Isolation of Kaempferol 3-*O*-Rutinoside from Kurdish Plant *Anchusa italica* Retz. and Bioactivity of Some Extracts

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Abstract: *Anchusa italica* Retz. is naturally grown in Iraqi Kurdistan Region, and it belongs to the Boraginaceae family. From a phytochemical point of view, the species *A. italica* has been almost neglected so far and it was thus considered worthy of study. In this study, the flavonoids were extracted from aerial parts of the plant using ethyl acetate, amyl alcohol, and *n*-butanol after defatting the plant by *n*-hexane and removing the pigments by chloroform. The residue obtained from evaporation of ethyl acetate extract was separated by reversed phase Medium Performance Liquid Chromatography (MPLC) to give a polar flavonoid. Standard UV experiments allowed for the identification of the aglycone moiety substituted pattern, while the sugar components and the interglycoside linkage was identified by different NMR spectra. The compound was eventually identified as kaempferol 3-*O*-rutinoside.

The other aim of this investigation is to study the antimicrobial activity for some extracts of the plant. It consists of two parts; the first part was antibacterial activity using four different bacteria (*E. coli, Staph. aureus, Bacillus sp.* and *Pseudo. aeruginosa*) via agar diffusion disc method, while the second part includes antifungal activity using two different fungi (*Pencillium puberulum* and *Aspergillus flavus*). This study showed that *Anchusa italica* is a good antibacterial plant especially aqueous and ethanolic extracts, at which the inhibition zone reaches (13 mm), while the aqueous extract was more effective compared with the ethanol extract for antifungal.

Keywords: Medicinal Plants, Boraginaceae, *Anchusa italica* Retz., Kaempferol 3-*O*-Rutinoside, Antimicrobial Activity

1. Introduction

Herbal medicine is a traditional or folk medicine practice based on the use of plants' seeds, berries, roots, leaves, bark, flowers, and plant extracts for medicinal purposes (Kraft, 2009; Amin et al., 2020). Plants are important sources of medicine for thousands of years and are the most important source of life-saving drugs for most of the worlds' population (Hussain et al., 2019; Tripathi & Tripathi, 2003). Herbal remedies are widely used for the treatment and prevention of various diseases that contain highly active pharmacological compounds (Saad et al., 2006; Abdullah et al., 2016; Amin et al., 2016). The knowledge about medicinal plant among the people of Iraq is based on hundreds of years of belief, observations, and a rich medicinal history (Alsamarkandi, 1985; Khalil, 1979). The Boraginaceae family comprises about 146 genera and 2000 species worldwide, while it is represented in wild Iraq by 26 genera and about 93 species (Al-Mussawy, 1987).

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Anchusa italica Retz. (*A. italica*) is a species of the flowering and perennial plant (Tutin et al., 1964) and has been used by many Kurdish people for cooking. The whole plant is antitussive, depurative, and diuretic. It is harvested when in flower and dried for later use. The dried and powdered herb is used as a poultice to treat inflammations. Use internally with caution, the plant contains the alkaloid cynoglossine which can have a paralyzing effect and carcinogenic (Al-Mussawy, 1987). Flowers are used like tea, and tonic to invalids and children, also they lower pulsation (Chiej, 1984).

A. italica is native throughout whole Europe, especially the southern and central parts such as Italy, Greece, Hungary, Romania, France, Portugal, Spain, Ukraine, and Russia. It is also found in Western Asia like; Iraq, Pakistan, Israel, Cyprus, Turkey, etc, Caucasus (e.g.: Azerbaijan, Soviet & Middle Asia: Kazakhstan etc.), and Tropical Asia (e.g.: Pakistan) (Bryant et al., 2011). The plant is present in Iraq especially it is widely distributed in Kurdistan region (Al-Mussawy, 1987) like; Kirkuk, Persian Foothill, Mosul, Upper Jazira, Amadia, Rawanduz and Sulaimaniya Districts (Al-Rawi & Chakaravarty, 1988).

The seeds of *Anchusa italica* Retz. are rich sources of many unsaturated fatty acids like; linoleic acid, oleic acid, γ-linolenic acid, eicosenoic acid and several saturated fatty acids like palmitic acid (Osw et al., 2017; López-Martínez et al., 2005; Guil-Guerrero et al., 2001). Also, the plant containing some other compounds like; triterpene glycosides (saponins), polyphenols (flavonoids) (Kuruüzüm-Uz et al., 2010) toxic and nontoxic pyrrolizidine alkaloids (Members, 2009) vitamin E, tannins (Khare, 2007) and nearly thirty elements (Osw & Hussain, 2020).

During literature review it was found that no earlier investigations had been done on *A. italica* in Iraq which is naturally grown in Kurdistan region. Thus, we decided to do the study of the plant in terms of extraction, isolation, and detection of natural compounds in the aerial parts of the plant, using different techniques such as: MPLC, ^{1}H-NMR, ^{13}C-NMR, DEPT ^{13}C-NMR and UV-Visible spectroscopy. The other aim of this investigation is to demonstrate the antimicrobial activity of *Anchusa italica* Retz. such as: antibacterial and antifungal activities.

2. Materials and Methods

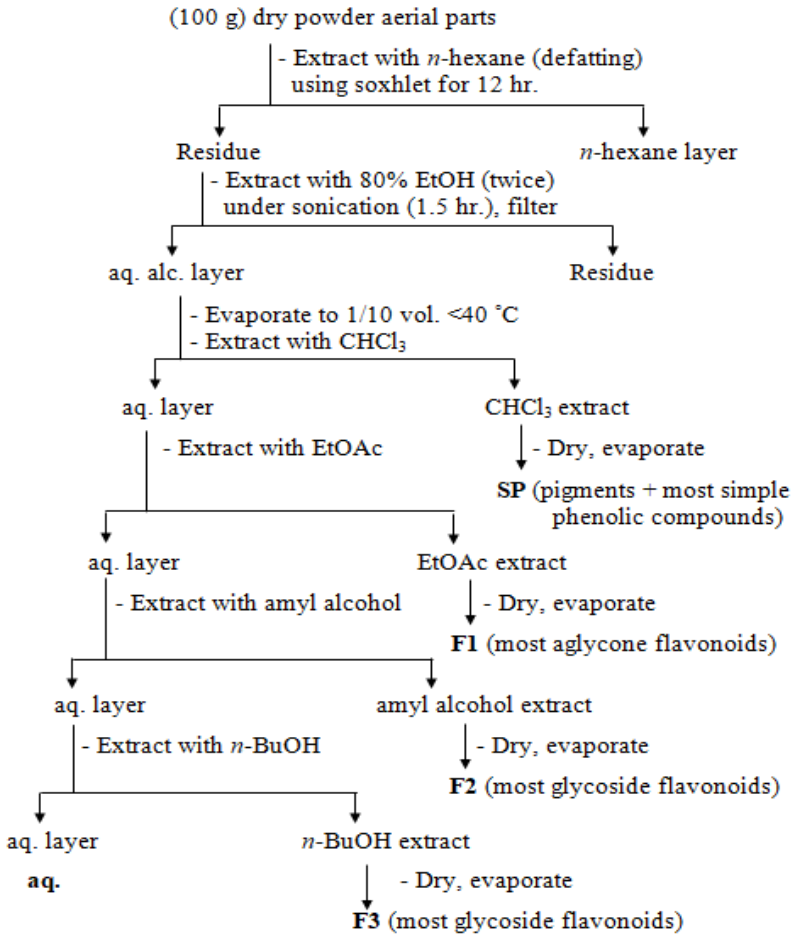
2.1 Plant Material (Collection)

Anchusa italica Retz. were collected during May 2009 from the Garota village (Safeen Mountain) which belongs to Shaqlawa-Erbil/Kurdistan region. The collected plant materials were identified and classified from ESUH (Education Salahaddin University Herbarium). The plant raw materials were washed and air-dried under shade at room temperature. After drying, the plant parts separately were ground into fine powder using a laboratory blender, passed through a 0.71 mm mesh sieve, to provide homogeneous powder for the analysis. Powdered materials were stored in dark bottles and maintained at room temperature until required for analyzes.

2.2 Extraction of Flavonoids (Harborne, 1998)

Generally, alcohols are the solvents of choice for the extraction of flavonoids. The most useful solvent for all types of flavonoids from living tissue is 70-80% alcohol (Goodwin, 1976). Dry powder aerial parts of the plant (100 g) were extracted with 300 ml of *n*-hexane using soxhlet apparatus for 12 hr. The defatted residue was drawn out from the thimble and extracted with 500 ml of 80% EtOH for (1.5

hr.) in an ultrasonic bath, and then filtered through the Buckner funnel. The residue was twice re-extracted with 500 ml of the same solvent. The extracts were combining and evaporated to (1/10 volume) using rotary evaporator at 40 °C. The remained extract was washed with 50 ml of $CHCl_3$ (five times) to separate the simple phenolic compounds and some pigments. The remained extract was further extracted with 50 ml (five times) of EtOAc, amyl alcohol and *n*-BuOH separately to obtain fraction (F1, F2 and F3) respectively, and an aqueous layer remained. The fractions F1, F2 and F3 were evaporated to dryness at low pressure and below 40 °C by using rotary evaporator (Scheme 1).



Scheme 1: Extraction of flavonoids

2.2.1 Examination of Flavonoids in (F1, F2, and F3) Extracts

To examine the complexity of flavonoid mixtures in (F1, F2, and F3) extracts (Scheme 1), a simple thin layer chromatography (TLC) has been performed. The chromatogram was run in different solvent systems as a mobile phase and hence some reagent sprayers were used to detect flavonoids.

The plates of Silica gel F254 (0.2 mm) thickness were heated in an oven at 126 °C for 4 min. The technique was established as a rapid method for initial screening of flavonoid compounds. The fractions (F1, F2, and F3) were applied with micropipettes along a line (2 cm) above the rim of the plate, for this purpose there are three different solvent systems were used as a mobile phase, but the most successful and efficient solvent system was *n*-BuOH–HOAc–H_2O (4:1:5, top layer) or (6:1:2).

The flavonoid compounds were detected by spraying the TLC plates with some reagents such as 5% alc. $AlCl_3$ (yellow-green fluorescence in UV), aq. $FeCl_3$–$K_3Fe(CN)_6$ (1:1) (blue on yellow background), Folin (blue on white background) and 5% alc. NaOH (intense yellow-green fluorescence develops when dried TLC is placed under UV light for 5-10 min).

2.2.2 Isolation of Kaempferol 3-*O*-Rutinoside in (F1) Extract

About 190 mg of (F1) extract was purified with an MPLC "Isolera ONE" (Biotage) with a reversed phase column (KP-C18-HS SNAP 12g) using the following condition:

Solvents: H_2O–MeOH; 0-2 min: 95:5; 2-27 min: gradient to 100% MeOH; 27-32 min: 100% MeOH; Constant flow rate: 12 ml/min; UV detector: 220 nm.

Some fractions were collected especially fraction (8 & 9) and then applied them on reverse phase TLC plates of Silica gel F254 (0.2 mm) thickness using MeOH–H_2O (3:2) as mobile phase, Figure (1). It appeared that the purification was not excellent but good enough to collect a fraction containing a pure sample of the lower yellow spot.

2.2.2.1 UV-Visible Spectral Analysis of Isolated Kaempferol 3-*O*-Rutinoside

The UV-Visible spectra of flavonoids need the following reagents (Mabry et al., 2012):

- Sodium methoxide (NaOMe): Freshly cut metallic sodium (0.25 g) was added cautiously in small portions to dry spectroscopic grade MeOH (10 ml).
- Aluminum chloride ($AlCl_3$): 50 g of fresh anhydrous reagent grade $AlCl_3$ was added cautiously to spectroscopic grade MeOH (10 ml).
- Hydrochloric acid (HCl): Concentrated HCl (5 ml) was mixed with distilled water (10 ml).
- Sodium acetate (NaOAc): Anhydrous powdered reagent grade NaOAc was used.
- Boric Acid (H_3BO_3): Anhydrous powdered reagent grade H_3BO_3 was used.

UV experiments on fraction 8-9 (lower yellow spot) were performed using a UV-Visible spectrophotometer (Jasco V-550). A stock solution of isolated flavonoid was prepared by dissolving a small amount of the flavonoid (about 0.1 mg) in about 10 ml of spectroscopic MeOH. All spectra were recorded with a wavelength variation of 100 nm/min and investigating the range from 200-500 nm.

Experiment 1: The MeOH spectrum of the stock solution was registered in standard condition (as specified above). The NaOMe spectrum was measured immediately after the addition of five drops of NaOMe to the stock solution which was used for the previous measure. Then the solution was discarded. Three λmax were recorded for the sample at [267, 304(sh) and 340 nm] and three λmax for (sample + NaOMe) at [275, 328 and 400 nm] Table (1) Figure (2, A).

Experiment 2: The $AlCl_3$ was measured immediately after the addition of six drops of $AlCl_3$ solution to 1 ml of a fresh stock solution of flavonoid. The $AlCl_3$/HCl spectrum was recorded immediately after the addition of three drops of the HCl solution to the cuvette containing the $AlCl_3$. Then the solution was discarded. Four λmax were obtained (for sample + $AlCl_3$) at [274, 305, 353 and 397 nm] and four λmax for (sample + $AlCl_3$ + HCl) at [275, 304(sh), 345 and 398 nm] Table (1) Figure (2, B).

Experiment 3: The NaOAc spectrum of the flavonoid was determined as follows. Excess NaOAc was added with shaking to a cuvette containing about 1ml of a fresh portion of the stock solution of the flavonoid. About 2mm layer of NaOAc remained on the bottom of the cuvette. Sufficient powdered

reagent grade H_3BO_3 was added with shaking to the cuvette containing the NaOAc to provide a saturated solution. We acquire three λmax for (sample + NaOAc) at [275, 311 and 384 nm] and also three λmax for (sample + NaOAc + H_3BO_3) at [267, 305(sh) and 352 nm] Table (1) Figure (2, C).

2.3 Antimicrobial Studies

2.3.1 Antibacterial Activity of *A. italica* Extract (Valgas et al., 2007)

The antibacterial activity of *A. italica* extracts was examined against four strains of standard bacteria namely *Escherichia coli* (G-ve), *Staphylococcus aureus* (G+ve), *Bacillus sp.* (G+ve) and *Pseudomonas aeruginosa* (G-ve) using agar diffusion disc method.

Agar diffusion disc: Five-millimeter filter paper discs (Whatman no. 3) were impregnated with 1 ml of each of the different plant (F) extract dilutions was used (20, 40, 60 and 80 mg/ml). The discs could remain at room temperature until complete diluent achieved evaporation and kept under refrigeration until ready to be used. Discs loaded with natural products were placed on the surface of the agar. The plates incubated for 24 hr. at 37 °C, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in (mm), Table (2).

2.3.2 Antifungal Activity of *A. italica* Extracts

Aerial parts of *A. italica* (50 g) are extracted with (300 ml) of EtOH in an ultrasonic bath (1.5 hr.). Filter the extract through Buckner funnel and then evaporate to dry under reduced pressure and 40 °C using rotary evaporator to obtain EtOH extract. The antifungal activity of EtOH extract was examined against two types of standard fungi namely *Pencillium puberulum* and *Aspergillus flavus*. The growth of the fungal mycelium was measured in (cm), Table (3).

2.3.2.1 Ethanolic Plant Extracts Sterilization and Dilution Preparation (Rios et al., 1987)

The stock solution of plant extract was prepared by adding 1 g of EtOH plant extract to 5 ml of DMSO then this stock solution was sterilized by using (Millipore filters 0.2 µm). The concentrations (3, 5 and 7 mg/ml) prepared from the stock solution then added to 500 ml of Czapek (Dox) Agar (CDA) and poured on to sterilized Petri dishes then inoculated by fungi, a sterilized Petri dish with no addition of plant extract (CDA medium only) used as control which was also inoculated by fungi.

2.3.3 Statistical Analyses

The analysis of data for bacteria was performed by using SPSS (Statistical Package for Social Science) software program. Factorial CRD (Complete Randomized Design) was used for comparison between the treatments and all data were presented as mean standard error and percentage values.

3. Results and Discussion

3.1 Extraction of Flavonoids

The flavonoid compounds were separated from *A. italica* aerial parts by extraction with ethyl acetate, amyl alcohol and then *n*-butanol from the defatted and removing other impurities as shown in Scheme (1). Simple thin layer chromatography (Silica gel F254) has been performed to examine the complexity of flavonoid mixtures in (F1, F2, and F3) extracts using BAW (4:1:5, top layer) as a mobile phase. Consequently, different reagent sprayers were used to detect flavonoids such as folin reagent response

(+ NH_3), 5% alc. $AlCl_3$ and 5% alc. NaOH [both response various colours fluorescence under UV lamp either at 254 or 366 nm)]. The best reagent was the first one. Also, the aq. $FeCl_3$–$K_3Fe(CN)_6$ (1:1) was used but it was not good and not colour fluorescence under UV lamp.

3.1.1 Isolation and Identification of Kaempferol 3-*O*-Rutinoside

The residue from evaporation of the extract (F1) (Scheme 1) was purified by reversed phase MPL chromatography using gradient system H_2O–MeOH. The eluted fractions then applied them on reverse phase TLC plates of Silica gel F254 (0.2 mm) thickness using MeOH–H_2O (3:2) as a mobile phase, Fig. (1, I). Another TLC plates for fractions 4 to 9 were obtained using the same system, Fig. (1, II) and then yellow spots were isolated from the fractions (8 & 9).

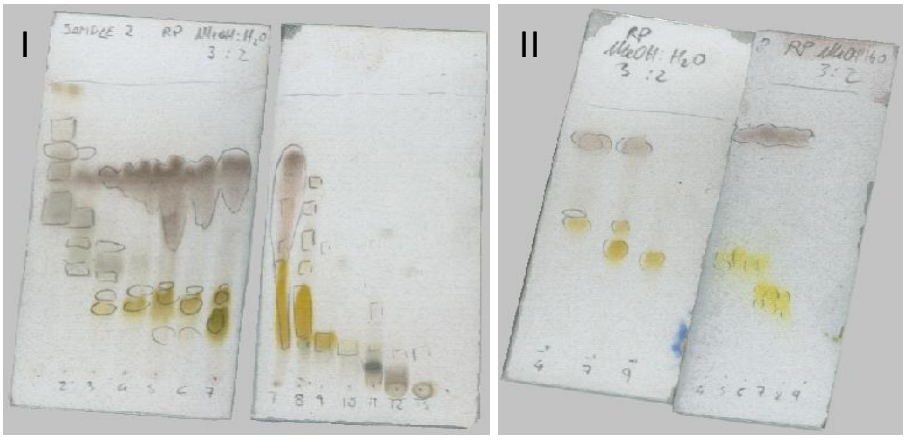


Figure 1: TLC plates for eluted fractions from (F1) extract using MPLC, I- whole fractions (1 to 13), II- fraction (4 to 9).

The isolated yellow spot was a polar flavonoid and the following analysis confirmed that this flavonoid was kaempferol 3-*O*-rutinoside.

3.1.2 Identification of Aglycone Moiety from Kaempferol 3-*O*-Rutinoside Using Some UV Experiments

Standard UV experiments allowed for the identification of the substitution pattern of the aglycone moiety. The UV spectrum of the methanolic solution of kaempferol 3-*O*-rutinoside exhibited two major absorption bands at 267 nm and 340 nm, which confirmed the flavonol structure. Bathochromic shift with NaOMe supported the presence of 4′-hydroxyl and with NaOAc indicated the 7-hydroxyl functions. The $AlCl_3$ and $AlCl_3$/HCl spectrum of isolated flavonoid showed the presence of a 5-hydroxyl group. This fact indicated that the 3-hydroxyl was absent or substituted. Table (1) and Fig. (2-A, B, and C) showed the UV data and spectra for kaempferol 3-*O*-rutinoside (Members, 2009).

Table 1: UV-Visible absorption spectral data of kaempferol 3-*O*-rutinoside isolated from *A. italic* (λ max (nm) MeOH and their shifts in different solvents)

Solvent / Shift Reagent	**kaempferol 3-*O*-rutinosid**
MeOH	340, 304(sh)*, 267
+ NaOMe	400, 328, 275
+ $AlCl_3$	397, 353, 305, 274
+ $AlCl_3$ + HCl	398, 345, 304(sh), 275
+ NaOAc	384, 311, 275
+ NaOAc + H_3BO_3	352, 305(sh), 267

*sh = Shoulder

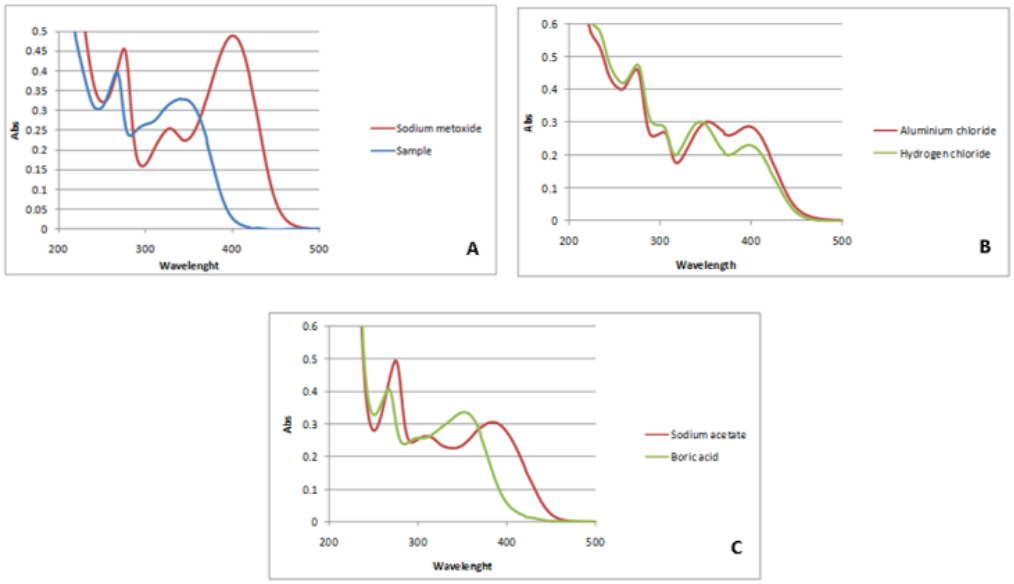


Figure 2: UV spectra for kaempferol 3-*O*-rutinoside; A- sample in MeOH + NaOMe, B- smaple + $AlCl_3$ + $AlCl_3$/ HCl and C- sample + NaOAc + NaOAc / H_3BO_3.

3.1.3 Identification of Kaempferol 3-*O*-Rutinoside by NMR Spectroscopy

Different NMR spectra have been taken to identify the isolated flavonoid. The ^{1}H-NMR spectrum of kaempferol 3-*O*-rutinoside in DMSO- d_6 Fig. (3) shows the following signals:

δ (ppm): 8.00 (2H, d, J=8.8 Hz, 2′-H and 6′-H), 6.88 (2H, d, J=8.8 Hz, 3′-H and 5′-H), 6.40 (1H, d, J=1.8 Hz, 8-H), 6.20 (1H, d, J=1.8 Hz, 6-H), 5.30 (1H, d, J=7.3 Hz, 1″-H), 4.37 (1H, bs, 1‴-H), 3.70 (1H, bd, J=10.0 Hz, 6a″-H), 3.68 (2H, m, 3‴-H and 5‴-H), 3.30 (1H, bd, J=10.0 Hz, 6b″-H), 3.24 (2H, m, 5″-H and 2‴-H), 3.21 (1H, m, 3″-H), 3.16 (1H, m, 2″-H), 3.10 (1H, m, 4‴-H), 3.05 (1H, m, 4″-H), 0.98 (3H, d, J=6.1 Hz, 6‴-H), and hence the signals of nine protons for nine hydroxyl groups of the compound interfere with the signals between (3.0-5.5) ppm. The ^{13}C-NMR spectrum of kaempferol 3-*O*-rutinoside in DMSO-d_6 Table (2) and Fig. (4) showed the signals of carbon atoms for isolated flavonoid. The reported reference (Kazuma et al., 2000) confirmed our results in both ^{1}H-NMR and ^{13}C-NMR data for isolated flavonoid compound (kaempferol 3-*O*-rutinoside). As a result, the data were very similar to a reference that mention here. The DEPT ^{13}C-NMR spectrum of kaempferol 3-*O*-

rutinoside in DMSO-d_6, Fig. (5) shows the following signals; δ (ppm): 130.8 (C-2′ and C-6′), 115.1 (C-3′ and C-5′), 101.3 (C-1″), 100.8 (C-1‴), 98.8 (C-6), 93.8 (C-8), 76.4 (C-3″), 75.7 (C-5″), 74.2 (C-2″), 71.8 (C-4‴), 70.6 (C-3‴), 70.3 (C-2‴), 69.9 (C-4″), 68.2 (C-5‴), 66.9 (C-6″), 17.7 (C-6‴).

Table 2: ^{13}C-NMR chemical shifts of kaempferol 3-*O*-rutinoside isolated from *A. italica*, (300 MHz; DMSO-d_6 compared with a reference (Kazuma et al., 2000))

Moiety	**C Positions**	**δ (ppm) kaempferol 3-*O*-rutinoside isolated from *A. italica***	**δ (ppm) kaempferol 3-*O*-rutinoside**
Kaempferol (aglycon part)	2	156.49	156.4
	3	133.20	133.1
	4	177.34	177.3
	5	161.17	161.1
	6	98.76	98.6
	7	164.34	164.0
	8	93.76	93.6
	9	156.80	156.8
	10	103.90	103.9
	1′	120.86	120.8
	2′ & 6′	130.85	130.8
	3′ & 5′	115.08	115.0
	4′	159.91	159.8
3-*O*-Glc (sugar at C-3)	1″	101.35	101.3
	2″	74.16	74.1
	3″	76.36	76.3
	4″	69.89	69.9
	5″	75.73	75.7
	6″	66.87	66.8
Rhamnosyl	1‴	100.76	100.7
	2‴	70.33	70.3
	3‴	70.58	70.5
	4‴	71.81	71.7
	5‴	68.23	68.1
	6‴	17.71	17.6

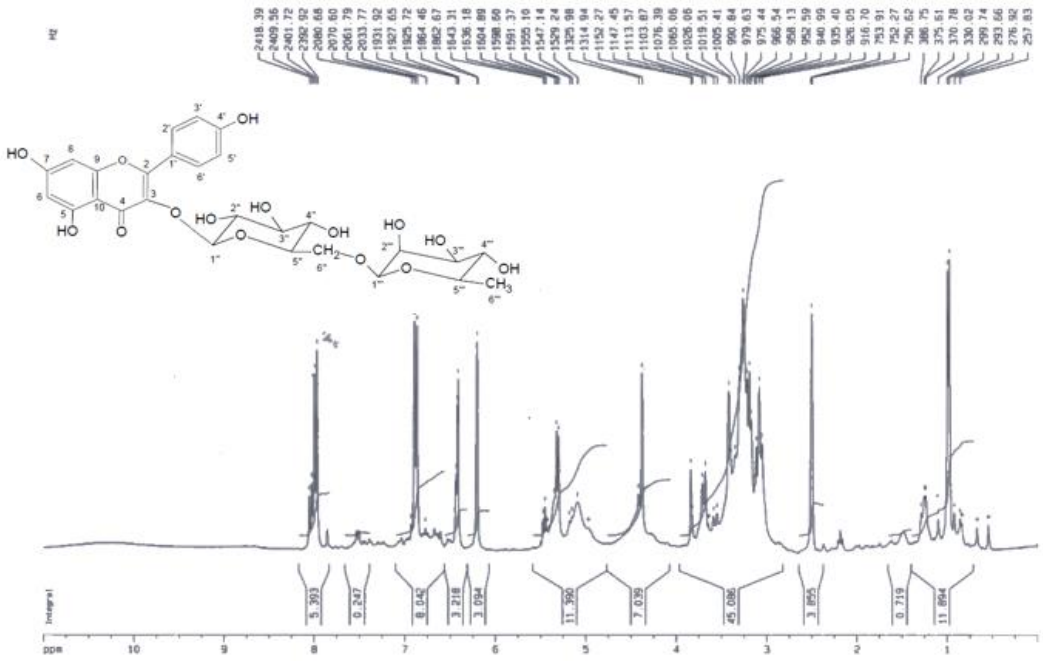


Figure 3: ^{1}H-NMR spectrum of Kaempferol 3-*O*-rutinoside

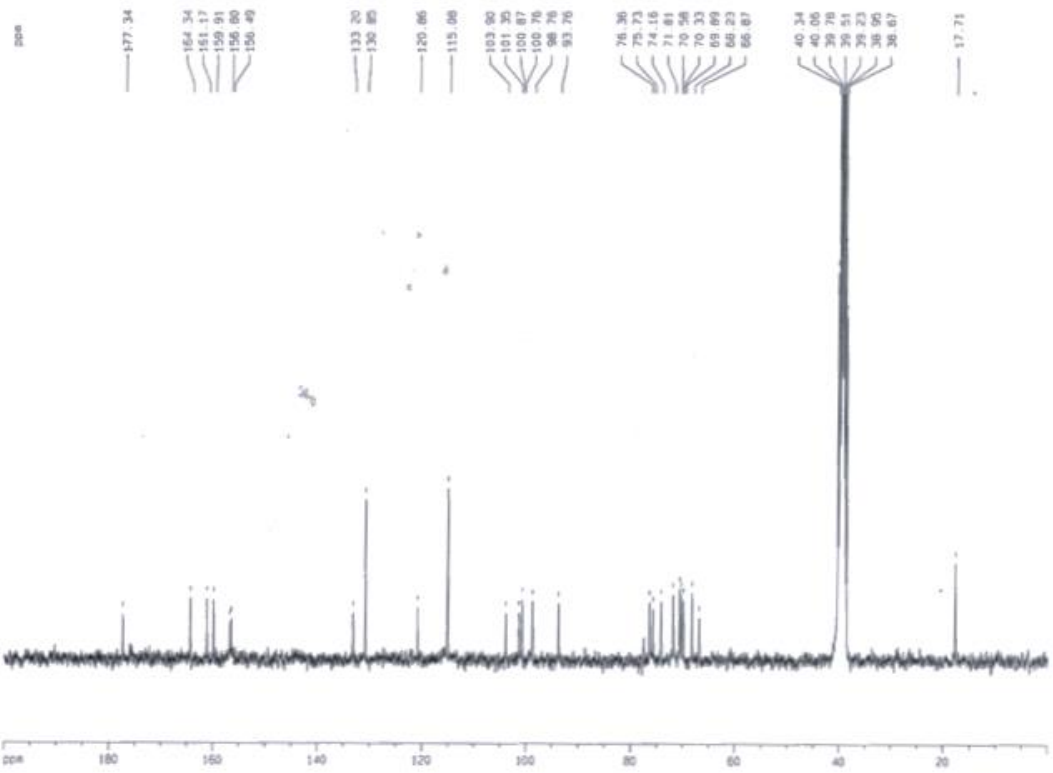


Figure 4: ^{13}C-NMR spectrum of Kaempferol 3-*O*-rutinoside

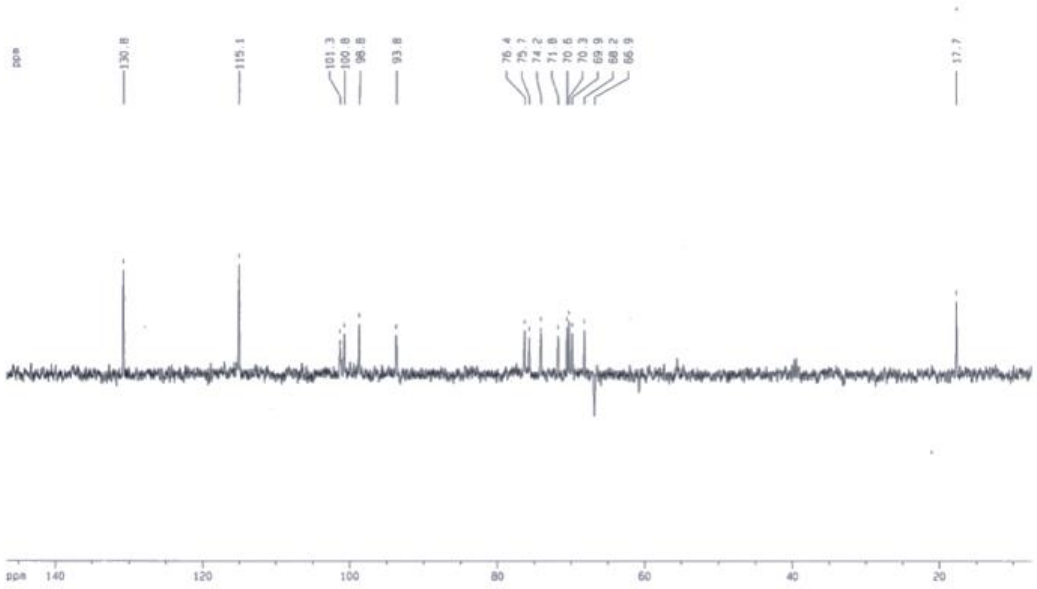


Figure 5: DEPT ^{13}C-NMR spectrum of Kaempferol 3-*O*-rutinoside

3.2 Antimicrobial Studies

3.2.1 Antibacterial Activity

I- Against *Staph. aureus*:

There are statistically significant differences ($p < 0.05$) regarding the antibacterial activity of different plant extracts. (The highest concentration of the extract (F2) was recorded the best antibacterial effect with inhibition zone about (11 mm), Table (3). Only two extracts (F1 and F2) were showed biological activity against *Staph. aureus* while the other extracts (SP, F3, and aq.) have no observed action as illustrated in Fig. (6)

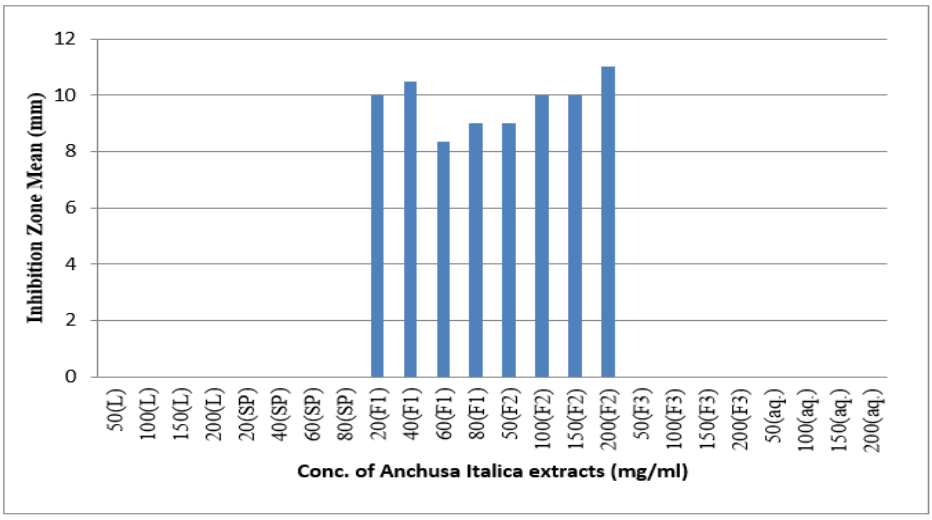


Figure 6: Effect of *A. italica* extracts with different concentrations on *Staph. aureus*

Table 3: Effects of *A. italica* extracts with different concentrations on four bacteria. (Mean ± S.E.)

Plant Extracts	Conc. (mg/ml)	Clear zone (mm)							
		Staph. aureus		*Bacillus sp.*		*Pseudo. aeruginosa*		*E. coli*	
		Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
L	50.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	150.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	200.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SP	20.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	40.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	60.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	80.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F1	20.00	10.00	0.58	12.00	1.00	10.00	1.00	8.00	0.00
	40.00	10.50	0.29	10.00	0.00	9.00	1.00	8.00	1.00
	60.00	8.33	0.33	8.50	0.50	10.00	0.00	8.00	1.00
	80.00	9.00	0.29	8.33	0.58	8.17	0.29	8.00	1.00
F2	50.00	9.00	0.29	9.00	0.50	9.00	0.50	10.00	0.00
	100.00	10.00	0.58	9.00	0.50	9.00	0.50	10.00	0.50
	150.00	10.00	0.58	11.00	1.00	11.00	0.00	10.00	0.50
	200.00	11.00	0.58	12.00	1.00	9.00	0.50	11.00	0.00
F3	50.00	0.00	0.00	9.50	0.50	9.00	0.50	9.00	0.50
	100.00	0.00	0.00	10.00	0.00	11.00	1.00	10.50	0.50
	150.00	0.00	0.00	10.67	1.53	12.00	1.00	10.00	1.00
	200.00	0.00	0.00	11.00	0.00	13.00	1.00	10.00	1.00
aq.	50.00	0.00	0.00	7.50	0.50	8.50	0.50	0.00	0.00
	100.00	0.00	0.00	11.00	0.50	11.00	0.50	0.00	0.00
	150.00	0.00	0.00	13.00	1.00	12.00	0.00	0.00	0.00
	200.00	0.00	0.00	13.00	1.00	13.00	0.50	0.00	0.00

L = Lipid extract, SP = Simple phenolic extract + pigments, F1 = Ethyl acetate extract, F2 = Amyl alcohol extract, F3 = *n*-butanol extract, aq. = Aqueous extract, S.E. = Standard error.

II- Against *Bacillus sp*.

The extracts which exhibited no effect on *Bacillus sp.* were (L and SP), while other remained extracts (F1, F2, F3 and aq.) were showed biological activity against *Bacillus sp.* as shown in Fig. (7). The last two concentrations of (aq.) extract recorded the best antibacterial influence with inhibition zone about (13 mm).

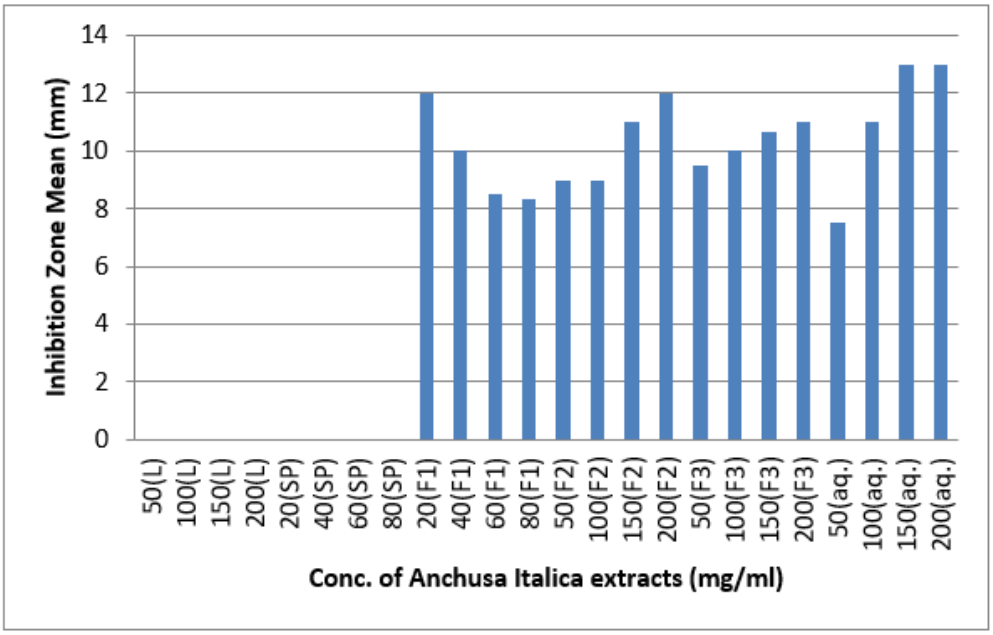


Figure 7: Effect of *A. italica* extracts with different concentrations on *Bacillus sp.*

III- Against *Psedo. aeruginosa*:

Most extracts showed biological activity against *Psedo. aeruginosa* except for two extracts (L and SP) which had no influences against *Psedo. aeruginosa*. The highest concentrations of extracts (F3 and aq.), at which the last two concentrations of them were recorded the best antibacterial effect with inhibition zone about (13 mm). The results show that there are statistically significant differences regarding the bactericidal activity of different plant extracts as shown in Table (3) and Fig. (8).

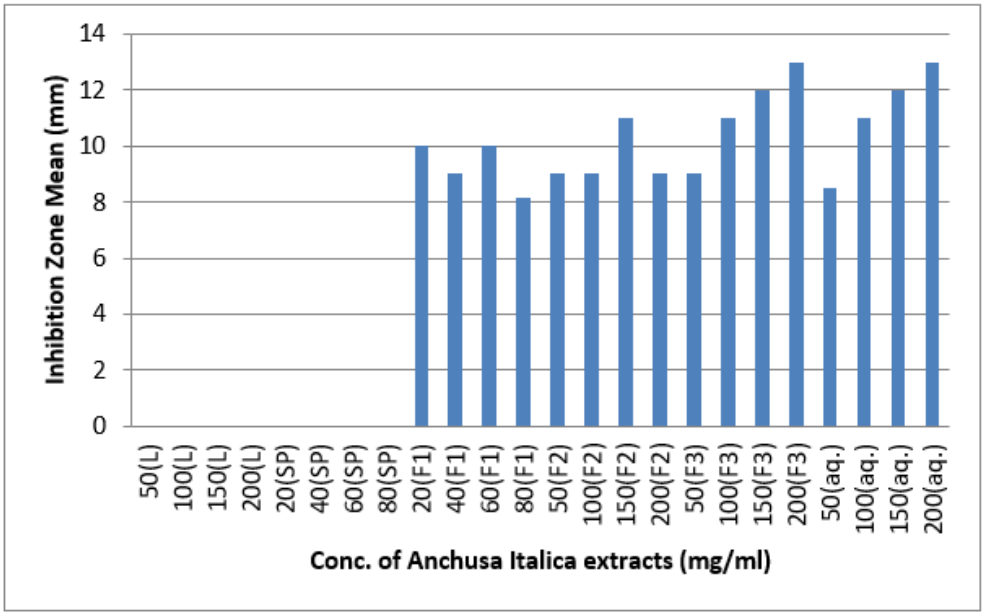


Figure 8: Effect of *A. italica* extracts with different concentrations on *Pseudo. aeruginosa*.

IV- Against *E. coli*:

Here the highest concentration of the extract (F2) was recorded the best antibacterial influence with inhibition zone about (11 mm). In general, there are four extracts (F1, F2, and F3) which were showed biological activity against *E. coli* whereas the other extracts (L, SP, and aq.) have no observed action as illustrated in Table (3) Fig. (9).

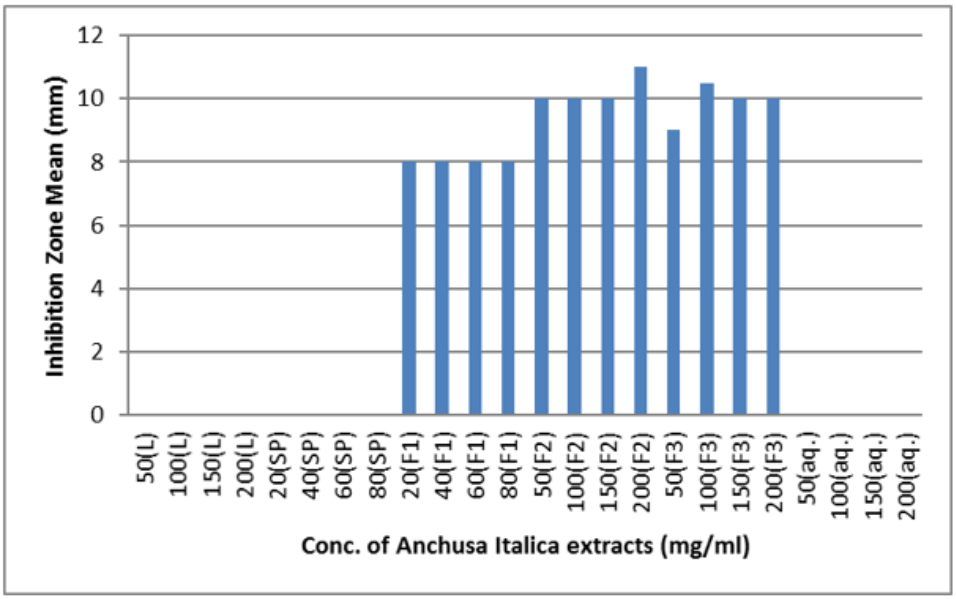


Figure 9: Effect of *A. italica* extracts with different concentrations on *E. coli*.

3.2.2 Antifungal Activity

The current results showed that the EtOH extract has a very weak antifungal influence against *Aspergillus flavus*. The extract could not inhibit the growth of *Aspergillus flavus* except for the highest concentration of EtOH extract which showed weak antifungal activity with a mean value (3.69 + 0.64 cm) comparing to control group (4.45 + 0.71) as shown in Table (4), Fig. (10).

In contrast to the previous result the antifungal action of EtOH extract was very clear against *Pencillium puberulum* Table (4). The aqueous extracts were more effective compared with the EtOH extracts. The best result was recorded in the highest concentration of aqueous extract with a mean value (1.09 + 0.24) as compared to control value (3.11 + 0.05) as illustrated in Fig. (10).

Table 4: Effects of *A. italica* aq. and EtOH extracts with different concentrations on *Aspergillu flavus* and *Pencillium puberulum* (Mean ± S.E.).

Extracts	Concentration (mg/ml)	Mycelium growth (cm)			
		Aspergillus flavus		*Pencillium puberulum*	
		Mean	S.E.	Mean	S.E.
Control	0	4.45	0.71	3.11	0.05
Aqueous	6.25	6.51	0.11	1.50	0.16
	7.5	5.81	0.62	1.60	0.50
	8.75	7.24	0.13	1.09	0.24
EtOH	3.75	6.44	0.26	2.75	0.05
	6.25	5.19	0.40	2.73	0.11
	8.75	3.69	0.64	2.89	0.34

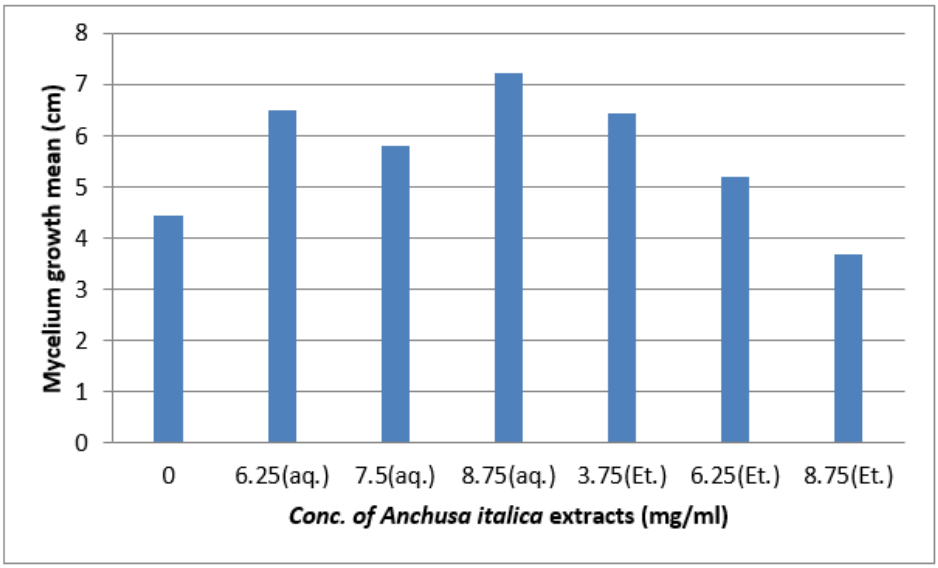


Figure 10: Effect of *A. italica* aqueous and ethanol extracts with different concentrations on *Aspergillus flavus*.

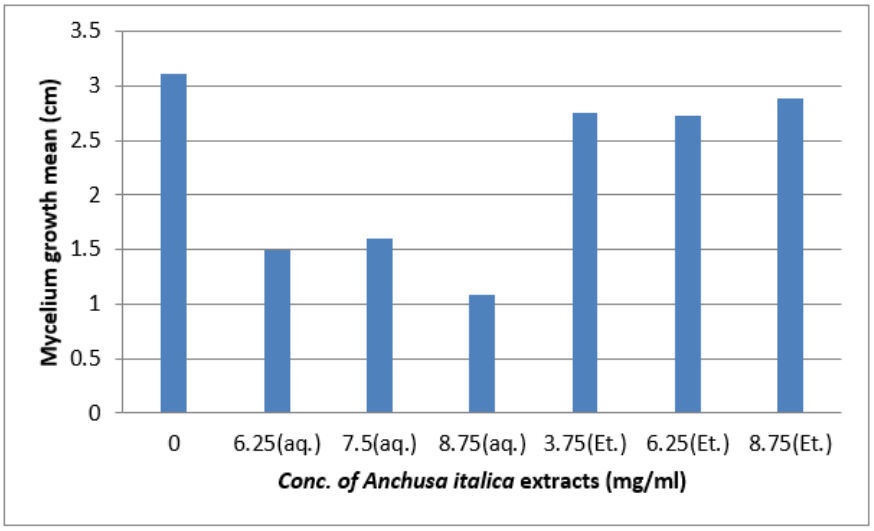


Figure 11: Effect of *A. italica* aqueous and ethanol extracts with different concentrations on *Pencillium puberulum*

4. Conclusion

The Kaempferol 3-*O*-rutinoside was isolated and purified from aerial parts of *Anchusa italica* using some different spectroscopic techniques. The plant contains antibacterial and antifungal active ingredients, in addition to other useful compounds, so we suggest for establishing more investigation on the plant parts, which could be used both medicinally and in the food industry.

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