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STUDY OF THE ROLE OF POLYPHENOLIC EXTRACT OF *CAPPARIS SPINOSA* L. LEAVES AS ACUTE TOXICITY AND ANTIBACTERIAL AGENT

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Abstract

The present study investigates the effect of polyphenolic extract of *Capparis spinosa* L. Leaves on acute toxicity in female rats and anti-bacterial *in vitro*. The working of this thesis involves the following terms: Analytical study involved extraction, isolation, and identification of polyphenolic content of *Capparis spinosa* L. extract. The UV-Vis Spectra and high-performance liquid chromatography of extracted polyphenols are proved the presence of (Gallic acid, Caffeic acid, Coumaric acid, Vanillic acid, Syringic acid, Ferulic acid, Chlorogenic acid, Rutin and Quercetin) in the extract. Acute toxicity: in vivo study included of the extract was performed on four groups of rats (6 rats in each group). After treatment of different concentrations of *Capparis spinosa* L. extract (25, 50 and 100 mg/kg B.W) and after 72 hr of treatment, no mortality in all rats of experiments can be observed. This indicated that extract is orally non-toxic. Antibacterial study: four types of bacteria, two Gram-positive (*Staphylococcus aureus*, *Bacillus subtilus*) and two Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) have been used to investigate the antibacterial activity of the extract. The extract is actively worked against both types of bacteria. Furthermore, the highest activity of extracted polyphenolic for *C. spinosa* against showed maximum activity against pathogens *E.coli* and *Pseudo* (gram-negative) (12mm) and minimum activity in *Staph* (gram-positive) (10mm) and inhibition zone against *Bacillus* (gram-positive) (11mm).

Key words: *Capparis spinosa* L., polyphenols, acute toxicity, anti-bacterial

Introduction

Phenolic compounds are also commonly referred to as polyphenolics, a term that originally meant "many phenolics" and probably referred to a compound having many phenolic groups (Wrolstad, 2005). Polyphenols are a wide and complex group of secondary plant metabolites, which can be defined as compounds possessing an aromatic ring bearing one or more hydroxy substituents (Bruneton, 1999). Structures of the compounds range from simple molecules such as phenolic acids to highly polymerized compounds like proanthocyanidins (Wrolstad, 2005).

Phenolic compounds are found in almost every plant-derived food, Fruits and vegetables contain several thousand structurally diverse phytochemicals, of which a large fraction are polyphenols (Scalbert and Williamson, 2000). The beneficial effects of polyphenols are mainly attributed to their antioxidant properties, many dietary polyphenols are known antioxidants (Surh, 2003; Pan and Ho, 2008) since they can act as chain breakers or radical scavengers depending on their chemical structures (Rice-Evans, 2001). Due to their antioxidant activity, polyphenol compound and fruit extracts have been reported to have positive effects on cancer, cardiovascular disease, immune disorders, microbial infections, neurodegenerative diseases and viral infections (Xu *et al.,* 2000).

Polyphenols occupies a unique place in science and the only class of biologically active natural products that result of their presence in the food and beverages derived from plants and inserted into the formulas of well-marketed cosmetics (Arakawa *et al*., 2004) Para pharmaceutical products (Bernaert *et al*., 2009). Polyphenols exhibit multiple pharmacological properties

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such as anti- microbial, anti-allergenic, anti-ulcerogenic, anti-neo plastic, and anti- inflammatory activities (Formica and Regelson, 1995). The effects of polyphenols therapeutically relevant for the biological systems, are: they reduce the scavenger properties for oxygen free radicals (Burns, 2000), they reduce platelet aggregability (Jeong *et al.,* 1999), and they decrease arterial blood pressure (Hiroko *et al.,* 2004). The bioavailability and biological properties of dietary polyphenols vary to a great extent and depend on their chemical structure (Loke *et al.,* 2008).

As a result, the antioxidant activity of phenolic acids increases the higher the degree of hydroxylation (Naczk and Shahidi, 2006). It is their role as dietary antioxidants that have received the most attention in recent literature (Laranjinha *et al.,* 2002; Lodovici *et al*., 2001). They are also known to exhibit antibacterial effects (Weston *et al*., 1999) antimutagenic and anti-inflammatory activities in bacteria and mammalian (Loarca-Pina *et al*., 1998).

Capparis spinosa L. *is well* known for its common name 'Capers' in different countries (Azaizeh *et al.,* 2003). This plant is also known as the caper bush, is a perennial winter deciduous species that bears rounded, fleshy leaves and large white to pinkish flowers (Ramezani *et al*., 2008). Extracts of different parts of *C. spinosa* have been shown to possess biological activity against a large number of pathogens (Chopra *et al*., 1996). Antifungal, antibacterial, anti-amoebic, and anti-worm activities have been demonstrated (Guba Bakshi *et al*., 1999), antidiabetic, antihyperlipidemic (Eddouks *et al*., 2005), antihypertensive, poultice (Baytop *et al*., 1984) antileishmania, antihepatotoxic and antiallergic activities (Trombetta *et al*., 2005).

Materials and methods

Study Plant

Capparis spinosa L. leaves were collected in May-2013 from Nasiriyah city in Iraq, then it was authenticated and specimen of plant was classified in biological department-college of science at university of Thi-Qar in Iraq by Asst. prof. Hayder Radhi. The leaves were cleaned, washed by distilled water, dried at room temperature for two weeks, ground as powder and kept in Dark glass containers for further use.

Chemicals

Chloroform, ethanol, sodium hydroxide, hydrochloric acid, ferric chloride, acetic acid, lead acetate, - naphthol, sulphuric acid, Potassium citrate, mercuric chloride, Potassium hydroxide, n-hexane.

Extraction of Polyphenols from *Capparis spinosa L* Leaves

(500g) of the powder dry leaves were defatted by washing several times with hexane (1L) at (60°C), then it was macerated with (800mL) of acetic acid (2% v/v), the mixture was placed in sterile conical flask volume (2000mL) and put in water bath (60°C) for 8h, then the extraction process done by reflex condenser. The mixture was heated at 50 C and then left to cool. The suspension was filtered by Buchner funnel by Whatman no.1 filter paper and use vacuum pump. The precipitate was canceled and the filtrate volume was measured then n-propanol was added into filtrate with the same volume of filtrate. Then (NaCl) added until to become solution supersaturated. Then, it was evaporator by using rotary evaporator until drying (Gayon, 1972).

Primary Qualitative Analysis

Isolated polyphenols were underwent a number of different tests such as:

Phenolic compounds test: was carried out by using (1%) ferric chloride. (Waterman and Mole, 1994).

Flavonoids test: was achieved by using(5N) alcoholic potassium hydroxide (Al- Assadi, 2001)

Tannins: was achieved by using (1%) lead acetate (Molan *et al.,* 1997).

Carbohydrates test: was done by using Molish´s reagent (Harborne, 1984).

Glycosides test: was carried out by using Benedict´s reagent (Harborne, 1984).

Alkaloids test: was done by using Wagner´s reagent (Harborne, 1984).

Saponin test: was carried out by using(5%) mercuric chloride (Harborne, 1984).

Triterpenoids test: was achieved by using concentrated sulfuric acid (Harborne, 1984).

Triterpenes and Sterols test: was achieved by using Liebermann- Burchard reagent. (Harborne, 1984).

Investigation of Polyphenolic Extract by UV-VIS (spectrophotometer)

The absorption spectra of plant constituents were measured in very dilute solution against a blank solvent by using an automatic recording spectrophotometer. The solvent was used for UV spectroscopy is water: the method was performed by using polyphenolic. The sample solutions absorbance (A), was recorded by measuring the range scan from 190nm to 800nm on a double beam UV-VIS spectrophotometer (Ikbal, 2004).

Investigation of Polyphenolic Extract by HPLC Technique

The extract was separated on FLC (Fast Liquid Chromatography) column, C-18, 3µm particle size (50 × 4.6mm ID), mobile phase were 0.1% acetic acid in Dionized water: acetonitrile (20:80V/V) using linear gradients from 0-100% B in 10 minutes, detection UV set at 275nm, flow rate 1.5 mL/min, the sequences of the eluted material of the standard were as follow, each standard was 25 µg/mL. 1.0g of the sample was weighted, then dissolved in 10 mL HPLC methanol, the sample shaking and agitated in ultrasonic bath for 10 minutes, then concentrated by evaporating the solvent with stream of liquid N_2 until reach 0.2 µm (supelco company cat No16534K) then 20 µL were injected on HPLC column. The concentration for each compound was quantitatively determined by comparison the peak area of the standard with that of the samples. The concentration for each compound was quantitatively determined by comparison the peak area of the standard with that of the samples. The separation occurred on liquid chromatography Shimadzu 10 AV-LC equipped with binary delivery pump model LC-10A Shimadzu, the eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer. (Ikbal, 2004).

Experimental Animal

Twenty four healthy adult female rats weighing (100-169g) of 9-10 weeks old were used in the present study. Animals were housed in the animal house of biology Dept. College of Education, Thi-Qar University. Experiments were achieved between September-2013 and October-2013. Animals were housed in iron boxes bedded with wooden chips. During the experimental period six animals were kept in each box and they were housed under standard laboratory conditions (12h light: 12h dark photoperiod (LD) at 22 ± 2ºC and relative humidity 45-55% (Coskun *et al.*, 2004). Animals were fed on standard rat pellets and tap water *ad libitum*. The standard pellet contains wheat 66.6%, soya 25.6%, and sunflower oil 4.4%, limestone 1.5%, salt 0.63%, methionine 0.158%, choline chloride 0.062% and trace elements 0.05% (Krinke, 2000).

Acute Toxicity Study OF *Capparis spinosa* L. Leaves Polyphenolic Extract

Administration of Laboratory Animals

Experimental animals were divided into four groups (6 rats in each group) upon the following designed:

- **Group A**: control (normal) that were treated with distilled water (D.W)
- **Group B**: Rats were treated with (25 mg/kg) of *Capparis spinosa L.* polyphenolic extract.
- **Group C**: Rats were treated with (50 mg/kg) of *Capparis spinosa L.* polyphenolic extracts.
- **Group D**: Rats were treated with (100 mg/kg) of *Capparis spinosa L.* polyphenolic extracts.

Acute toxicity studies were performed according to the organization for economic co-operation and development (OECD/OCDE) guidelines. The animals fasted for 4hrs with free access to water only. The polyphenolic extract was administered orally as above doses and mortality if any was observed for 3 days(Ecobichon, 1997).

Investigation of Antibacterial Activity of Polyphenolic Extract

Culture Media

Muller Hinton Agar was supplied from Himedia company (India), it was used as a culture medium and was prepared to depend on information determining by manufacturing company.

Pathogenic Bacterial Strains

Staphylococcus aureus and *Bacillus subtilus* (positive to Gram stain), *Escherichia coli* and *Pseudomonas aeruginosa* (negative to Gram stain) were used as pathogenic bacteria strains and they were identified in Biology Dept. College of Science, Thi- Qar University.

Assessment of Antibacterial Activity of Extract

Bacteria suspension of each tested bacteria (107 CFU/mL) was spread onto the surface of Muller-Hinton agar plates. Eight mm cork borer was used to punch wells into the plates and 100 µL of each extract dissolved in DMSO (10 mg/mL) as well as were applied to each well. The plates were incubated for 18 h at 37°C. The inhibition zones diameter for each extract was measured and the phenol coefficient was calculated (NCCLS, 1999).

Results and Discussion

Capparis spinosa showed contain a number of antioxidants phytochemicals such as flavonoids and other polyphenols (Aslanturk and Tulay, 2009).

The chemical qualitative analysis test results are shown in table 1 which indicate the presence of polyphenols, flavonoids, and glycoside but the carbohydrate, tannins, saponins, alkaloids, terpenoids, terpenes and sterols gave a negative test, this ensures that polyphenolic compounds are pure. From table 1, it

was found that glycosides because polyphenols are found normally connected with saccharide units. Fig. 1 illustrates the UV-Vis spectra of the polyphenolic extract with max at (202nm) and another peak at (280nm), these peak absorbed at (202) for π-π^* electronic transitions due to the founding of multi double bonds in aromatic rings for these compounds and another peak at (280) which is of low intensity appear in wavelength longer than the first peak due to n-π^* electronic transitions for non bonding electrons for oxygen atoms that are present in these compounds. This result is similar to the result of Roomi, *et al*., (2013).

The plant's extracts were analyzed to estimate their contents of polyphenol compounds. The identification of each compound was based on retention time in comparison with pure commercial standards (Shindalkar *et al.,* 2005). Fig. 2 and Fig. 3 explained that the retention time of sample agrees with the retention time of the standard for most contents in the extract.

In this study and from HPLC results it was found that polyphenol extract contains some important compounds that include (gallic acid, caffeic acid, coumaric acid, Vanillic acid, Syringic acid, and ferulic acid, Chlorogenic acid, Rutin and quercetin) as shown in Fig. 3 and the structures of these compounds were shown in table 2. The peaks of the mentioned chromatogram also pointed to presence of some unknown compounds that are thought represent derivatives of polyphenolic compounds.

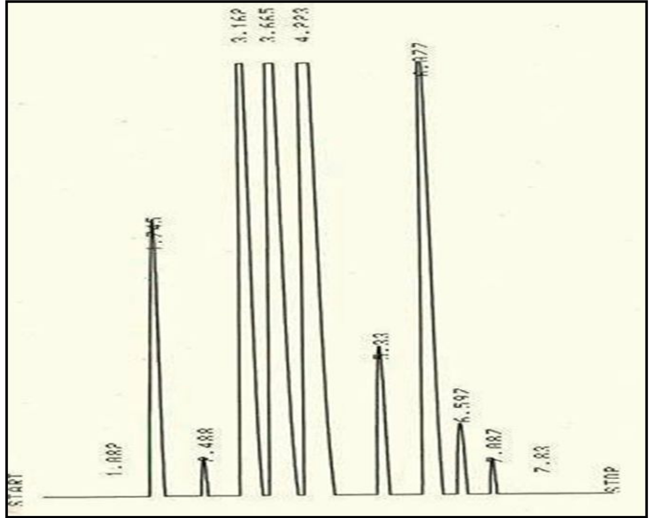

Fig. 3. HPLC chromatogram of *Capparis spinosa L* phenolic extract.

Table 1: Preliminary qualitative analysis tests of polypenols isolated from *C. spinosa* leaves

Reagent	Test result	Chemical Notes	Conclusions
FeCl3(1%)	+	Formation of bluish green colour	Phenols are present
Alcohol KOH (5N)	+	yellow precipitate	Flavonoids are absent
Wagner	-	No reddish-brown precipitate	Alkaloids are absent
Pb(Ac)2	-	No light brown precipitate	Tannins are absent
Molish	-	No Formation of violate ring	Carbohydrate are present
Benedict	+	Formation of red precipitate	Glycosides are present
HgCl2	-	No white precipitate	Saponins are absent
Conc. H2SO4	-	No purple red color	Terpinoids are absent
Liebermann Burchard	-	No green colour	Terpenes and sterols are absent

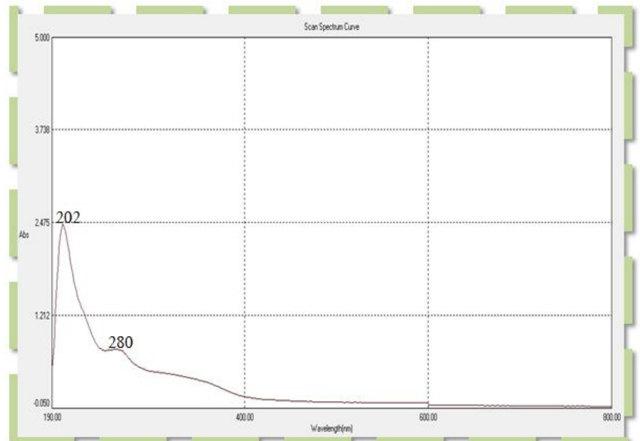

Fig. 1. Absorption spectrum of polyphenol by UV- SCAN in Water.

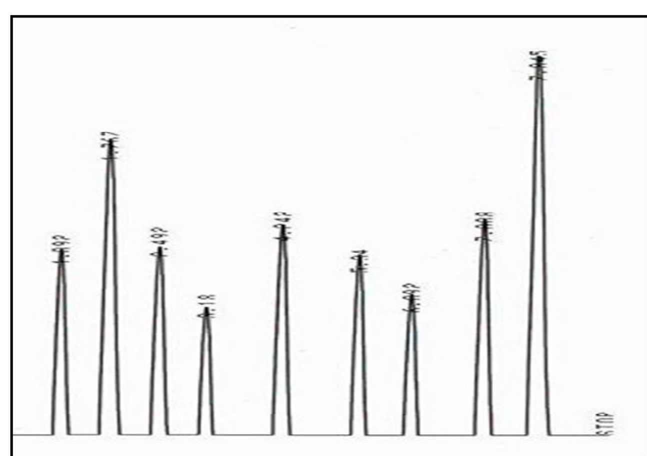

Fig. 2. HPLC chromatogram of standard polyphenolic compounds.

Table 2: Concentration of standard polyphenolic compounds.

seq	Polyphenolic contents in the standard	Retention time (min)	Area
1	Chlorogenic acid	1.09	40854
2	Caffeic acid	1.77	55315
3	Gallic acid	2.49	44057
4	Quercetin	3.18	39424
5	Syringic acid	4.24	45691
6	coumaric acid	5.34	39763
7	Ferulic acid	6.09	37854
8	Vanillic acid	7.08	43141
9	Rutin	7.85	66993

Table 3: Concentration of polyphenolic compounds in polyphenolic extract.

seq	Polyphenolic contents in the extract	Retention time (min)	Area	Conc. of polyphenolic compounds µg/mL
1	Chlorogenic acid	1.08	22004	40.395
2	Caffeic acid	1.75	54240	73.542
3	Gallic acid	2.49	32976	56.136
4	Quercetin	3.16	76898	146.289
5	Syringic acid	4.22	121635	199.659
6	P-coumaric acid	5.33	45022	84.918
7	Ferulic acid	6.08	65018	128.82
8	Vanillic acid	7.09	34199	59.454
9	Rutin	7.83	29482	33.003

Table 5: Acute toxicity effect of *Capparis spinosa L.* polyphenolic extract.

Groups	Number of rats	Number of death after 72 h
Group A	6	0
Group B	6	0
Group C	6	0
Group D	6	0

Acute Toxicity Study

These results indicated that the doses (25, 50 and 100 mg/kg) Polyphenolic extract for *Capparis spinosa L.* leaves employed for acute oral toxicity studies were found to be non-toxic, no abnormal symptoms and no death of the rats was observed as shown in tables 5. According to the OECD (2001) polyphenolic and flavonoids extracts can be classified as nontoxic since the limited dose of acute toxicity is generally considered to be 5.0 mg/kg BW (Assam *et al.,* 2010). Dietary polyphenols are generally regarded as safe based on their long history of use in the diet and / or as traditional medicines, it is becoming increasingly apparent that these compounds could have deleterious effects (i) at pharmacological concentrations, (ii) in certain vulnerable populations, and (iii) in certain diseases or pharmaceutical contexts (Schilter *et al.,* 2003. According to OECD guidelines for acute oral toxicity, an LD_{50} dose of 2000mg/kg and above is characterized as unclassified and hence the drug is found to be safe (Chidambaram *et al.,* 2007).

Antibacterial Properties of Polyphenolic Extracts

The results of the *in vitro* antibacterial activity of polyphenolic extract are determined by diameters of inhibition zones as shown in table 3-5. Polyphenolic extract for *Capparis spinosa L* leaves showed maximum activity against pathogens *E.coli* (gram-negative) (12mm) and equal inhibition zone against *Pseudo* (gram-negative) (12mm), which is in agreement with the study of (Veronica *et al.,* 2011), and minimum activity in *Staph* (gram-positive) (10mm) and inhibition zone against *Bacillus* (gram-positive) (11mm) this could be attributed to the cell envelope including cytoplasmic membrane and cell wall components' structural differences between Gram-positive and negative bacteria (Hugo, 1998). Moreover, antimicrobial agents make contact with the cell envelope first, the structural difference plays a key role in susceptibility.

The control (Ciprodar) showed maximum activity against *Staph* (30mm) and against *Pseudo* (25mm).

The broad antimicrobial activity of the extracts in this study can be attributed to the presence of various bio-actives components such as phenolic acids, glycosides and various flavonoids (Marjorie, 1999).

Polyphenols have been reported to exhibit antibacterial activities (Haslam, 1996). The inhibition of microorganisms by polyphenolic compounds may be due to iron deprivation or hydrogen bonding with vital proteins such as microbial enzymes (Scalbert, 1991). Polyphenols are well documented to have microbicide bacteria (Cowan, 1999). Oxidized polyphenols also have inhibitory activity against bacterial growth (Cowan, 1999; Field, 1992). polyphenols interact with microbial membrane proteins, enzymes and lipids, thereby altering cell permeability and permitting the loss of protons, ions, and macromolecules (Tamba *et al*., 2007). Phenolic acids are antimicrobials and are directly involved in the response to micro-organisms. Indeed, their concentration raises after plant infection (Shahidi and Naczk, 1995), and the phenolic acid content of vegetables produced by organic or sustainable agriculture is higher than that of vegetables grown without stress, such as those grown in conventional or hydroponic conditions (Manach *et al*., 2004).

Flavonoids are known to retard the growth of microorganism through inhibiting their nucleic acid

synthesis, cytoplasmic membrane function, and energy metabolism (Thiem and Grosslinka, 2000; Tim and Lamb, 2005). Extracts of various medicinal plants containing phenolic and flavonoids have been previously reported to possess antimicrobial activity (Ayaz *et al*., 2008; Rahman and Moon, 2007).

Ciprodar (Ciprofloxacin hydrochloride), the IUPC name is 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1, 4-dihydroquinoline-3-carboxylic acid. The structure of ciprodar shown in Fig. 4:

Ciprofloxacin hydrochloride will also kill bacteria in both the active and inactive growth phases (Wolfson and Hooper, 1985). In addition, regular feeding schedules are not interrupted when using the antibiotic (Schmitt *et al*., 1988). The values of inhibition zones of ciprodar against both Gram-positive and Gram-negative of this study as shown in table 6, reflect the antimicrobial activities of ciprodar.

Conclusion

At the end of this thesis points below can be

Table 4: Names and Structure of polyphenolic compounds in polyphenolic extract.

Organizational name	Structure	subject
(1*S*,3*R*,4*R*,5*R*)-3-{[(2*Z*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy} -1,4,5-trihydroxycyclohexanecarboxylic acid	HO, CO_2H, HO, OH, O, OH, OH	Chlorogenic acid
3-(3,4-Dihydroxyphenyl)-2-propenoic acid	HO, HO, O, OH	Caffeic acid
3,4,5-trihydroxybenzoic acid	O, OH, HO, OH, OH	Gallic acid
2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one	OH, OH, HO, O, OH, OH, O	Quercetin
4-hydroxy-3,5-dimethoxybenzoic acid	COOH, H_3CO, OCH_3, OH	Syringic acid
3-(4-hydroxyphenyl)-2-propenoic acid	O, OH, HO	P-Coumaric acid
3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid	H_3CO, HO, O, OH	Ferulic acid
4-Hydroxy-3-methoxybenzoic acid	O, OH, O, CH_3, OH	Vanillic acid
2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[á-L-rhamnopyranosyl-(1'!6)-â-D-glucopyranosyloxy]-4*H*-chromen-4-one	HO, OH, OH, HO, OH, OH, O, O, H_3C, HO, HO, OH	Rutin

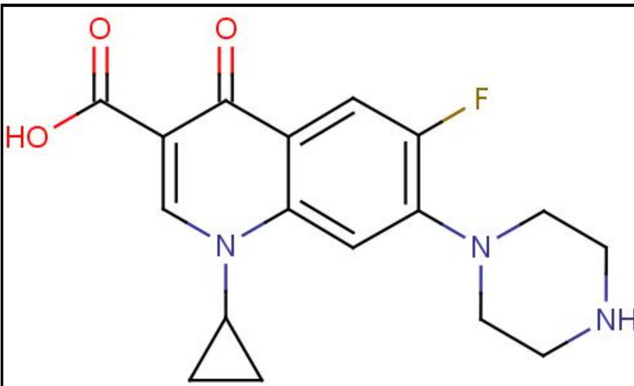

Fig. 4. Structure of high active ciprodar

Table 6: Diameters of inhibition zone (mm) for extract

Organisms	Zone of inhibition in mm		
	Gram stain	Polyphenolic extract	Control (ciprodar)
E. coli	-	12	18
Pseudomonas	-	12	25
Staph. aureus	+	10	30
Bacillus	+	11	18
DMSO		0	

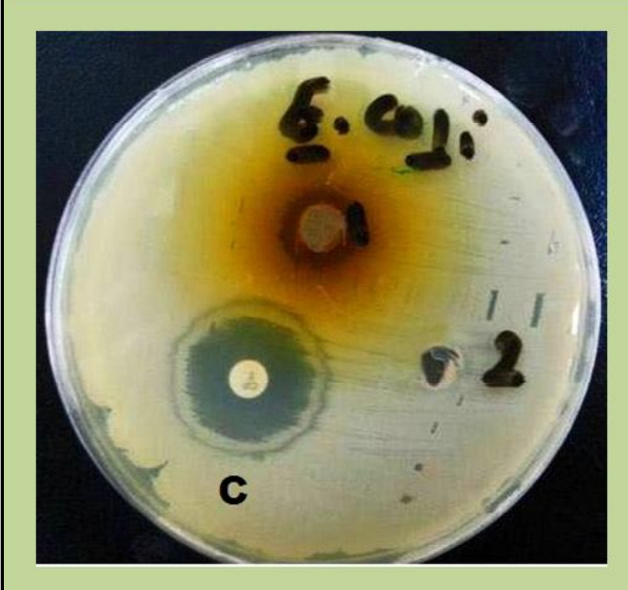

Fig. 5. The activity of Polyphenolic extract against *E.Coli* bacteria *E.Coli: Escherichia coli.*

1: polyphenol, 2: standard (DMSO), C: control (Ciprodar)

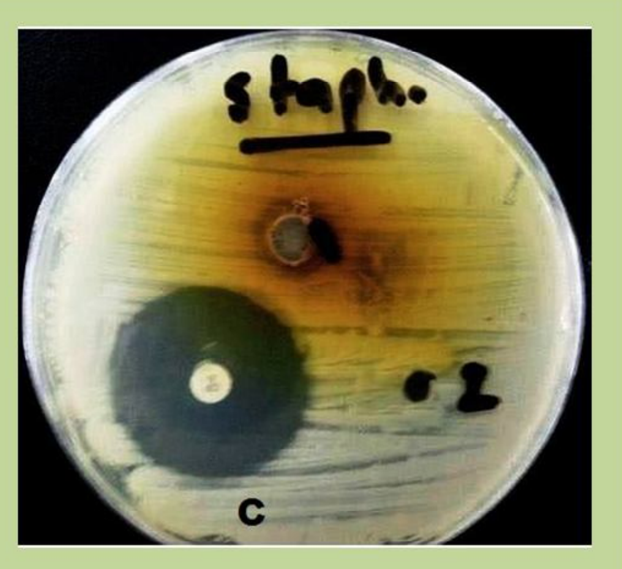

Fig. 6. The activity of Polyphenolic extract against (*Staph.*) bacteria *Staph*: *Staphylococcus aureus*.

Fig. 7. The activity of Polyphenolic extract against (*Pseudo.*) bacteria *Pseudo*: *pseudomonas aeruginosa*.

concluded:

- Polyphenolic extract isolated from *Capparis spinosa* L. leaves. in this study giving the immediate yields.
- Compounds that exist in each extract identified by using UV-Visible spectrum, also appearance of several peaks indicating the existing of phenolic acids.
- Compounds are determined through using the HPLC technique, wherein polyphenolic extract seven phenolic acids one flavonoid and one glycoside (gallic acid,caffeic acid, coumaric acid, Vanillic acid, Syringic acid, and ferulic acid, Chlorogenic acid, Rutin and quercetin) have been extracted from *Capparis spinosa L.* leaves.
- The extracted compounds were tested for their acute toxicity properties and it was not appearance any toxic effect on the rats so it is safe.

The extracted compounds have been tested for their inhibitory activities in comparison with various bacteria, these extracts have irregular effects on various bacteria

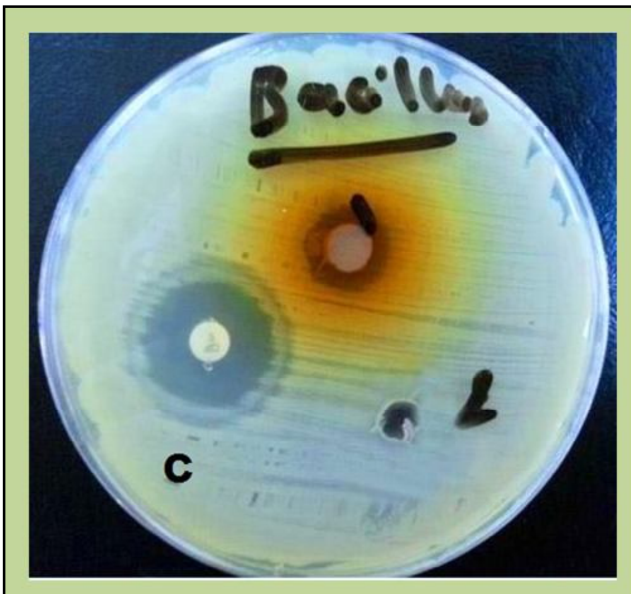

Fig. 8. The activity of Polyphenolic extract against (*Bacillus*) bacteria *Bacillus* :*Bacillus amyloliquefaciens*

compared with control.

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