D-glucopyranosyl), (8) Luteolin 7-O-[2-(â-D-apiofuranosyl)-â-D-glucopyranoside-8-C-R-L-arabinopyranoside, (9) Kaempferol, (10) Coumarins

3.3. Phenolic Compounds

Results of FLC (fast liquid chromatography) analysis indicated the presence of ten phenolic compounds in *P. nigrum* (Table 7) and Figure 7 and Figure 8. All the isolated compounds appeared to have different retention time. Chrysophanol-1-O-B-glucopyranoside (55.08 µg mL^{-1}) and Trens–p-sinapyl-â-D-glucopyranoside (147.4 µgmL^{-1}) were the highest phenolic compounds in *P. nigrum*, while Anthraquionone (4.76 µgmL^{-1}) and Trans-p-feruloyl-â-D-glucopyranoside (3.68 µg/mL) were the lowest concentration in *P. nigrum*.

4. Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. Structurally, the plant extracts contains at least two classes of secondary metabolites namely alkaloids, phenols. However, it should be noted that the activity does not depend on the number of classes of detected bioactive compounds, but mostly on their concentration. The inhibitory activity of *P. nigrum* was previously reported against some bacteria such as *Staphylococcus aureus, Bacillus cereus, Streptococcus faecalis, Pseudomonas aeruginosa, Salmonella typhi* and *Escherichia coli* [16]. Some studies showed that the pharmacological and biology properties were mostly due to alkaloids [17]. The inhibitory activity of the system originated from the synergistic actions of both capsaicin and 2-dihdrocapsaicin. While the capsaicin microcapsules displayed potential antimicrobial applications in the food storage [18]. The phytoconstitutes of *P. nigrum* fruits include volatile oil, other minor alkaloids such as piplartin, piperlogumine, piperidine, starch, resin and pungent alkaloid piperine. The piperine present in the skin and seed of the black pepper which is responsible for the antimicrobial activity [19,20].

Generally, the reason could be attributed to the presence of extra outer membrane in their cell wall acting as barrier for the compound(s) to diffuse into the bacterial cells. Or may be genetic content of their plasmids [21] or to the differences in the composition and the mechanism of action of the bioactive compounds [22].

The alkaloids sanguinarine, berberine, jatrorrhizine and palmatine are known to inhibit the multiplication of bacteria, fungi and viruses [23]. Therefore the antibacterial activity observed in the present investigation is attributed to the alkaloids berberine, palmatine and jatrorrhizine which have been widely known to occur in different species of this genus. Otherwise the biological phenolic compounds activities are related to the molecules structures; by their hydroxyl groups or by phenolic ring, phenolic compounds have capacity to link with proteins and bacterial membrane to form complexes [7].

The variation in the inhibition among the gram positive and gram negative bacteria is due to the cell wall and cell membrane compositions. However the differences in sensitivity between Gram-positive and Gram-negative bacteria to the extract can probably be attributed to the structural and compositional differences in membranes between the two groups [24]. The Gram-negative bacteria have an outer membrane that serves as an impermeable barrier for many small molecules.

The resistance between bacteria from same spices may due to antibiotic resistance genes and the later may be on chromosome or on plasmids. It is well known that

plasmids are major vectors for the dissemination of both antibiotic resistance and virulence determinants among bacterial populations [25]. The exchanging of genetic material between microorganisms through transformation, conjugation or transduction processes or may by mobile genes (transposons) have been proposed as a major contributor in the rapid evolution of microorganisms resistant to antibiotics [26]. On the other hand, using inaccurate concentration of antibiotics or drug or unnecessary of medicine appointment leads to the resistance of sensitive bacteria. In addition to weakening the immunity system in some human due to poor nutrition or heredity factors make bacteria to be more resistant [26].

The above results open the possibility of finding new clinically effective drug and could be useful in understanding the relationship between traditional cures and current medicines. An understanding of these properties would be invaluable in the development of alternative, natural, and safe methods of controlling bacterial infections. The crude extract of *P. nigrum* demonstrated a significant antibacterial activity against the microorganisms investigated and could therefore be added to the potential list of antibacterial agents.

This result suggests the need for further studies on this substance to identify, isolate, characterize and elucidate the structure of the active ingredient(s) using some spectroscopic techniques such as nuclear magnetic resonance (NMR), infrared spectrophotometry (IR) and mass spectrometry (MS).

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