Journal of Drug Design and Medicinal Chemistry
2018; 4(2): 16-21
http://www.sciencepublishinggroup.com/j/jddmc
doi: 10.11648/j.jddmc.20180402.12
ISSN: 2472-355X (Print); ISSN: 2472-3576 (Online)

In Vitro Antimicrobial Activity and GC-MS Analysis of Seed Extracts from *Pimpinella anisum* L

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To cite this article:
Ayat Ahmed Alrasheid, Babeker Siddig Abdallah, Amar Osman Ali. *In Vitro* Antimicrobial Activity and GC-MS Analysis of Seed Extracts from *Pimpinella anisum* L. *Journal of Drug Design and Medicinal Chemistry*. Vol. 4, No. 2, 2018, pp. 16-21.
doi: 10.11648/j.jddmc.20180402.12

Received: June 1, 2018; **Accepted**: July 1, 2018; **Published**: July 24, 2018

Abstract: *Pimpinella anisum* L. (family Apiaceae) is widely used for curing variety of ailments. The objective of this study was to evaluate the antimicrobial activity and chemical composition of chloroform and ethanol extracts from the seed of *Pimpinella anisum* against standard microorganism. This plant has been used as a traditional treatment for several diseases such as microbial infections. Extracts were evaluated for their effectiveness against four bacterial strains including both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria as well as fungal species (*Candida albicans* and *Aspergillus niger*) using disc diffusion method. The chloroform extract showed the highest activity against *B.subtilis* (11 mm), *E. coli* (16mm) and *P. aeruginosa* (14.5 mm) but the same result against *S. aureus* in two extracts (15.5 mm). All extracts exhibited high antifungal activity against *C. albicans* and *A. niger* with inhibition zone ranging from (14.5 to 20 mm). The quantitative analysis of chemical composition determined by Gas Chromatography–Mass Spectrometry (GC-MS). The result showed high amounts of Butanoic acid, 2-methyl-, 2-methoxy-4-(2-propenyl) phenyl ester (23.21%) and Anethole (12.52%).

Keywords: *Pimpinella anisum* Seed, Antimicrobial Activity, GC-MS, Chemical Composition, Anethole

1. Introduction

Anise (*Pimpinella anisum*), is an annual important spice and medicinal plant belonging to the family of Apiaceae, and native to Mediterranean region. Today, anise seeds are an important natural raw material which is used for pharmaceutics, perfumery, food and cosmetic industries [1]. In recently, this spice plant has drawn more consideration of consumers due to the antimicrobial, antifungal, insecticidal, and antioxidtaive effect of this herb on human health [2]. The world production of anise essential oil amounts to 40-50 tons per annum. The most significant importing countries of anise oil are the USA and France. Russia, Spain and Poland are among the largest producers of anise oil. There is no distillation of anise oil and no production of *trans*-anethol in many of thecountries which cultivate the crop [3]. The drug as well as the essential oil is characterized by carminative, mild expectorant, diuretic, antiseptic as well as antispasmodic effects [4, 5]. Its fruits known as aniseed were used as traditional medicine in china as early as in the 5th century [6]. In addition to its medicinal value, the fruits and oil have been used in food industry, such as cookie, candy, toothpaste, liquor and in some alcoholic drinks like pernot, pastis, and anisette for flavorings. Also it is added in American tobacco products because of its aromatic characteristics [7, 8]. Anise fruits known also as aniseed contain 1.5 - 5.0% essential oil with *trans*-anethole, a phenylpropanoid, as predominant component [9]. In addition, the essential oil of the anise fruits contains also small quantity of estragol, anisaldehyde, γ-himachalene and *cis*-anethole [10-13]. In European countries consumption of anise fruits is more than its production so the amount of

imported anise fruits reached about 2000 in 2004. Among other countries Germany remains the largest spice importer of anise [14]. This stimulates the cultivation of anise in European countries including Germany. Because of anise favors warm climatic conditions throughout the growing season it is cultivated particularly in subtropical regions [15, 16]. The quality of anise is determined mainly by the essential oil content and its composition. For both quality parameters it is necessary to determine the environmental factors under which they give higher yields and better quality [17]. The yield may noticeably vary depending on ecological conditions such as temperature, precipitation and soil fertility. Previous studies showed that, the effects of row spacing, water supply, fertilization, sowing time, sowing density on anise seed yield and quality were studied under field and greenhouse conditions [18-20]. The cultivation of anise in Germany is rather limited due to problems such as poor establishment of plant stand in the spring and lower yield in autumn. Because of its sensitivity to low temperatures the sowing of anise in Germany cannot be carried out in early spring. On the other hand delayed sowing under warmer conditions in spring may lead to shortening the growing cycle which decreases the amount of UV radiation intercepted by the crops which may reduce the formation of reproductive organs. Seed rate has important effect on yield and yield components such as the number of branches, number of umbels, number of fruits per plant, fruit weight per plant and 1000-fruit weight [17]. As the higher plant densities affect negatively the yield and yield component, so optimal seed rate is very important for maximum seed yield. Plant spacing is an important factor in determining the microenvironment in the anise field. The optimization of this factor can lead to a higher yield in the crop by favorably affecting the absorption of nutrients and exposure of the plant to the light. Additionally, aniseed plants can be infected by several fungal pathogens observed under practice cultivation. One of the most important pathogens in anise cultivation in Germany is the fungus *Passolaram alkoffii*. The symptoms of this infection are characterized by cylindrical light brown spots with dark veins and later the whole leaves can be colored brown. The infection starts at the lower parts of the plants at the underside of the leaves. Later the whole leaves, stems, flowers and seeds can be infected. The seeds get dark color which comes from stomata of the fungus which reduced the quality of seeds.

1.1. Taxonomy and Botanical Description of Pimpinella anisum L

Anise is belonging to the family of Apiaceae which consists 300-455 genera and 3000-3750 species distributed in the northern hemisphere [21, 22]. Members of this family have alternate leaves, widening at the base into a sheath that clasps the stem. The stems of these family members areoften furrowed. The compound flowers are determined in umbels. The rays of themain umbel produced a secondary umbel with the flower bearing pedicels. The flowers of this family have 5 petals and 5 stamens. The fruits form below where the petals and stamen originate. Fruits or seeds are in pairs, commonly conspicuously ribbed, and sometime winged. The genus *Pimpinella* L. consist 150 species spread in Eurasia and Africa, more than 16 of which present in Europe. The family Apiaceae can be familiar by certain characters that are generally found in the group including the herbaceous nature ofthe family; the frequent occurrence of compound leaves; small flowers, with a small number of floral parts arranged in whorls and grouped in shaped inflorescences. The genus includes herbaceous annual, biannual, or perennial plants, usually with a fine hair covering. From medicinal and agricultural point of view, only few species are economically significance, these are including, *Pimpinella anisum* L., *P. major*, *P.saxifraga* L., *P. peregrine*L. and *P.diversifolia* L. [23].

1.2. Morphological Characteristics

Anise plant reaches a maximum height of 30-70 cm with ternately pinnate leaves. Very small and white flowers are born in compound umbels which distributed into 7 to15 rays. The leaves of anise plant at the basal part are simple, 1.3-5.1 cm long and shallowly lobed, while leaves top on the stems are feathery pinnate divided into numerous leaves [24]. The fruit of anise is pyriform or ovoid laterally compressed which 3-5 mm in length and 2-3 mm wide. The color of anise fruits is greyish-green to greyish-brown with a sweet smell. Every fruit contains two carpals both containing an aniseed. The seed is small and curved, about 0.5 long and greyish-brown. The pericarp is broadly ovoid, five ridged with short hairs and various vittae [1]. The essential oil is located in the schizogenic oil ducts of anise fruits, and shoots [25].

2. Materials

2.1. Sample Preparation

The plant materials were harvested and immediately washed with distilled water. The plant material was air dried at room temperature (about 30°C) for approximately 5 days. When dried, the material was ground to a course powder using a pestle and mortar and stored in a clean container ready for analysis. The powder of *Pimpinella anisum* was extracted sequentially with chloroform and ethanol at room temperature for 48 h. Extracts were first filtered through Whatman No. 4 filter paper. After filtration, the extracts were vacuum concentrated.

2.2. Antimicrobial Activity

2.2.1. Test Strains and Culture Media

Standard strains of microorganism were used in this study and were obtained from Medicinal and Aromatic Institute of Research, National Research Center, Khartoum. The bacterial species used were the Gram-negative bacteria; *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and the Gram-positive bacteria; *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923). Fungal species were *Candida albicans*(ATCC 7596) and

Aspergillus niger(ATCC 9763). Bacteria were grown in Mueller Hinton Agar and fungi were grown in Sabouraud Dextrose Agar. The concentration of bacterial suspensions were adjusted to 108 cells/ml, and that of fungal suspensions to 107 cells/ml.

2.2.2. Culture Media

a. Mueller Hinton Agar

Thirty eight grams of the powder of Mueller Hinton agar were weighed, dissolved in 1 liter of distilled water and allowed to soak for 10 minutes. The medium was placed in water bath to dissolve, swirled to mix and sterilized by autoclaving for 15 minutes at 121°C, cooled to 47°C mixed well then poured into sterile Petri dishes.

b. Sabouraud Dextrose Agar

Sixty two grams of the powdered Sabouraud dextrose agar, was weighed, dispersed in 1 liter water and allowed to soak for 10 minutes, swirled to mix then sterilized by autoclaving for 15 minutes at 121°C, cooled to 47°C, mixed well then poured in to sterile Petri dishes.

3. Methods

3.1. Antimicrobial Activity

3.1.1. Assay for Antibacterial Activity

The disc-diffusion assay [26] with some modifications was employed to investigate the inhibition of bacterial growth by plant extracts. Plant extracts were resuspended in extracting solvent at a concentration of 20mg/ml. Base plates were prepared by pouring 15 ml Mueller-Hinton (MH) agar into sterile Petri dishes. About 0.1 ml of the standardized bacterial stock suspension 10^8–10^9 C.F.U/ ml were streaked on Mueller Hinton agar medium plates using sterile cotton swab. Sterilized filter paper disc (6 mm diameter) were soaked in the prepared extracts, and then were placed on surface of the test bacteria plates. The plates were incubated for 24 h and the diameters of the inhibition zones were measured.

3.1.2. Bioassay for Antifungal Activity

The same method described for bacteria will be adopted. To test antifungal activity, Sabouraud Dextrose Agar was used. The inoculated medium will be incubated at 25˚C for two days for the *Candida albicans* and three days for *Aspergillus niger*.

3.1.3. Statistical Analysis

Antimicrobial activity experiment was performed in replicates and data were presented as means ± standard deviation (SD). Statistical analysis for all the assays results were done using Microsoft Excel program (2010).

3.2. GC-MS Analysis

The qualitative and quantitative analysis of the sample was carried out by using GM/MS techniquemodel (GC/MS-QP2010-Ultra) from japans 'Simadzu Company, with serial number 020525101565SA and capillary column (Rtx-5ms-30m×0.25 mm×0.25μm).The sample was injected by using split mode, helium as the carrier gas passed with flow rate 1.61 ml/min, the temperature program was started from 60c with rate 10c/min to 300c as final temperature degree with 2 minutes hold time, the injection port temperature was 300c, the ion source temperature was 200c and the interface temperature was 250c.The sample was analyzed by using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 26 minutes. Identification of components for the sample was achieved by comparing their retention times and mass fragmentation patents with those available in the library, the National Institute of Standards and Technology (NIST).

4. Results and Discussion

4.1. Antimicrobial Activity

The antibacterial activity of the chloroform and ethanol extracts from seed of *Pimpinella anisum* was determined against the Gram positive *B. subtilis* and *S. aureus* and the Gram negative *E. coli* and *P. aeruginosa* and two fungi; *A. niger* and *C. albicans* using the disc diffusion method. Results are presented in Table (1). Different extracts showed variable activity against the tested bacteria. Generally, the chloroform extract showed higher antibacterial activity than the ethanol extract. But the ethanol extract showed high antifungal activity than chloroform extract. The crude extract found active in this study could be useful for the development of new antimicrobial drugs. The chloroform extract showed the highest activity against *B.subtilis*(11 mm), *E. coli* (16mm) and *P. aeruginosa* (14.5 mm) but the same result against *S. aureus* in two extracts (15.5 mm).All extracts exhibited high antifungal activity against *C. albicans* and *A. niger* with inhibition zone ranging from (14.5 to 20 mm). The highest result showed in ethanol extract against *C.albicans* (20 mm) and (14 mm) with chloroform extract. Both extracts showed same inhibition zone against *A.niger* (19 mm). When compared, we found the results quite different with that related study reported by Akhtar [27], results showed the extracts of *Pimpinella anisum* were tested *in vitro* against 4 bacterial species by the disc diffusion method. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherchia coli* and *Klebsiella Pneumoniae*. Only aqueous and 50% (v/v) methanol extract exhibited fair antibacterial activity against all the test bacteria whereas acetone and petroleum ether extract were not observed to inhibit the growth of any of the test bacteria. variation of results may be due to solvents which used in extraction and the source of plant under study. Results is same with that reported by Huda[28], who foundThe petroleum ether and chloroform extracts were found active against *Pseud. aureginosa* and the two fungi, *C. albicana* and *Aspergillus niger*.

Table 1. *Antimicrobial activity of seed extracts of Pimpinella anisum.*

Extract20 mg/ml	MDIZ (Mean diameter of growth inhibition zone, mm)					
	Bacteria strain				Fungi strain	
	B.subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C.albicans***	***A.niger***
Chloroform	11±0.00	15.5±0.7	16±0.00	14.5±0.7	14.5±0.7	19±0.0
Ethanol	10±0.00	15.5±0.7	15.5±0.7	10±0.00	20±0.00	19±1.0

Interpretation of results: MDIZ (mm): < 9 mm Inactive; 9-12 mm Partially active; 13-18mm Active;>18 mm: Very active.

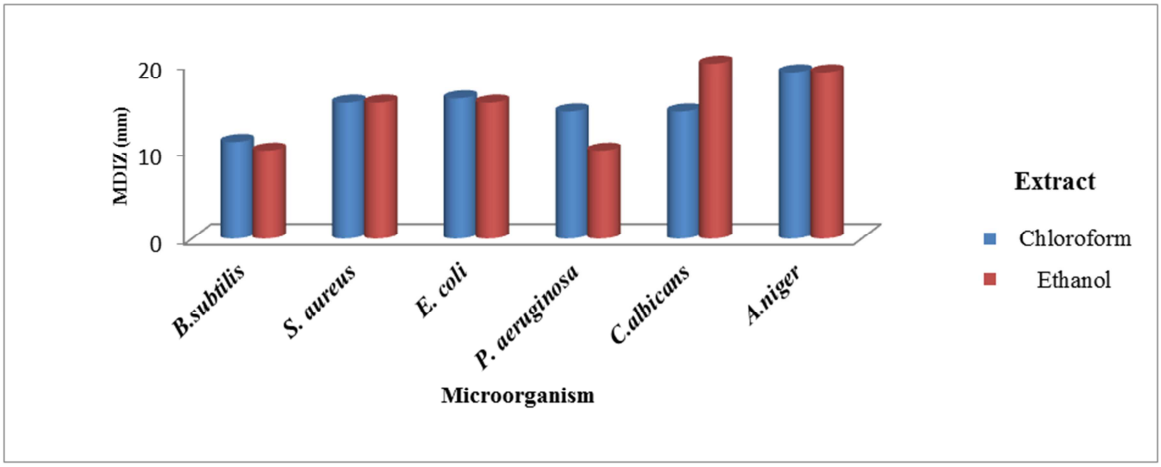

Figure 1. *Antimicrobial activity of seed extracts of Pimpinella anisum.*

4.2. GC-MS Analysis

Besides the analysis of main components of anise extract by GC-MS was used for the analysis of components of anise extract which were present in minor quantity. For identification of these components. The retention time of the desired substance can be placed between the retention times of two adjacent homologous alkanes which were already determined (Table 2). The retention index is a good comparison to identify the samples of unknown substance. The most important components Butanoic acid, 2-methyl-, 2-methoxy-4-(2-propenyl) phenyl ester (23.21%) and Anethole (12.52%) as active component.

Table 2. *Chemical composition of ethanol extract of Pimpinella anisum seed using GC-MS.*

No.	Compound name	R-time	Formula	M/W	Ret. Index	Area%
1	Undecane	5.723	$C_{11}H_{24}$	156	1115	0.43
2	Nonanal	5.817	$C_9H_{18}O$	142	1104	0.11
3	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	7.020	$C_{10}H_{18}O_2$	170	1197	0.11
4	Mequinol	7.477	$C_7H_8O_2$	124	1090	0.30
5	Benzaldehyde, 4-methoxy-	8.029	$C_8H_8O_2$	136	1171	6.53
6	Anethole	8.430	$C_{10}H_{12}O$	148	1190	12.52
7	1,4-Benzenedimethanol,.alpha.-methyl-	9.698	$C_9H_{12}O_2$	152	1411	2.49
8	2-Propanone, 1-(4-methoxyphenyl)-	9.759	$C_{10}H_{12}O_2$	164	1318	1.29
9	trans-Z-.alpha.-Bisabolene epoxide	9.985	$C_{15}H_{24}O$	220	1531	1.52
10	Benzeneacetic acid, 4-methoxy-.alpha.-oxo-	10.606	$C_{10}H_{12}O_2$	164	1318	0.64
11	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-	10.706	$C_{15}H_{24}$	204	1494	0.20
12	Isolongifolene, 4,5-dehydro-	10.920	$C_{15}H_{22}$	202	1398	0.14
13	Estragole	10.987	$C_{10}H_{12}O$	148	1172	0.30
14	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	11.058	$C_{15}H_{24}$	204	1407	1.42
15	2-Allyl-1,4-dimethoxybenzene	11.085	$C_{11}H_{14}O_2$	178	1361	0.63
16	5-Methoxyindane	11.257	$C_{10}H_{12}O$	148	1236	0.14
17	Phenol, 2-methoxy-4-(1-propenyl)-	11.337	$C_{10}H_{12}O_2$	164	1410	0.25
18	1H-1,2,3,4-Tetrazole-1,5-diamine, N(1)-[(2-ethoxy-3-methoxyphenyl)methyl]-	11.546	$C_{11}H_{16}N_6O_2$	264	0	1.38
19	3-tert-Butyl-4-hydroxyanisole	11.685	$C_{11}H_{16}O_2$	180	1417	0.79
20	S-(p-Methoxy benzoyl)thiohydroxylamine	11.933	$C_8H_9NO_2S$	183	1613	0.25
21	2-Tridecenal, (E)-	11.979	$C_{13}H_{24}O$	196	1510	0.74
22	2-Hydroxy-2-(4-methoxy-phenyl)-N-methyl-acetamide	12.124	$C_{10}H_{13}NO_3$	195	1744	4.80

No.	Compound name	R-time	Formula	M/W	Ret. Index	Area%
23	(4-Methoxyphenyl)(2-methylenecyclohexyl)methanol	12.206	$C_{15}H_{20}O_2$	232	1872	1.25
24	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)	12.324	$C_{15}H_{24}O$	220	1536	0.53
25	Lanceol, cis	12.515	$C_{15}H_{24}O$	220	1743	0.18
26	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	13.108	$C_{15}H_{22}$	202	1524	0.49
27	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	13.286	$C_{10}H_{12}O_3$	180	1538	0.95
28	Aromadendrene oxide-(2)	13.528	$C_{15}H_{24}O$	220	1462	1.06
29	Benzenemethanol,.alpha.-ethyl-4-methoxy-	13.663	$C_{10}H_{14}O_2$	166	1344	1.17
30	2-Butyl-1-methyl-1,2,3,4-tetrahydronaphthalen-1-ol	13.709	$C_{15}H_{22}O$	218	1737	0.27
31	Acetic acid, 3-(2,2-dimethyl-6-methylene-cyclohexylidene)-1-methyl-butyl ester	14.337	$C_{16}H_{26}O_2$	250	1682	0.40
32	Butanoic acid, 2-methyl-, 2-methoxy-4-(2-propenyl)phenyl ester	15.084	$C_{15}H_{20}O_3$	248	1786	23.21
33	Butanoic acid, 2-methyl-, 4-methoxy-2-(3-methyloxiranyl)phenyl ester	15.677	$C_{15}H_{20}O_4$	264	1848	15.58
34	Methoxsalen	17.203	$C_{12}H_8O_4$	216	1901	0.63
35	11,14-Eicosadienoic acid, methyl ester	17.469	$C_{21}H_{38}O_2$	322	2292	0.29
36	9-Octadecenoic acid (Z)-, methyl ester	17.469	$C_{19}H_{36}O_2$	296	2085	0.29
37	1-Hexen, 2-(p-anisyl)-5-methyl-	19.099	$C_{14}H_{20}O$	204	1482	0.55
38	Benzenepropanol,.gamma.-(ethylamino)-.beta.,4-dimethyl-.alpha.-phenyl-, methyl ester	19.383	$C_{19}H_{25}NO$	283	2309	1.83
39	2H-Pyran, 2-(2-heptadecynyloxy)tetrahydro-	19.682	$C_{22}H_{40}O_2$	336	2453	2.81
40	Tetracontane	20.852	$C_{40}H_{82}$	562	3997	1.13
41	Ethanone, 2-hydroxy-1,2-bis(4-methoxyphenyl)-	22.015	$C_{16}H_{16}O_4$	272	2243	0.99
42	Dotriacontane	22.361	$C_{32}H_{66}$	450	3202	3.00
43	Tetrapentacontane	23.767	$C_{54}H_{110}$	758	5389	6.22

A gas chromatogram and mass spectrometer of anise extract are given above in Table 2. The qualitative and quantitative analysis of the sample was carried out by using GC/MS technique model (GC/MS-QP2010-Ultra) from japan'Simadzu Company, with serial number 020525101565SA and capillary column (Rtx-5ms-30m×0.25 mm×0.25µm). The sample was injected by using split mode, helium as the carrier gas passed with flow rate 1.61 ml/min, the temperature program was started from 60c with rate 10c/min to 300°C as final temperature degree with 2 minutes hold time, the injection port temperature was 300°C, the ion source temperature was 200c and the interface temperature was 250°C. The sample was analyzed by using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 26 minutes. Identification of components for the sample was achieved by comparing their retention times and mass fragmentation patents with those available in the library, the National Institute of Standards and Technology (NIST).

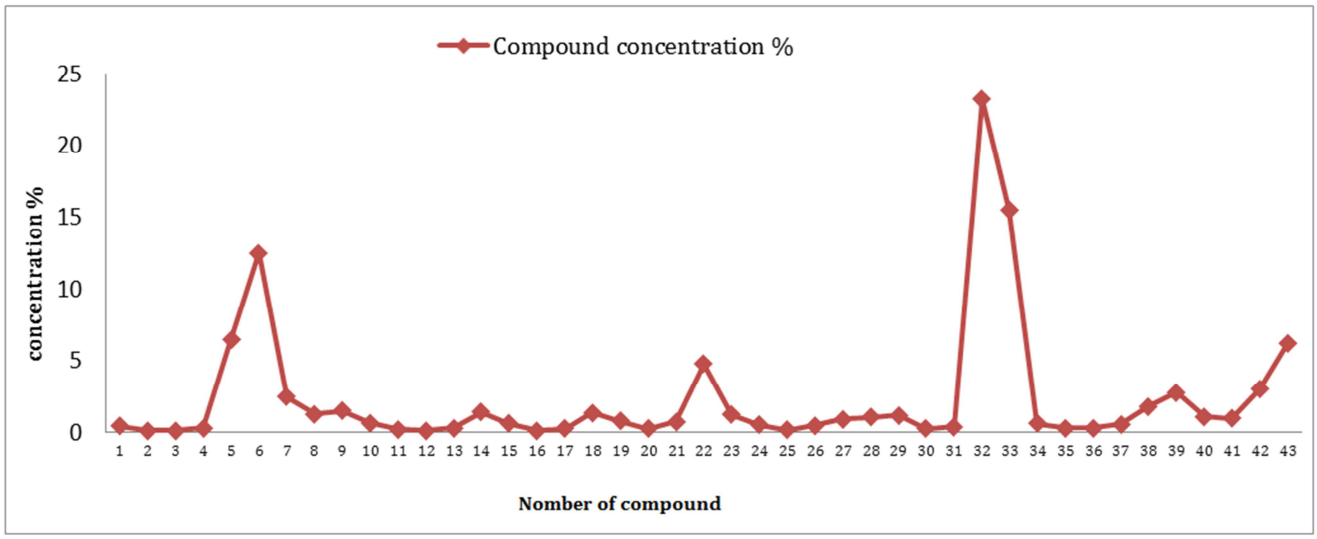

Figure 2. *The Compound percentage of ethanol extract of Pimpinella anisum seed.*

In recent years there has been an increasing interest in the activities of phytopharmaceutical products and biologically active substances of plant origin. Anethole is a type of aromatic compound that occurs widely in nature in essential oils. It is such a substance used from ancient times in traditional medicine in many countries. Nowadays it is widely used in food and beverage industry . Its wide spread use and accessible price justify carrying out extensive scientific research in order to support the traditional uses of anethole with scientific evidence [29].

5. Conclusion

The seed extracts of *Pimpinella anisum* showed the various degree of inhibitory activity against different species of bacteria and fungi. Results obtained justified the use of the seed of *Pimpinella anisum* as antimicrobial therapy in traditional medicine in Sudan and the neighboring countries. The present results indicate that anethole can be identified and determined by GC-MS, Further investigations to determine the other medicinal active compounds of the plant and experimental as well as clinical studies are warranted.

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