

Chemical investigation and Antibacterial Activity of Leaves Extracts of *Artemisia absinthium*

Sitotaw Ewunu

Wollo University

Sisay Awoke (✉ sisay.awoke@wu.edu.et)

Wollo University

Mesfin Getachew

Addis Ababa Science and Technology University, Addis Ababa

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Abstract

Plants are among the most important common sources of potentially valuable new drugs. Therefore, to investigate biological activity of medicinal plants are used to develop new drugs. *Artemisia absinthium* L. is an aromatic plant of the family Asteraceae, and is known by the common names wormwood and absinthe. The study of this research was aimed to chemical investigation, essential oil analysis and antibacterial activity of *A. absinthium*. The plant was collected in different local area of north Wollo, Woldiya. Maceration technique was used for extraction. Isolation of essential oil was done using Cleavinger apparatus. Column chromatography was used to isolate major compounds. In this present study, the major plant secondary metabolites present in *A. absinthium* was screened, the major constituents the plant was investigated and antibacterial activity of the crude extracts was performed. The results of phytochemical screening showed the presence of alkaloid, flavonoid, carbohydrate, tannin, quinines, protein, phenol and terpenoids. The identified components account for 56% of the oil. The oil contains camphor as a major constituent and account for 41% of the oil. From column chromatography two compounds, chamazulene and davanone were isolated and identified on the bases of their ^1H and ^{13}C NMR spectra. Antimicrobial assay of the crude extracts of *A. absinthium* against gram negative (*E. coli* and *K. pneumonia*) and gram positive (*L. monocytogen* and *S. aureus*) pathogenic microorganisms showed that a significance activity. Different concentrations of methanol and chloroform were tested and maximum zone of inhibition was found at 200mg/ml in both extracted and zone of inhibition was decreased with decreasing concentrations.

Introduction

The plant kingdom is the most essential to human well-being in providing basic human needs [1]. Medicinal plants play an important role in human life for therapeutic purposes and popularized worldwide [2]. More than 391,000 plant species are reported as used for medicinal purposes in the world. Plants have been used as a source of medicine in Ethiopia from the ancient time to treat different disease [3]. About 800 plant species are found in Ethiopia [4] and about 10% are believed to be endemic and 14% are used as medicinal plants [5]. It is reported that leaves are the most widely used plant parts followed roots, flowers, fruits and seeds, 48%, 33%, 9%, 9%, respectively. Most common methods of using for medicine application are squeezing, grinding, boiling, chewing, crushing and tying. Fresh plant parts of medicinal plant are commonly used for traditional preparation [6].

The two broad classes of medicine, modern and traditional medicines, are synthesized by pharmaceutical industries and produced based on guidelines of natures from generation to generation, following local traditions and belief, respectively [7]. About 25% of modern drugs contain one or more active ingredients from plants [8]. Potentially valuable new drugs are derived from plants sources. Therefore, to investigate biological activity of medicinal plants are used to develop new drugs [9, 10].

Earlier studies indicate that many plant extracts have antimicrobial activity (both antibacterial and anti-fungal activity), antioxidant, anti-carcinogenic activity, analgesic activity, antipyretic activities, insecticidal

properties and anti-coccidial activity [11]. Medicinal plants have been tested for biological, antimicrobial and hypoglycemic activity. They have also tested for anti-ulcerogenic, anti-helminthic, hepatoprotective and anti-leishmania [12]. Many plants also produce secondary metabolites such as phenolic compounds, essential oils and saponins [11]. Secondary metabolites contribute significantly towards the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anti-carcinogenic, anti-malarial, anti-cholinergic, anti-leprosy activities [13]. Chemical compounds with a broad spectrum of biological activities such as alkaloids [14–16], flavonoids [16–19], phenols [17, 20], tannin [16, 19, 21, 22] and terpenoids [22] have been reported.

Human beings used plants for the purpose of disease control and prevention for all time [23, 24].

In Ethiopian diseases such as gonorrhea, cough, inflammation, spasm, thrombosis, mental illness, eye disease, toothache, urinary retention, stomach problems, leprosy and ascariasis have been commonly treated by traditional medicine. *A. absinthium* is commonly used in food industry in the preparation of a preservatives, bitters and spirits. Moreover, biological properties such as anthelmintic activity, antimicrobial activity, antioxidant activity have been reported. The characteristic bitterness of wormwood is due to the presence of sesquiterpenes lactones such as absinthin and anabsinthin [25–27].

This research was conducted on the photochemical screening and anti-bacteria activities of *A. absinthium* and its antibiotic role. The reason why interested to this is the aromatic plant used to the fragrant leaves are used as a fumigant or spread together with grasses to give a pleasant odor during church or coffee ceremonies. The most important characteristics of traditional herbal treatments are their crude preparations and the active principles of many drugs found in plants are secondary metabolites. Therefore, this study was carried out to test the presence of secondary metabolites as component of biological active *A. absinthium* extract and also in at a particular area of N. Wollo zone of Ethiopia they don't well identify the medicinal advantage of the plant. The main objective of the study was to undergo screening tests of secondary metabolites and to evaluate antibacterial activities of methanol and chloroform extracts of the leaves *A. absinthium*.

Materials And Methods

Chemicals and apparatus

Distilled water, chloroform, methanol, dimethyl sulfoxide (DMSO), 5% ferric chloride solution (FeCl₃), hydrochloric acid, sulfuric acid, sodium hydroxide, copper sulphate, ammonia, glacial acetic acid, lead acetate solution, 10% ferric chloride solution (FeCl₃), iodine, acetone, n-hexane, potassium iodide, n-butanol were chemicals used in the study. The apparatus used were separatory funnel, filter paper (What man No. 1), pipettes, water bath, beakers, electronics balance, Clevenger apparatus, flask, measuring cylinder, rotary evaporator, ruler, pencil, spatula, heating mantel, refrigerator, mortar and pestle, aluminum foil, glass ware, Petri dish, incubator, McFarland density meter, tong, sieve, crucible, mask, autoclave, micro litter, cork-borer,

Collection of plant material and extraction

Fresh leaves of *A. absinthium* were collected from natural (wild) growing population located in N. Wollo, Woldiya, it is 520 Km from the capital city, Addis Ababa, Ethiopia. It was identified by Professor Sebsibe Demissew at Ethiopian National Herbarium, Addis Ababa University and deposited with voucher number AAU-Her 203/2017. All the plant experiments were in compliance with relevant institutional, national, and international guidelines and legislation. The fresh leaves of *A. absinthium* were washed with tap water to remove fragments and dust particles and then air-dried at room temperature for ten days. The air-dried leaves were finally crushed into a uniform powder with the aid of mortar and pestle. The powder obtained was stored in airtight container and kept in the refrigerator at 4°C until use for analysis. 100 g of the ground leaves was separately soaked in 300 ml of different solvents (petroleum ether, chloroform and methanol) and left on a platform shaker for about 72 h and filtered using Whatman No.1 filter paper. Petroleum ether, chloroform and methanol extracts were dried using rotary evaporator to obtain crude extract. The dried extracts were then stored in labeled sterile screw capped bottles at 5°C in airtight vials until use [28].

Phytochemical screening

Detections of common secondary metabolites were performed for methanol and chloroform leaves extract of *A. absinthium* using the preceding analytical procedures steroids [29, 30], saponins [30–32], phenols [29, 33, 32], flavonoids [30, 33, 34], alkaloids [31, 33], 35], tannins [30, 33, 36], proteins [33, 37], carbohydrates [30, 31], quinones [35], terpenoids [38, 39], gums and mucilages [40], coumarins [40], anthraquinones [35], glycosides [32].

Extraction and analysis of oil from *A. absinthium*

The powdered aerial parts of the plant material were subjected to hydro-distillation for one and half hours using a Clevenger-type apparatus. The percentage composition of the essential oils was computed from GC and GC/MS peak areas. Qualitative analysis was based on comparison of retention times with the standard or isolated compounds and the corresponding data in the literature [41]. GC analysis of the essential oil was performed using an HP6890 GC, coupled with an auto sampler equipped with a capillary column HP-5 (5% phenyl methyl siloxane), 30.0 m x 320 µm internal diameter, film thickness 0.25µm. Nitrogen was used as the carrier gas at a flow rate of 0.8 ml/min. The oven temperature was held at 50 0C, and then programmed to 210 0C at a rate of 3 0C/min. The injection and FID temperatures were kept at 210 0C and 270 0C, respectively.

Isolation of compounds from leave of *Artemisia absinthium*

Isolation of constituents of the methanol extract of *A. absinthium* was performed by column chromatography using silica gel (mesh: 70–230). Analytical TLC were run on a silica gel 60 F254 (Merck), 20 x 20 cm per coated plate and components were detected by spraying anisaldehyde reagent (anisaldehyde: H₂SO₄: AcOH: EtOH; 0.5:0.5:0.1:9). 400 mg of the extract was packed on column and

eluted with petroleum ether and CHCl_3 . The combined fractions 6–8 (46 mg) from CC contained the blue component and gave a single spot on TLC. This was identified as chamazulene based on its spectra. And also fractions 25–27 were combined after chromatographed on a column of silica gel and petrol-EtOAc (8:2) solvent system to isolate davanone.

Test Organisms

Both gram positive and gram negative bacteria strains; namely *E. coli*, *S. aureus*, *L. monocytogenes* and *K. pneumoniae* were used. The test microorganism was grown on nutrient agar at 37°C for 24 hrs. The standard 0.5 McFarland was prepared by taking two to four groups in normal saline solution following standard procedure [42].

Bacterial Media And Working Solution Preparation

Muller Hinton Media (36.0 g) was mixed with 500 mL distilled water and then sterilized in autoclave at for 15 minutes. The sterilized media were poured into Petri dishes. About 70.0 mL freshly prepared sterile Mueller Hinton Agar (MHA) media is poured into 150.0mm diameter agar plate and allowed to cool at room temperature. Crude extracts (0.5 g) were dissolved in dimethyl sulphoxide (DMSO) until the volume of a solution became 1 mL to get 0.5 g/mL stock solutions. Different concentrations of extracts (60,130,200 mg/mL) were prepared after dilution of the stock solution with DMSO and NaCl (0.85 g) was dissolved in 100.0 mL of distilled water to get 0.85% of NaCl solution.

Determination Of Inhibition Zone

The antibacterial activities of plant extracts were tested using paper disc diffusion method. Within 15 min. of adjusting the turbidity of the inoculums suspension to 0.5 McFarland standard, a sterile cotton swab was dipped in to adjusted microbial suspension, rotated gently and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums from the swab. The swab is streaked to the entire surface of the MHA plate three times by rotating approximately 60° each time to ensure even distribution of the inoculums. Petri-plates were left for 5 minutes at room temperature [43]. Different dilute concentrations of the extracts (200, 130 and 60 mg/ mL) were prepared to fill the paper discs using micropipette after dissolved in DMSO [44]. Chloramphenicol (5.0 µg/disc) and DMSO (100 µL) were used as positive and a negative control, respectively. After placement of the plant extracts and controls. The plates were incubated at 37°C for 24 hours. The complete zone of inhibition was measured in millimeter and was judged by the naked eye using ruler. All tests were performed in triplicate for each bacterial species. The mean zone of inhibition and standard error of the mean (Mean $\pm$ SEM) were calculated.

Results And Discussion

Percentage yields of extracts

In the preparation of crude methanol, chloroform and petroleum ether extract from the dried leaves of *Artemisia absinthium* different percent yields were obtained. Among the solvents used for extract, methanol gave the highest and petroleum ether has the lowest percent yields. The yield of extraction by maceration in different solvents was calculated by equation shown below respectively and summarized in Table 1.

$$\% \text{Yield of crude extract (w/w)} = \frac{\text{mass of extract (gram)}}{\text{total mass of plant material used}} \times 100\%$$

Table 1
Yield of plant extracts in both crude extract

S.No	Types of extract	Yield of extract (%)
1	Petroleum ether Crude extract	10
2	Chloroform Crude extract	13
3	Methanol crude extract	17

Calculating yield of extracts were serious tasks behind phytochemistry, because biological active component of medicinal plant depends on yield. In the preparation of crude methanol extract from the dried leaves of *Artemisia absinthium* a yield 13.415% was obtained. According to the previous study, it is agreeing with reported by [45]. A yield of 12% [46], which extracted by 80% methanol. But, yield of crude methanol extract of test plant disagree with the previous studies which were reported by a yield of 11.4% [47]. The observed difference may be due to the amount of solvent used, on previous studies 80% methanol and 20% water was used but, in this study normal methanol without water was used. The duration of maceration, on previous work were soaked for 3 days but, in this study were soaked for 5 days. This study also disagrees with reported by [48] a yield of 5.44% due to the type of solvent. From this previous study was used acetone as solvent but, in this work used methanol as solvent.

Finally, it disagrees that was reported by a yield of 8.4% [49]. Due to the type of solvent which used water as a solvent. Generally, methanol is good solvent in this study to increase yield of extraction that compared the previous study using 80% methanol, chloroform and aqueous extract. The observed difference may be due to the weight of fresh plant material that was used. The yield solvent maceration also depends on polarity of solvent which showed maximum yield for more non polar and more polar solvent.

Phytochemical Screening Tests Of Extracts

Preliminary phytochemical analysis of the crude methanol extract revealed the presence of alkaloids, flavonoids, terpenoids, phenol, tannins and oxalate in *A. absinthium* leaves and absence of

anthraquinone, saponins, steroids and quinine was investigated in the sample extract by different test methods (Table 2).

The existence of these phytochemicals in the extracts may contribute the plant to be known of its medicinal use especially for antimicrobial activity. Tannin, alkaloids and flavonoids have been reported potential ingredients towards the treatment intestinal disorders [48], pain killer and inflammation, respectively [49].

The findings of the present study agreed with previous studies; for chloroform extracts and Hussein Haji et. al in 2016 for aqueous extracts reported that, leaves of *A. absinthium* possess tannins and phenols [38, 47]. In addition to the previous work, this study showed the presence of alkaloid, flavonoids, terpenoids and oxalate in methanol extract. This implies that methanol is the best solvent compared to chloroform and water for extraction of *A. absinthium*.

Phytochemical of determination of *A. absinthium* showed that the presence of constituents like alkaloids, flavonoids, tannins, quinones, steroids, phenols, terpenoids and absence of constituents such as carbohydrates, saponins, glycosides, anthraquinone, coumarins, gums, protein and amino acid in methanol extract. Chloroform extract showed the presence of alkaloids, flavonoids, carbohydrates, tannins, glycosides, quinones, terpenoids, steroid, coumarins, amino acid, phenols anthraquinones and absence of constituents such as saponins, glycosides, protein and gums.

According to [25] the phytochemical analysis of *A. absinthium* showed the presence of saponins, flavonoids, phenols, tannins, quinones, steroids methanol ethanol extract and acetone extract showed the presence of phenols, tannins, quinones, alkaloids, carbohydrates. And also according to [49] the presence of alkaloids, flavonoids, carbohydrates, tannins, quinines, proteins, phenols and terpenoids.

Table 2
Phytochemical test result of leaf extracts of *A. Absinthium*

Secondary Metabolites	Petroleum ether	chloroform	Methanol
Alkaloid	+	+	+
Flavanoid	+	+	+
Carbohydrates	-	+	-
Tanins	-	+	+
Saponins	-	-	-
Glycosides	-	-	-
Quinones	+	+	+
Anthroquinone	-	+	-
Steroid	+	+	+
Coumarins	-	+	-
Amino Acid	-	+	-
Protein	-	-	-
Gums	-	-	-
Phenol	+	+	+
Terpenoid	+	+	+

+ = present - = absent

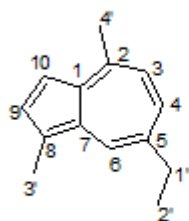
In the course of this study, the essential oil obtained from *A. absinthium* was studied. The oil was obtained using hydro distillation and the yields was 0.5% and the oil was aromatic. The major components of the oil were identified. The essential oil was analyzed by GC and constituents of the oil were identified by using RT and NMR. The identified components account for 56% of the oil. The oil contains camphor as a major constituent and account for 41% of the oil. Figure 1 shows the GC of was overlapped with camphor and essential oil *A. annua*.

From column chromatography two compounds, chamazulene and davanone were isolated and identified on the bases of their ¹H NMR spectra. The spectra obtained were comparable with literature values (Table 3) [50].

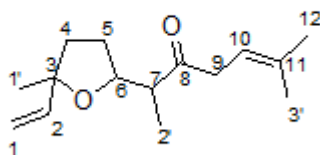
Table 3

¹HNMR spectra of chamazulene and davanone compared with literature value

Position	¹ HNMR, δ (ppm)			
	Chamazulene	[50]	Davanone	[50]
1	-	-	5.19, 4.98 (<i>trans</i>) 4.98 (<i>trans</i>)	5.04
2	-	-	5.88	5.78
3	6.90	6.55		
4	7.38	7.15		
5	-	-		
6	8.14	7.93	3.26	3.88
7	-	-	2.68	2.60
8	-	-		
9	7.20	7.05		
10	7.60	7.45		
11	-	-		
12	-	-	1.64	1.57
1'	2.87	2.71	1.27	1.17
2'	1.38	1.33	0.99	0.88
3'	2.67	2.55	1.77	1.70
4'	2.85	2.75		



Chamazulene



Davanone

The GC of these two isolated compounds were overlapped with the oil (Fig. 1). The oil is composed of 0.6% chamazulene and 14% davanone.

Antibacterial activity

Determination of zone of inhibition for the extract of the plant material against clinical pathogenic bacteria was effective. Crude methanol and chloroform extract of this plant was active both gram positive bacteria and gram negative bacteria due to the nature of cell wall of bacteria. The anti-bacterial activity of crude methanol extract results is presented in Tables 3. The antibacterial activity of plant extracts was performed at concentrations of 60, 130 and 200mg/mL. The maximum zone of inhibition for the crude methanol extract was achieved for *S. aureus* (11.77 ± 0.86) at the concentration of 200mg/ml, while the minimum antibacterial activity was obtained (0) at a concentration of 60 mg/ml and 130mg/ml in the same organism. *L. monocytogenes* showed antibacterial activity both the concentration of 60mg/ml, 130mg and 200 mg/ml respectively (12.89 ± 0.62 , 11.62 ± 0.6 and 11.22 ± 0.36). and gram negative *E. coli* did not show antibacterial activity at any concentration shown in Table 4. *K. pneumonia* showed antibacterial activity both the concentration of 60mg/ml and 200 mg/ml respectively (12.9 ± 0.56 , and 12.26 ± 0.1)

Table 4

Mean inhibition zone of methanol extract of *Artemisia absinthium* at different concentration and different test bacteria

Extracts	Concentration (mg/ml)	Zone of inhibition in Mean $\pm$ SD			
		<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>E. coli</i>	<i>K. Pneumonia</i>
Methanol	60	-	11.22 ± 0.36	-	-
	130	-	11.62 ± 0.6	-	12.26 ± 0.1
	200	11.27 ± 0.89	12.89 ± 0.63	-	12.9 ± 0.56
Chloroform	60	-	11.33 ± 0.33	-	-
	130	-	11.36 ± 0.67	-	10.77 ± 0.34
	200	13.23 ± 0.35	12.02 ± 0.79	-	12.37 ± 79
Chlora.	1000 μ g/ml	9.03 ± 0.85	$8.07 \pm 0.1.6$	9.45 ± 0.44	-
DMSO	100 μ L	-	-	-	-
Chlora. = Chloramphenicol DMSO = dimethyl sulfoxide Conc. = Concentration					

According to Table 3 the extracts isolated from *Artemisia absinthium* showed antibacterial activity against three of the tested bacteria which was indicated by clear zone of inhibition. These are matched with the previous researches has antimicrobial action. [42]. The results also that showed the anti-bacterial activities of *Artemisia absinthium* extracts revealed concentration dependent inhibition zone. In this

biological active compound, zone of inhibition in all tested bacteria increased with increasing of the extract concentration, this is might be due to the reason that, as increasing the concentration of extract increased activity of secondary metabolites, in which chemicals are depend on concentration behind phytochemistry.

The above Table showed that, *A. absinthium* extracts showed high antibacterial only *L. monocytogen* at all concentration, while *Staphylococcus aureus* showed only at 200 mg/ml which implies it is concentration dependent and highly drug resistance bacteria. Therefore, zone of inhibition of these bacteria is effective at the concentration of 200 mg/ml based on this work. But, *E. coli* did not show antibacterial activity at all concentration due to their structural membrane. Gram positive bacteria are more susceptible towards plants extracts as compared to gram negative bacteria [49]. This is because the fact that the cell wall in gram positive bacteria is a single layer and the gram negative cell wall is multilayered structure [42, 47].

For the present study, crude extracts of leaves of *A. absinthium* which showed antibacterial effect against gram positive bacteria (*L. monocytogen*) at all concentrations. However, methanol extracts of *A. absinthium* did not show any zone of inhibition for gram negative bacteria (*E. coli*). This result agrees with the previous reports in which both methanol and chloroform extract could not inhibit the growth of gram negative bacteria. Preliminary qualitative phytochemical screening showed the antibacterial activity of methanol extract due to the presence of alkaloid, terpenoids, flavonoids, tannins and phenol compound by integration.

In Table 3 methanol and chloroform extract had greater zone of inhibition than positive control chloramphenicol. This difference is may be integration effect of different phytochemical of plant extract but, positive control is the most isolated and purified clinical isolated drug those isolated pathogens. The anti-bacterial activity of crude chloroform extract results are presented in Tables 3. The antibacterial activity of plant extracts were performed at concentrations of 60, 130 and 200mg/mL. The maximum zone of inhibition for the crude chloroform extract was achieved for *S. aureus* (13.23 ± 0.35) at the concentration of 200mg/ml, while the minimum antibacterial activity was obtained (0) at a concentration of 60 mg/ml and 130mg/ml in the same organism. *L. monocytogen* showed antibacterial activity both the concentration of 60 mg/ml, 130 mg/ml and 200 mg/ml respectively (12.02 ± 0.79 , 11.33 ± 0.33 and 11.36 ± 0.67). and gram negative *E. coli* did not show antibacterial activity at any concentration shown in Table 4. *K. pneumonia* showed antibacterial activity both the concentration of 60mg/ml and 200 mg/ml respectively (12.37 ± 0.79 , and 10.77 ± 0.34). Moreover, chloroform extract at a concentration 200 mg/ml showed high inhibition zone than methanol extract at the same concentration towards *S. aureus*, *L. monocytogen* and *K. Pneumonia*, respectively, 13.23 ± 0.35 , 12.02 ± 0.79 and 12.37 ± 79 .

Conclusion

In this study phytochemical analysis of methanol, chloroform and petroleum ether extracts of *A. absinthium* leaves showed the presence of major secondary metabolites. Alkaloids, flavonoids, quinones,

steroids, phenols and terpenoids were found in all of the crude extracts and saponins, glycosides and gums were not detected in all extracts. Both essential oil and GC analysis revealed that chamazulene and davanone are the major components of the plant. The crude extracts exhibited strong activity against the selected bacterial strains. Several studies have shown that due to synergy effect crude extracts had strong and consistent inhibitory effects against various pathogens. Among all samples analyzed in this work, the MeOH extract was the most effective as an antibacterial agent. The antibacterial activity has been attributed to the presence of some active constituents in the extracts. The results of antibacterial activities of this study clearly indicated that *A. absinthium* has a potential to inhibit tested human pathogenic bacteria.

Declarations

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Conflict of interest

Authors declared that there is no conflict of interest on this article

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

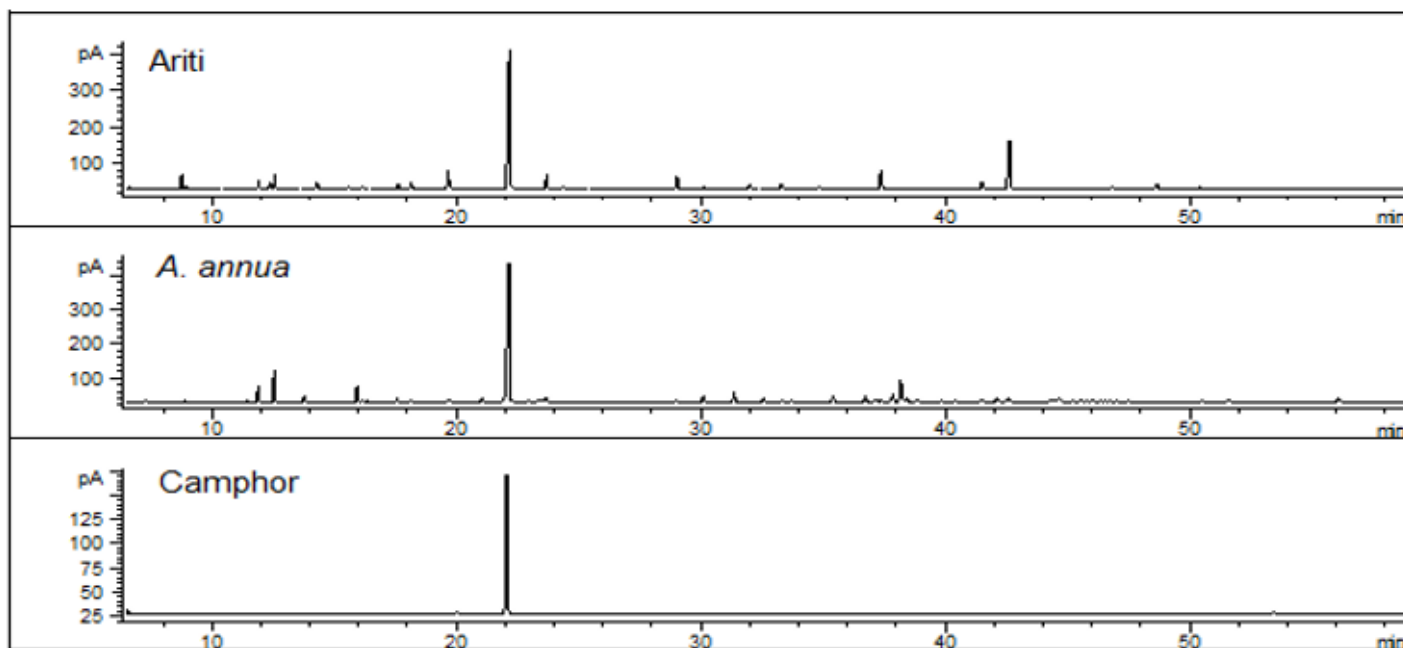


Figure 1

GC of *A. absinthium* oil, *A. annua* and camphor