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Inhibitory Efficacy of Geranium stepporum L. extracts Against Some Species of Bacteria and Fungi

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Abstract

Inhibitory efficacy of Geranium stepporum L. extracts (aqueous, ethanol and methanol) against two gram positive pathogenic bacteria (Staphylococcus aureus, Streptococcus lactis), two gram negative (Escherichia coli and Proteus mirabilis) and four pathogenic fungi (Microsprum canis, Trichophyton mentagrophytes, Pencillium chrysogenum and Fusarium oxysporeum) was studied, tannins and flavonoids contents by using HPLC technique was identified as well glycosides and tannin extracted to testing its biological activities. The results showed that the examined plant contains all the active compounds that were detected, the glycoside with 15.64mm diameter was the most effective against the studied bacteria compared to the negative control and the other studied extracts, Proteus mirabilis with 11.06 diameter is the most affected by the extracts compared to the rest examined bacteria, likewise the results showed that the extracts of water (hot and cold), alcohol (ethanol, methanol, and methanol) and glycosides were more effective against the examined fungi than tannin extract, the species T. andagrophytes with 12.11mm and F. oxysporeum with 11.48mm diameter were most affected by all extracts compared to other tested fungi, quercetin, rutin, kaempferol, and tannins were found to have different concentrations. The quercetin at 1.45ppm and rutin at 1.23ppm. were the most concentrated than other studied compounds, the results also seemed a difference in the concentration of active compounds between the entire aerial plant parts and underground parts.

Key word: phytochemistry; active compounds; abstracts; Geranium stepporum.

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1. Introduction

The genus Geranium L. consist of 430 species, which belongs to the family Geraniaceae, perennials or annuals herbs or shrubs, widely distributes in the temperate and subtropical regions, wich they has economic value as ornamental plants and as industrial sources [28, 17, 13], has a wide spread in northern Iraq [16], commonly used in the field of traditional medicine due to its medicinal properties, specifically for its volatile oils and physiological roles [23, 2,15], ethanol extract of the whole plant parts of G. stepporum has an inhibitory effect against fungal species (Candida albicans, C. parapsilosis and C. krusei), some gram positive bacteria (Staphylococcus aureus, Enterococcus faecalis) and gram negative bacteria Escherichia coli, Pseudomonas aeruginosa [21], methanol extract (entire plant) of G. tuberosum, G. lasiopus, and G. purpureum has anti-fungal effect against Candida albicans, C. kuse and C. parapsilosis [26], the study of [23] indicated to that the species G. lucidum L. contains types of volatile oils (Chrysanthenone, Trans-Pinocarveol, Camphor, E, Z) 2,6-Nonadienal, Pinocamphone, Benzoic acid Octaneic acid, Borneol, cis-Linalool oxide (pyranoid), trans-linalool oxide (pyranoid), Isopinocamphone, Terpinen-4-ol, p-Cymen-8-ol, α-Terpineol, Dodecane, Myrtenol, Decanal, Phenylacetic acid Nonanoic acid, Tridecane, Decanoic acid, 1-Tetradecene, Tetradecane, Tetrahydrogeranyl, acetone, Dodecanal, and Monoerpenoid) as well as showed the aromatic extracts effectiveness of these two species for inhibition of some bacterial species (positive gram: S. aureus, Clostridium perfringens, C. sporogenes, Bacillus Subtilis, Sarcina lutea, and Micrococcus flavus) and negative gram (E. coli, Klebsiella pneumoniae, K. pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella enterica, Candida albicans, Saccharomyces cerevisiae and Aspergillus restrictus) and fungi (Penicillium chrysogenum and fumigatus).

Therefore, this study aims to:

1 - Identify the anti - fungal effectiveness of water and alcohol extracts against pathogenic fungi.

2. Recognizing of the effective chemical content (flavonoid and tannin) of the G. stepporum by using HPLC technique.

3 - Separation of some effective compounds and study their antifungal activity against studied species.

2. Material and Methods

Plant Sampling:

Plant specimens of G. stepporum were collected from two different districts of Sulaymania governorate (Piramagroon and Tawila- northeast Iraq) from March-May 2015 and 2016, collected plants were washed and dried at room temperature away from the light (to avoid optical oxidation), they were grinded and placed in dark sealed cans and kept in frozen until use.

2.1. Aqueous Extraction

Water extraction was carried out according to the study [3] with some modification as follows:

1. extraction of hot and cold water were done by mixing 40gm of plant powder (aerial plant parts) with 160 ml distilled water (1: 4 weight: volume) heated to boiling point 100C°, then shaking and leave to cool down
2. mixture put in the refrigerator for 24 hours, then filtrated through several layers of medical gauze, and filtrated again by the Buchner funnel using filter papers Whatmann No. 1 to get rid of non-powdered parts and fiber to obtain liquid raw plant extract.
3. mixture put it in the rotary evaporator at a temperature not exceeding 40C°, that working on the basis of evaporation under rarefied pressure to obtain a thick layer of extract.
4. the extract was dried at laboratory temperature (30-35C°) then put in bottles with an airtight lid and kept frozen until use.

2.2. Ethanol Extraction

Methods of [3 and 11] was followed for ethanol and methanol extraction, as follows:

1. dissolving 20gm of plant powder (aerial plant parts) in 200ml of ethanol 80% (1:10 weight:volume) with shaking.
2. mixture placed in the refrigerator for 24 hours to soak.
3. extract was filtered through several layers of gauze and filtered again by filter papers (Whatman. No. 1) to get rid of the uncrushed plant parts and fibers
4. extract put in rotary evaporator at a temperature not exceeding 40C° to obtain a thick layer of extract
5. extract was dried at room temperature and put it in airtight lid bottles and kept freeze until use.

2.3. Glycoside Extraction

Methods of [27] was followed:

1. 10gm of plant powder was added to 100 ml of ethanol 80%, placed in the refrigerator for 24 hours and filtrated to get ethanol.

2. the extract was concentrated by rotary evaporator to one third of its volume, 50ml of ether and 5ml of lead acetate solution (0.3 molarity) was added to it by separation funnel to pull the water layer, this process was repeated three times and dried the water layer withdrawn at a temperature of 30C° until the full drying then put the extracts in bottles with an airtight lid and kept freeze until use.

2.4. Tannin extraction

The procedure of [2] was followed.

1. 0.5gm of plant powder was mixed with 50ml of distilled water and boiling for 30 minutes

2. extract was filtrated and put in centrifuge (2000 cycle / minute) for 20 minutes.

3. the floating part was transferred to volumetric flask (100 ml) and add 20ml of lead acetate solution (4%) and complete the volume with distilled water to (100 ml).

4. the extract was filtrated and dried in oven (60C°) until the full drying and then put the extracts in airtight lid bottles and kept freeze until use

2.5. Chemical detections of active compounds

Glycosides and tannins according to [25], resins [19], flavones and alkaloids [8] and saponins and phenols [12 and 1] were detected.

2.6. Sampling preparation for flavonoid and tannin content assessment

1mg of dry plant specimens was dissolved in 150 ml of chloroform and placed on a hotplate for 10 hours, 0.5gm of tartaric acid was added to it and transferred to ultrasonic device for 2 hours then filtrated by filter paper 0.45mm and placed in rotary evaporator to remove the solvent, methanol was added to the remaining for injection into HPLC [24].

2.7. Separation conditions

1. HPLC column: C18 (25 x 4.6mm I.D. 5μm).

2. Mobile phase A = MeOH: Acetic Acid: D.W (10: 2: 88) m B = MeOH: Acetic Acid: D.W (90: 3: 7) ml

3. Wavelength 348 nanometers

4. Flow ratio: 1 ml/min.

5. Temperature 28C°

6. Standard concentration 3mg/ ml.

2.8. Antifungal activity

Methods of [18] was followed in the preparation and sterilization of stock solution in which 1gm of dry plant powder was dissolved in 5ml of distilled to get 200mg/ml of stock solution that sterilized by filtration to remove the bacterial contaminants, this solution was used as a source for preparation of the dilutions 25, 50 and 100mg/ml.

Four species of pathogenic fungi, Microsprum canis, Trichophyton mentagrophytes, Pencillium chrysogenum and Fusarium oxysporum, were directly activated prior to use, agar diffusion method was used [7] to observe the sensitivity of the fungi to the concentrations plant extracts (25, 50 and 100mg/ml) and potatoes dextrose agar media was used for the growing of the tested fungi.

3. Results and discussion

3.1. Chemical detection of active compounds

The results showed that the aerial plant parts was contained on glycoside, tannins, resins, flavonoid, saponin, phenols and alkaloids (table 1).

Table 1: chemical detection of active compounds in G. stepporum

active compounds	Detection method Used reagent	Detection result	G. stepporum
glycosides	Benedict's reagent Fehling reagent	red precipitate red precipitate	+ +
tannins	lead acetate 1%	gelatinous white precipitate	+
resins	boil alcoholic extract and add water acidifier	lees	+
flavonoids	ethanol 50% and potassium hydroxide 50%	yellow color	+
alkaloids	Dragandrov Meyer Wagner	orange precipitate white precipitate brown precipitate	+ + +
saponin	shake the aqueous extract mercury chloride	thick foam for a long time white precipitate	+ +
phenols	ferric chloride	green color	+

3.2. Antifungal activity of G. stepporum extracts

Table 2 seemed that the concentration 100mg/ml of the glycoside extract with 17.33mm diameter is the most effective in inhibiting bacteria compared to control 1, 2 and 3 and all extracts concentrations, the concentration of 100mg/ml of ethanol extract with 15.33 mm inhibitor diameter and glycoside with 16mm were the most effective in inhibition of S. aureus compared to control 1 and 3 and all extracts concentration.

The control treatment 2 is the most effective in inhibiting the bacteria with 20mm diameter, concentration of 100mg/ml of methanol with 17.33mm inhibitor diameter is the most effective in inhibiting St. lactis compared to control 1, 2 and 3 and all extracts concentrations, as for E. coli, the concentration of 100mg/ml of the glycoside with 20.67mm diameter is the most effective in inhibiting bacteria compared to control 1, 2 and 3, in P. mirabilis the concentration of 100mg/ml of the glycoside with 19.33 mm diameter is the most effective inhibition compared to control 1 and 3 and all extracts concentrations, as well as there is no significant difference between the concentration of the extract above and control 2, it is also noted from the table that the glycoside with 15.64mm is the most effective in inhibiting bacteria compared to control and the e examined extracts while P. mirabilis with 11.06mm was the most affected by the extracts compared to the rest of the studied bacterial species.

Table 2: inhibitory impact of G. stepporum extracts against examined bacterial growth (mm)

Extract	Concentration mg/ml	Fungi				Average concentration	Average extract
		P. mirabilis	E. coli	St. lactis	S. aureus		
Hot aqueous	25	A 10 cd	A 10 d	A 10.67 cd	A 10 e	10.134 ef	c 10.267
	50	A 10 cd	A 10 d	A 10.33 cd	A 10 e	10.266 ef	
	100	B 10 cd	B 10 d	B 12 cd	B 10 e	10.400 ef	
Cool aqueous	25	A 6 e	B 0 g	B 0 f	B 0 g	1.20 j	f 2.623
	50	A 8 de	B 0 g	A 8 g	B 0 g	3.20 ij	
	100	A 10 cd	C 0 g	B 7.33 e	C 0 g	3.47 ij	
Ethanol	25	C 11.33 bc	A 16 b	C 12 bc	B 14 bcd	13.466 cd	b 13.822
	50	B 12 bc	A 14.67 bc	B 12.67 bc	A 14.67 bcd	13.736 cd	
	100	B 11.33 bc	A 15.33 bc	A 14 ab	A 15.33 bc	14.264 bc	
Methanol	25	A 12 bc	A 12 cd	A 12 bc	A 10.67 e	11.468 de	b 12.088
	50	B 12 bc	B 10.67 de	A 14 ab	B 11.33 de	11.866 de	
	100	B 12 bc	B 12.67 cd	A 17.33 a	B 11.33 de	12.93 cde	
Glycoside	25	B 14.67 b	A 16.67 b	B 14 ab	B 13.33 cde	14.400 bc	a 15.643
	50	A 18 a	B 16 b	B 15.33 ab	B 13.33 cde	15.198 abc	
	100	A 19.33 a	A 20.67 a	B 14.67 ab	B 16 bc	17.33 a	
Tannin	25	A 10.67 cd	B 3.33 f	A 10 cd	B 2 fg	5.60 hi	e 6.267
	50	A 10.67 cd	B 7.33 de	A 10 cd	C 2 fg	6.40 gh	

	100	A 10.67 cd	B 5.33 ef	A 10 cd	B 4 f	6.80 gh	
control	Distilled water 1	A 0 f	A 0 g	A 0 g	A 0 g	0 k	f 0
	Tetracycline 2	A 19 a	B 16.67 b	C 7 e	A 20 a	15.67 abc	b 12.17
	Nystatin 3	B 4.67 e	A 10 d	A 10 cd	A 10 e	8.93 fg	
Average bacteria		9.15 A	10.8 A	9.683 A	9.650 A		

* the numbers represent the average of three replicates

- similar large horizontal letters mean that there are no significant differences between them at the probability level (0.05).

- similar small vertical letters mean no significant differences between them at the level of probability (0.05).

3.3. Antifungal activity of G. stepporum extracts

Table 3 indicated to the impact of the different extracts with concentrations 25, 50 and 100mg/ml in the inhibition of M. canis, T. mentagrophytes, P. chrysogenum and F. oxysporeum, with inhibition diameter (mm) compared to the control 1 and 2. The concentration of 100 mg/ml of the ethanol with 10.58 mm diameter and glycoside with 10.16 mm and two concentrations 50 and 100mg/ml of methanol with 9.83 and 10.16mm diameter respectively were the most effective in inhibiting fungi compared to control 1, concentrations of the extracts and control 2 is the most effective in inhibiting the fungi with a diameter of 33mm, concentration 100mg/ml of hot water extract with 10mm and ethanol with 10mm diameter and concentrations 25, 50 and 100mg/ml of cold water extract with 10, 10 and 10 mm, and methanol extract with 10, 10 and 11mm are most effective in inhibition of M. canis compared to control 1 and all extracts concentrations, there was no significant difference among the concentrations of the six extracts in the inhibition of T. mentagrophytes, concentrations 50 and 100mg/ml of hot water extract with 10 and 10.33mm diameter and cold water extract with 9.67 and 10mm diameter and concentration 100mg/ml of ethanol with 10mm diameter and methanol with 10mm diameter are most effective in inhibiting P. chrysogenum compared to control 1 and all extracts concentrations. The concentrations 25, 50 and 100 mg/ml of the hot water with 10, 10 and 10mm diameter, glycoside with 10, 10 and 10mm diameter and concentrations 50 and 100mg/ml of the tannin with 10 and 1 mm diameter are the most effective inhibition in F. oxysporeum compared to control 1 and extracts concentrations, as well results revealed that the hot water with 9.74mm, cold water extract with 9.24 mm, ethanol with 9.22mm, methanol extract with 9.77 mm and the glycoside with 9.16mm are the most effective in inhibiting the studied fungi compared to the tannin, there are no significant difference among M. canis T. mentagrophytes, P. chrysogenum and F. oxysporeum affected by concentrations of G. stepporum extracts. The effectiveness of water extracts is due to the water-soluble active compounds, especially flavonoids and glycosides [15], antimicrobial activity of hot water extract may be due to tannins because hot water is the best solvent used for its isolation, may also be due

to gallic acid, which is found in G. lucidum, that dissolved in hot water 60C° [22 and 9], the results of current study are agreed with the study of [21] that showed the effect of the water extract of the entire plant (G. stepporum) against the fungal species (Candida albicans and C. parapsilosis and C. krusei) and the study of [26], seemed the water extract activity of (whole plant) of G. tubersum. G. lasiopus and G. purpureum against species Candida albicans, C. krusei and C. parapsilosis. The inhibitory efficacy of ethanol and methanol of tested plant is mainly due to the soluble compounds, especially phenols such as flavonoids (quercetin and kampferol) or flavonoidic glycosides (rutin) or due to their interferon or with other active soluble compounds in alcohol, the study of [22] indicated to that the antimicrobial efficacy was due to the flavonoid content of ethanol from tannins, rutin, quercetin and kampferol, as such the study [4] referred to that this activity is due to polyphenols (tannins and flavonoids), antimicrobial activity of methanol is due to volatile oil content of active compounds such as previfolin carboxylic acid, ellargic acid, previfolin protocatechuice acid, gallic acid, methyl gallate ester, caffeic acid, perivilin and carboxylic acid [22 , 10], however this does not rule out the effect of alkaloids dissolved in alcohols [14].

Table 3: inhibitory impact of G. stepporum extracts against examined fungal growth (mm)

Extract	Concentration mg/ml	Fungi				Average concentration	Average extract
		F. oxysporeum	P. chrysogenum	T. mentagrophytes	M. canis		
Hot aqueous	25	A 10 b	AB 9.33 bc	A 10 b	A 8.33 bcd	9.415 bc	b 9.749
	50	A 10 b	A 10 b	A 10 b	A 8.67 bcd	9.668 bc	
	100	A 10 b	A 10.33 b	A 10.33 b	A 10 b	10.1650 b	
Cool aqueous	25	B 6 cd	A 9.33 bc	A 10 b	A 10 b	8.832 bc	b 9.249
	50	B 7.33 bcd	A 9.67 b	A 10.33 b	A 10 b	9.332 bc	
	100	B 8 bc	A 10 b	A 10.33 b	A 10 b	9.582 bc	
Ethanol	25	C 4.67 d	B 7.67 bc	A 10 b	AB 8.67 bcd	7.75 cd	b 9.228
	50	C 7.67 bcd	B 9 bc	A 10 b	BA 9.67 bc	9.085 bc	
	100	A 9.67 bc	A 10 b	A 10.67 b	A 10 b	10.085 b	
Methanol	25	A 9.33 bc	B 8 bc	A 10 b	A 10 b	9.332 bc	b 9.778
	50	A 9.67 bc	A 9.67 bc	A 10 b	A 10	9.835 b	

					b		
	100	A 9.67 bc	A 10 b	A 10 b	A 10 b	10.168 b	
Glycoside	25	A 10 b	B 7.67 bc	A 10 b	B 6 cd	8.415 bc	
	50	A 10 b	B 7.67 bc	A 10 b	B 8 cd	8.918 bc	b 9.167
	100	B 10 b	B 9.67 bc	A 12 b	C 9 bcd	10.168 b	
Tannin	25	A 9 b	B 6.33 bc	A 9.33 b	C 0 e	6.17 d	
	50	A 10 b	B 7.67 c	A 9.67 b	C 5.33 d	8.17 bc	c 7.640
	100	AB 10 b	B 833 bc	A 10.67 b	C 5.33 d	8.58 bc	
control	Distilled water 1	A 0 e	A 0 d	A 0 c	A 0 a	0 d	d 0
	Nystatin 2	A 32 a	A 33.33 a	A 32.67 c	A 33 a	33.00 a	a 33
Average bacteria		9.15 A	10.8 A	9.683 A	9.650 A		

* the numbers represent the average of three replicates

- similar large horizontal letters mean that there are no significant differences between them at the probability level (0.05). - similar small vertical letters mean no significant differences between them at the level of probability (0.05).

3.4. Effective chemical content of G. stepporum

The table 4 showed that the G. stepporum contains all studied active compounds, quercetin at 1.45ppm and rutin at 1.23ppm are the most compared to tannins, rutin and kampferol in aerial plant parts, It was found that rutin at 0.47ppm concentration was the most concentrated compared to the rest of the studied compounds, regarding underground plant parts it was found that rutin with 0.47ppm is the most compared to the rest studied compounds.

Table 4: effective chemical content of G. stepporum

No.	Plant parts G. stepporeum	Active compound ppm.				
		Tannins	Quercetin	Catechin	Rutin	Kampferol
	Entire plant	0.26	1.45	0.32	1.23	0.06
	Underground parts	+	+	+	0.47	+

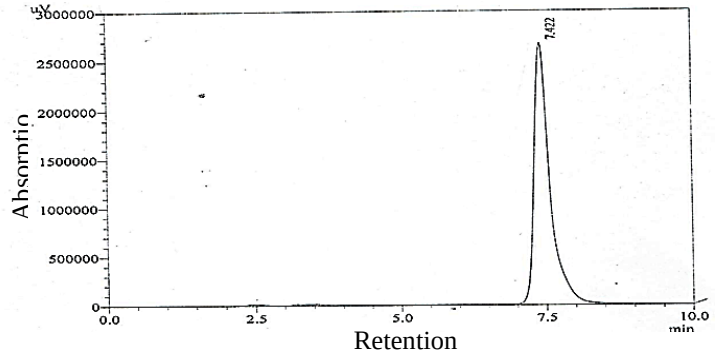

Figure 1: Tannins standard curve by HPLC of studied

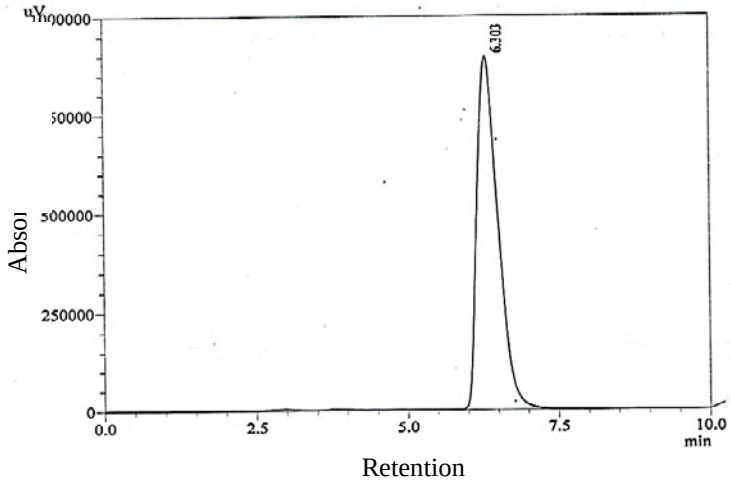

Figure 2: Quercetin standard curve by HPLC of studied

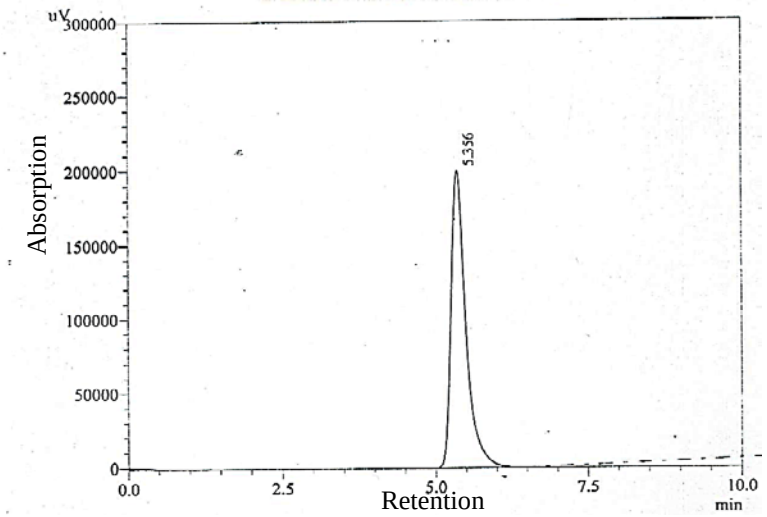

Figure 3: Catechin standard curve by HPLC of studied plant

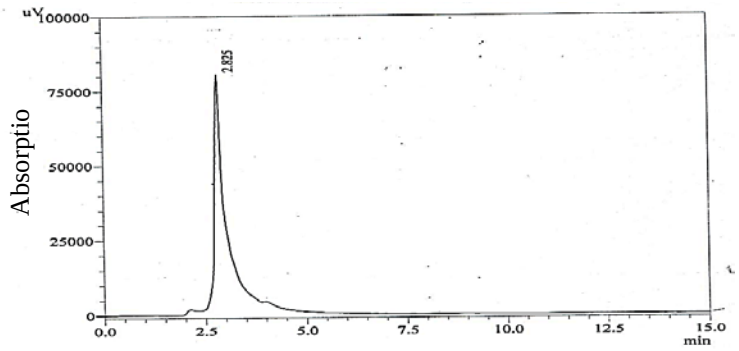

Figure 4: Rutin standard curve by HPLC of studied

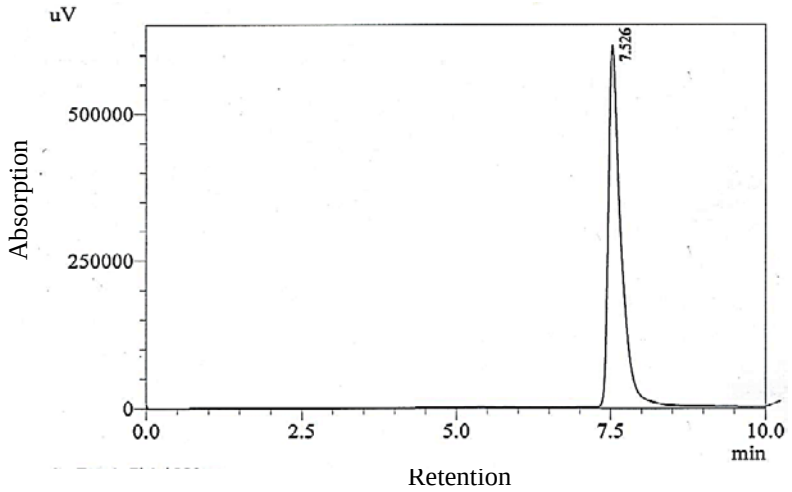

Figure 5: Kampferol standard curve by HPLC of studied

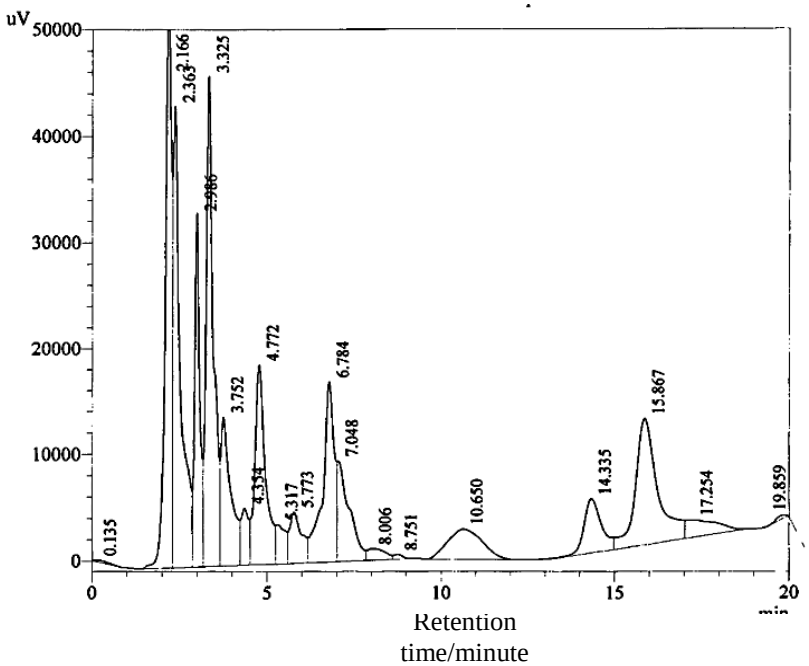

Figure 6: HPLC curve of *G. stepporum* (entire plant)

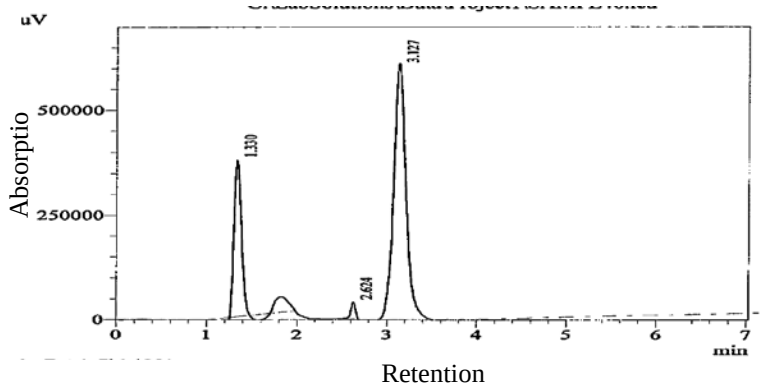

Figure 7: HPLC curve of *G. stepporum* (tuber

The results agree with the others such study of [21] which showed that the aerial plant parts of the G. stepporum contains quercetin-3-O-(3-O-gallolyl) β-galactopyranoside and flavonoids (quercetin 3-O-β- Glucopyranoside, quercetin 3-O-β-galactopyranoside, quercetin 3-O-6 (-O-gallolyl) -β-glucopyranoside and quercetin 3-O-6 (-O-gallolyl)-β-galactopyranoside, likewise the study (Öhretoulu and his colleagues 2011) seemed that the entire plant above the soil of G. purpureum contains the compounds: [4-oxy-5-hydroxy-6-methyl-3,4-dihyropyran-2H -2-pyranoside] and Geranioside B (quercetin 3-3-(4-oxy-5-hydroxy-6-methyl-3 and 4-dihyropyran-2H-2-pyranoside), quercetin, kaempferol, quercetin 4-O-β-glucopyranoside, quercetin 3-O-B-glucopyranoside, aemferol-3-O-β-glucopyranoside and gallic acid derivative (methyl gallate, hydrolysable tannin pusilagin), the study of [4] showing that the aerial plant parts of the G. pusillum contains hexahydroxybiphenyl-D-galactopyranoside 3 and6-O-gallolyl-1.

4. Conclusion

This study concluded that the concentration 100mg/ml of the glycoside extract with 17.33mm diameter is the most effective in inhibiting bacteria and the concentration of 100mg/ml of ethanol extract with 15.33 mm inhibitor diameter and glycoside with 16mm were are the most effective in inhibition of S. aureus. The control treatment 2 is the most effective in inhibiting the bacteria with 20mm diameter and concentration of 100mg/ml of methanol with 17.33mm inhibitor diameter is the most effective in inhibiting St. lactis, as for E. coli, the concentration of 100mg/ml of the glycoside with 20.67mm diameter is the most effective in inhibiting bacteria, in P. mirabilis the concentration of 100mg/ml of the glycoside with 19.33 mm diameter is the most effective inhibition , whileP. mirabilis with 11.06mm was the most affected by the extracts compared to the rest of the studied bacterial species. Regarding the fungi, the concentration of 100 mg/ml of the ethanol with 10.58 mm diameter and glycoside with 10.16 mm and two concentrations 50 and 100mg/ml of methanol with 9.83 and 10.16mm diameter respectively were the most effective in inhibiting fungi, concentrations of the extracts and control 2 is the most effective in inhibiting the fungi with a diameter of 33mm, concentration 100mg/ml of hot water extract with 10mm and ethanol with 10mm diameter and concentrations 25, 50 and 100mg/ml of cold water extract with 10, 10 and 10 mm, and methanol extract with 10, 10 and 11mm are most effective in inhibition of M. canis, there was no significant difference among the concentrations of the six extracts in the inhibition of T. mentagrophytes, concentrations 50 and 100mg/ml of hot water extract with 10 and 10.33mm diameter and cold water extract with 9.67 and 10mm diameter and concentration 100mg/ml of ethanol with

10mm diameter and methanol with 10mm diameter are most effective in inhibiting P. chrysogenum. The concentrations 25, 50 and 100 mg/ml of the hot water with 10, 10 and 10mm diameter, glycoside with 10, 10 and 10mm diameter and concentrations 50 and 100mg/ml of the tannin with 10 and 1 mm diameter are the most effective inhibition in F.

oxysporeum compared to control 1 and extracts concentrations. The effectiveness of water extracts is due to the water-soluble active compounds, especially flavonoids and glycosides, antimicrobial activity of hot water extract may be due to tannins because hot water is the best solvent used for its isolation, may also be due to Gallic acid, which is found in G. lucidum, that dissolved in hot water 60C° , The inhibitory efficacy of ethanol and methanol of tested plant is mainly due to the soluble compounds, especially phenols such as flavonoids (quercetin and kaempferol) or flavonoidic glycosides (rutin) or due to their interferon or with other active soluble compounds in alcohol. Therefore, such studies need to scrutinize the concentrations and testing further extracts qualitatively and quantitatively to take advantage of them later medically.

5. Recommendations

This study recommends additional researches include other investigations of plant extracts and detection of further plant chemical compounds that can be used in the future to combating pathogenic bacteria and fungi.

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