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SEPARATION AND IDENTIFICATION OF ANTIMICROBIAL AGENTS OF SOME ESSENTIAL OIL EXTRACTS AND ITS APPLICATION IN SALTED FISH

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ABSTRACT: The antimicrobial activities of the ethanol, methanol and hexane extracts of 3 plants species were studied. The extracts of camomile (*Anthemis nobili L.*), garlic (*Allium sativum L.*) and fenugreek (*Trigonella foenumgraecum L.*) were tested in vitro against 8 microbial species and strains such as *Bacillus subtilis, Listeria monocytogenes, Micrococcus spp, Staphylococcus aureus, Aspergillus orizea, Aspergillus niger, Penecillium sp. and Saccharomyces cerevisiae* by paper disc technique.

The yield of plant oil extracts of samples with methanol was more than ethanol and hexane, respectively. Some antimicrobial compounds of essential oil samples were separated with thin layer chromatography to identify the active components. The major active components of the tested extracts were identified based on R_f value , color test under UV lamp, and identified by Gas Chromatography – Mass Spectrometry (GC-MS). Crude oil extracts of plant samples were added to salted sardine fish as antimicrobial agents.

The hexane extract of all plant samples and methanol, ethanol extracts of garlic and fenugreek showed various antimicrobial effects on microorganisms listed above, while that methanol and ethanol extract of camomile , garlic and fenugreek showed various antimicrobial effects against *Bacillus subtilis, Listeria monocytogenes, Micrococcus spp,* and *Staphylococcus aureus.* All garlic oil extracts showed various antimicrobial effects on all mold and yeast species and strains listed above .The total viable count (CFU/g) of salted

sardine fish treated with natural extracts of camomile , garlic, and fenugreek were lower than total viable count in control treatment (untreated fish). This treatments lead to an increment reduction of total contamination level in addition their effect against food pathogens. Results of GC-MS showed the presence of some compounds as natural antimicrobial in hexane extract of garlic such as quinoline, 5,6,7,8-tetrahydro -3-methyl; acetamid,2,2,2-trifluoro (trifluoroaceta); ethanthioic acid , s-ester; beta-amino-trans,trans-5,9-cyclododecadiene and 9-octadecenoic acid. Also, GC-MS showed many other unknown compounds which may play an important positive role as antimicrobial agents. Finally, camomile , garlic, and fenugreek could be used as sources for natural oil extract and could be used as antimicrobial agents in some foods to reduce the total contamination level . In addition their effect against food pathogens is of a great value.

Key words: **Antimicrobial, camomile, fenugreek, garlic, extract, essential oil.**

INTRODUCTION

Various medical plants have been used for years in daily life to treat disease allover the world. Egypt is an important floristic center internationally because of its geographic location, climate and the presence of the thousands natural plant species. In the preparation and commercialization of food stuffs, the aim is always to obtain a product of maximal quality and well preserved as possible before reaching the consumer (Tome *et al.,* 2001). The use of natural plant species as antioxidants and antimicrobial agents in processed foods is a promising alternative to the use of synthetic compounds, because of increasing consumer interest in natural food additives (Yin and Cheng, 1998 and Cheah and Gan, 2000).

Camomile(*Anthemis nobili L.*) grows in fields and many other places throughout England, Europe, Russia and Asia, and is naturalised in Australia and the US. Camomile flowers contain 0.3-2% volatile oil including bisabolol; bitter glycosides (anthemic acid); flavone glycosides (anthemidin),coumarins

(including umbelliferon and herniarin), phenolic carboxylic acids, polysaccharides, mucilage, choline, amino acids, tannins, malic acid (Hyperhealth, 1996). Blue chamazulene is formed from the sesquiterpene lactone matricin during steam distillation (Mills, 1993).Modern science has shown that camomile flowers and their constituents have several therapeutic effects including: anti-inflammatory, spasmolytic, vulnerary, antimicrobial, antibacterial, mild sedative, carminative, antiseptic, anticatarrhal (Hyperhealth, 1996).

Garlic [*Allium sativum*] is among the oldest of all cultivated plants. It has been used as a medicinal agent for thousands of years. It is a remarkable plant, which has multiple beneficial effects such as antimicrobial, antithrombotic, hypolipidemic, antiarthritic, hypoglycemic and antitumor activity (Mazza, 2001 and Thomson and Ali, 2003). Garlic contain some important compounds and their biological activities such as isothiocyanates and organosulfur compounds. All of these components, may reduce risk heart disease (cholesterol lowering) and cancer, antimicrobial, antioxidant (Mazza, 1998; Mazza and Oomah, 2000). Modern research has shown garlic to have broad-spectrum antimicrobial and antiviral activity against a wide range of bacteria, viruses, worms and fungi (Tsao and Yin 2001). All bacteria tested by Ross *et al.* (2001) which included both gram-negative and -positive bacteria and pathogenic forms, were susceptible to garlic materials (garlic oil, garlic oil sulfides and garlic powder).

Fenugreek has been used both as a medicine and as a food spice in Egypt, India, and the Middle East. It was traditionally recommended for the treatment of wounds, bronchitis, digestive problems, arthritis, kidney problems, and male reproductive conditions (Muralidhara, Narasimhamurthy *et al.*, 1999). The name comes from *Foenum-graecum*, meaning Greek hay, the plant being used to scent inferior hay. The name of the genus, *Trigonella*, is derived from the old Greek name, denoting 'three-angled,' from the form of its corolla. The seeds of fenugreek have been used medicinally all through the ages and were held in high repute among the Egyptians, Greeks and Romans for medicinal and culinary purposes (Nature One Health, organization, fenugreek.

htm). Fenugreek seeds contain several compounds such as 4-hydroxyisoleucine, trigonelline, as well as certain aromatic compounds and steroidal substances that have not been found in other plants. All of these components, alone or in combination, provide this plant with a number of pharmacological, and therapeutic properties including glucose and cholesterol lowering effects (Mazza, 2001).

So this study was designated to investigate the antimicrobial activities of ethanol, methanol and hexane extracts of the 3 plant species being camomile (*Anthemis nobili L.*), garlic (*Allium sativum L.*) and fenugreek (*Trigonella foenumgraecum L.*) and its application in salted fish as natural food additives to obtain a product of maximal quality and well preserved.

MATERIALS AND METHODS

1-Materials:

1. Camomile (*Anthemis nobili L.*), garlic (*Allium sativum L.*) and fenugreek (*Trigonella foenumgraecum L.*) were obtained from locally market in Zagazig city, Sharkia Governorate, Egypt (2004). 100g of each plant part were ground and extracted with 1000ml of each solvent i.e. ethanol, methanol and hexane (1:10 w/v) in a round bottom flask at room temperature (30°C) for 24 hours under vigorous agitation. The mixture was filtered by using Whatman No.1 and the residue was extracted again with 100 ml of the same solvent under the same condition. The filtrates from the two extractions were combined and the solvent were subsequently evaporated under vacuum using rotary evaporator at 50°C until dry film of extract was formed. The yields of extracts were determined according to Wu, *et al.* (1994).The yield extract was dissolved in about (25×2ml) of each solvent and kept in refrigerator until its used and analysis.

2. Commercial essential oils of camomile, garlic and fenugreek were obtained from Captain Company for Extracting Natural Oils, Herbs and Cosmetics, Egypt on 2004.

3. Sardine fish processing:

10kg of sardine (*Sardinella sp.*) fish were obtained from local market, Sharkia Governorate, Egypt on 2004. Fish samples were then washed with tape water. Head, tail, fins and viscera of the

fish were removed and discarded, fish flesh was washed by tape water and sliced into transverse slices fillets as mentioned by El-Shawaf (2000). All samples were put in glass jars container and covered with brine solution (20%NaCl) as above treatment using 1000, 2000, 3000ppm from different extracts as additive to brine solution . Cured fish samples stored at room temperature until determination microbiological test after 0, 15, 30 days and sensory evaluation was carried out at the end of salting period (15 days).

2. Methods:

1. Thin layer chromatography (TLC):

Camomile, garlic, and fenugreek extracts were spotted on TLC silica GF_{254} plates (sizes 20×20, thickness 0.25mm and activation at 105°C for 2 hours) and using chloroform: ethyl acetate: formic acid (50: 40: 10 v/v/v) as a solvent system . The plates were examined under ultra violet (UV) lamp (365 nm) according to Pratt and Miller, (1984) and the components were marked for **R*f***(Rat of flow) value by the following equation :-

$$\textbf{R}f \text{ value} = \frac{\text{The distance of sample}}{\text{The distance of solvent}}$$

2. Gas Chromatography -Mass Spectrometry (GC-MS) :

The obtained extract (hexane extract of garlic as the better antimicrobial than other extracts) was identified by Gas Chromatography – Mass Spectrometry (GC-MS). The analysis was operated in Laboratory Center, Dept. of Food Industries, Fac. of Agric., Cairo Univ., using HP Model 6890 GC-MS Spectrometer.

Gas Chromatography conditions:

- Hewelett Packared Model 6890.
- Temperature program: oven 50 to 260 °C with rate 8 °C /min. Injection 290°C;Detector 300°C.
- Carier gas: Helium at 0.8 cm/min.
- Capillary Column: Carbowax, Length 80 m, Thikness 0.3.

Mass Spectrometry conditions:

- Hewelett Packared Model 5973 Mass Selective detective.
- Scanning mode (m/z 40 to 650).
- Identification of separated compounds was by using standard library (NIST Version 2.0).
- Sample concentrated with nitrogen gas prior to dryness,

then sample was subject to sililylation with TMSI Mixture (Supelco company Catalog No. R- 402170 LA 97702).

3.Microorganisms :

- *Staphylococcus aureus* and *Listeria monocytogenes* were obtained from Dairy Dept., Fac. of Agric., Mansoura Univ., Egypt.
- *Bacillus subtilis, Micrococcus spp, Aspergillus orizea, Aspergillus niger , Penecillium sp.,* and *Saccharomyces cerevisiae* were obtained from Dept. of Microbiology, Fac. of Agric., Mansoura Univ., Egypt.

4. Media for microbiological examinations :

- *Staphylococcus aureus* was plated with Staphylococcus medium No.110 (Difco,1974).
- *Listeria monocytogenes* was plated with NAB (Nalidixic acid blood) agar medium according to Beerens and Tahon-Castel, (1966).
- *Aspergillus orizea, Aspergillus niger* ,and *Penecillium sp.* were plated with Potato Dextrose Agar (PDA) was used according to Adekunl and Ayeni (1974).
- *Bacillus subtilis, Micrococcus spp,* and *Saccharomyces cerevisiae* were plated with nutrient agar medium.

5.Antimicrobial activities :

All the obtained extracts were injected into paper discs (6 mm diameter) in amount of 20 µl. to determine its effect on pathogenic and non-pathogenic microorganisms using minimum inhibition concentration "MIC" technique during their growth at 30°C for 48 hours and 5 day for fungi. Discs injected with 20 µl of pure ethanol, methanol and hexane served as negative control (6mm). At the end of the period, the inhibition zones formed on the media were measured with a transparent ruler in millimeter.

The sensitivity of each organism for the different extracts was recorded as mentioned by El-Shawaf and Gomaa (2000) as follows:

- Zones diameter > 15 mm: highly sensitive.
- Zones diameter 5-15 mm: moderate sensitive.
- Zones diameter 1-5 mm: slightly sensitive.
- No Zones considered to be insensitive.

6. Total viable count :

The pour plate technique for the microbiological analysis. plate counts were performed on nutrient agar for fermented fish medium according to APHA (1992). After serial dilutions and inoculation, plates were incubated at 30°C for 48 hours before counting. The average of triplicate reading were taken as mentioned by El-kotry *et al.,* (1994). Numbers of colony forming unit (CFU) were counted and reported as log CFU/g (cell for colony).

7. Sensory evaluation:

Sensory evaluation for all investigated samples of cured fish were evaluation by a taste panel of 10 well trained members. The samples were tested for color, odor, taste and overall acceptability as mentioned by El-Sharbiny (1996).

RESULTS AND DISCUSSION

1. Yield of camomile, garlic and fenugreek extraction:

Camomile, garlic, and fenugreek samples were subjected to successive extraction with many solvent different in their polarity. The yield of purified crude extract by ethanol, methanol and hexane is shown in Table 1. Results appears that higher the polarity of the solvent the higher of yield. Also, results indicate that the efficiency of the solvents on the extraction was in the order of methanol > ethanol > hexane. These results are in agreement with those obtained by El-Shawaf (2000) who reported that methanol is widely used and effective solvent for extraction depending on its high polarity.

2. Antimicrobial activities of different extracts:

The antimicrobial activities of the obtained extracts of camomile, garlic, and fenugreek compared with control (injected with only pure ethanol, methanol and hexane) and commercail sample as standard control are shown in Table 2.

It can be seen that the extracts of camomile showed antimicrobial activity against *Micrococcus spp.* as very high sensitive (14-23 mm) zone. The hexane extract have high effect on *Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes* as moderate sensitive (6-10mm) zone. The methanol and ethanol extracts of chamomile did not inhibit *Aspergillus*

Table 1: Effect of solvent type on yield percent of crude oil extract

Type of plant Material*	Yield %		
	Ethanol	Methanol	Hexane
Camomile	6.57	16.33	2.31
Garlic	4.48	6.07	3.74
Fenugreek	4.54	11.80	3.57

*sample: solvent 1:10 (w/v) ratio in all materials.

orizea, Aspergillus niger, Penecillium sp., and *Saccharomyces cerevisiae* as insensitive (0 mm) zone. Modern research has shown that camomile flowers and their constituents have several therapeutic effects including: antimicrobial, antiseptic, antibacterial and anti-inflammatory (Hyperhealth, 1996). All camomile extracts were higher sensitive against all tested microorganisms in comparison to commercial sample.

Garlic extracts showed various antimicrobial activities (6-19mm) inhibition zone against the microorganisms tested. The hexane extract showed higher inhibition rate on *Bacillus subtilis, Micrococcus spp.,* and *Penecillium sp.* as very high sensitive (19,16and15 mm) and *Listeria monocytogenes* as highly sensitive (13mm) while *Staphylococcus aureus, Aspergillus orizea, Aspergillus niger,* and *Saccharomyces cerevisiae* as moderate sensitive (8,8,7 and 9 mm) inhibition zone, respectively. The methanol and ethanol extracts showed 13 and 10 mm inhibition zone (highly sensitive) against *Micrococcus spp.*, respectively. While they showed a 6 to 8 mm inhibition zone (as moderate sensitive) against *Bacillus subtilis, Listeria monocytogenes, Staphylococcus aureus, Aspergillus orizea, Aspergillus niger, Penecillium sp.* and *Saccharomyces cerevisiae*. These results are in agreement with those obtained by Ross *et al.* (2001). They reported that all bacteria tested, which included both gram-negative and -positive bacteria and pathogenic forms, were susceptible to garlic materials (garlic oil, garlic oil sulfides and garlic powder). All garlic extracts were higher sensitive against the tested microorganisms in comparison to commercial sample.

Table 2: The antimicrobial activities of the crude oil extracts of camomile, garlic,and fenugreek.

Microorganisms	Inhibition Zone (mm / 20 µl.)											
	Camomile				Garlic				Fenugreek			
	A	B	C	D	A	B	C	D	A	B	C	D
Bacillus subtilis	7*	6	6	7	-	6	19	6	-	7	9	6
Listeria monocytogenes	6	6	7	6	6	7	13	6	-	-	-	-
Micrococcus spp.	14	16	23	11	13	10	16	9	12	13	10	8
Staphylococcus aureus	6	8	10	6	6	7	8	6	-	-	12	7
Aspergillus orizea	-	-	6	-	8	7	8	-	-	-	6	-
Aspergillus niger	-	-	7	-	6	7	7	-	-	-	8	6
Penecillium sp.	-	-	6	-	6	8	15	-	-	7	7	6
Saccharomyces cerevisiae	-	-	7	-	7	8	9	-	-	8	10	6

Extraction solvent : A: Methanol, B: Ethanol, C: Hexane, D: Commercial Sample as standard control .
* This value was determined by difference from control (6mm) of eash .
(15-20 mm) : very high sensitive,
(10-15 mm) : highly sensitive,
(5 - 10 mm) : moderate sensitive,
(1 - 5 mm) : slightly sensitive,
Nil (-) : Insensitive

All fenugreek extracts showed antimicrobial activity against *Micrococcus spp.* as highly sensitive (10-13 mm) zone. The hexane extract have high effect on *Staphylococcus aureus*, and *Saccharomyces cerevisiae* as highly sensitive (12 and10 mm) zone, respectively. While ethanol extracts showed no inhibition zone (insensitive) against the above listed microorganisms. All fenugreek extracts were higher sensitive against all tested microorganisms in comparison to commercial sample.

This study indicated that the hexane extract of all plant samples and methanol, ethanol extract of garlic and fenugreek showed various antimicrobial effects on microorganisms listed above, while that methanol and ethanol extract of camomil, garlic, and fenugreek showed various antimicrobial effects against *Bacillus subtilis, Listeria monocytogenes*, *Micrococcus spp.*, and *Staphylococcus aureus.* These results are in good agreement with the data reported by Furuya *et al.* (1997), El-Shazly *et al.* (2002), Ates and Erdogrul (2003), and Velickovic *et al.* (2003).

According to the results camomile, garlic, and fenugreek could be used as a new sources for natural oil extracts as antimicrobial agents in foods.

3. TLC Chromatography of various extracts:

Data in Table 3 shows the identification of component of various extracts on TLC under ultra violet light at 365 nm using chloroform: ethyl acetate: formic acid (50: 40: 10 v/v/v) as a solvent system.

Data showed that methanolic extracts of camomile and fenugreek contain about five components separate and high resolution appear on TLC under UV different in its color and R_f value, where, the high molecular weight appear at R_f = 0.103(green color) and R_f =0.248 (red color) respectively, the lower molecular weight appear at R_f =0.963 (red color) and R_f = 0.921(green color) respectively. Also methanolic extracts of garlic contain two components R_f =0.191and 0.829 (green color). While ethanolic extracts of camomile contain about four components appear under UV different in its R_f value and color. The compounds had low molecular weight was appear at R_f =0.971(red color), high molecular weight was appear at R_f = 0.126(green color). Also, ethanolic extracts of garilic and fenugreek

Table 3: Separation and fractionation compounds of crude oil extracts by different solvents on TLC and its characteristic under UV lamp at 365 nm.

Type of plant material	Fraction On TLC	Extracts							
		Methanolic		Ethanolic		Hexane		Commercial sample	
		R_f	Color	R_f	Color	R_f	Color	R_f	Color
Camomile	1	0.103	Green	0.126	Green	0.159	Green	0.438	Red
	2	0.329	Green	0.312	Green	0.324	Green	0.750	Violet
	3	0.609	Green	0.893	Violet	0.874	Violet	-	-
	4	0.918	Violet	0.971	Red	0.943	Red	-	-
	5	0.963	Red	-	-	-	-	-	-
Garlic	1	0.191	Green	0.157	Green	0.176	Green	0.533	Green
	2	0.829	Green	0.857	Green	0.746	Green	0.818	Green
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
Fenugreek	1	0.248	Red	0.761	Green	0.764	Green	0.727	Green
	2	0.303	Green	0.909	Green	0.939	Green	0.818	Green
	3	0.424	Green	-	-	-	-	-	-
	4	0.471	Red	-	-	-	-	-	-
	5	0.921	Green	-	-	-	-	-	-

contain two compounds R_f=0.157, 0.857 (green color), and R_f =0.761, 0.909 (green color), respectively.

Ethanolic extracts was lower than methanolic extracts, of all plant samples with the same solvent system depended on the polarity. Also, hexane extracts of camomile had four compounds was appear under UV at 0.159 (green), 0.324 (green), 0.874 (violet) and 0.943 (red), respectively depended on polarity and non polarity of compounds which soluble in hexane and insoluble in hexane. On the other hand, hexane extracts of garlic and fenugreek had two only compounds was appear under UV at R_f = 0.176, 0.764 (green) and R_f = 0.764, 0.939 (green).

Finally, the commercial samples had only two compounds was appear under UV at 0.438(red), 0.750 (violet) in camomile, R_f = 0.533, 0.818(green) in garlic and R_f = 0.727, 0.818(green) in fenugreek.

The methanolic extracts of camomile and fenugreek had high number of compounds appear under UV. Methanolic extracts had high solubility of compounds than other solvents, depend on the polarity of methane (El-Shawaf, 2000).

4. GC- MS of garlic hexane extract:

Identification of the isolated compounds were confirmed by matching the electron impact GC-MS spectra with those of a known standard as shown Table 4.The compound in Fig. 1b was quinoline, 5, 6, 7, 8- tetrahydro-3-methyl chief aromatic constituents which confirmed with the a known stander Fig. 1a.

Table 4 illustrates that garlic extract contains trifluoroaceta (acetamid, 2, 2, 2- trifluoro); ethanthioic acid, S-(2-methylpropyl) ester; beta-amino-trans, trans-5,9-cyclododecadiene as chief aromatic constituents and 9-octadecenoic acid (z)-, methyl ester (linoleic: unsaturated fatty acid) as shown in Fig. 2b, 3b, 4b and 5b compared with a known stander Fig. 2a, 3a, 4a and 5a respectively. These results are in good agreement with the data reported by Mazza, (2001) and Thomson and Ali (2003). On the other hand, our data showed that linoleic (unsaturated fatty acid) is present at low concentration.

5. Total viable count (TVC):

The evaluation of total viable count of salted fish (sardine) during storage as effected by

Table 4: GC- MS for separation of hexane extract of garlic as antimicrobial substances

Compounds	R.T.*	Area %	Ions(M/Z)	Matching %	Structure
Quinoline,5,6,7,8-tetrahydro-3-methyl	2.51	35.36	028712-62-1	100.0	Fig(1)
Acetamid,2,2,2-trifluoro (trifluoroaceta)	5.45	24.20	000354-38-1	67.9	Fig(2)
Ethanthioic acid, S-(2-methylpropyl)ester	1.88	6.55	002432-37-3	18.38	Fig(3)
Beta-amino-trans,trans-5,9-cyclododecadiene	18.97	6.25	072193-55-6	17.55	Fig(4)
9-octadecenoic acid (z), methyl ester (linoleic)	19.25	1.49	000112-62-9	4.19	Fig(5)

* R.T. : Retention Time.

ethanolic extract for camomile, hexane extract for garlic and methanolic extract for fenugreek at 1000, 2000, 3000ppm was illustrated in Table 5. The total viable count at zero time was not considerably effected. A considerable increase in total viable count was noticed after 15 days storage in the control, treatments No. 3, 5 and 7 with progressively increasing up to 30 days of storage. These results were in agreement with Achinewhu and Oboh, (2002) and Paludan, *et al.* (2002), they indicated that TVC of microorganisms were slightly decreased in fermented and unfermented sardinella with decreasing pH from 6.5 to 4.3.

On the other hand, treatments No. 1,2,4,6,8 and9 showed a little increased in total viable count up to the end of storage period. The least total viable count was found in samples of treatment No.9 which contained 3000ppm methanolic extract for fenugreek followed by treatment No.4 (1000ppm hexane extract for garlic), treatment No.8 (2000ppm methanolic extract for fenugreek) and No.2 (2000ppm ethanolic extract for camomile) in decreasing order .

Search Libraries: D:\DATABASE\WILEY275.L Minimum Quality: 0

Unknown Spectrum: Apex minus baseline at 0 minutes
Integration Params: current RTEINT parameters

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
2	2.51	35.63	D:\DATABASE\WILEY275.L			
			Quinoline, 5,6,7,8-tetrahydro-3-me	33312	028712-62-1	37
			6-Amino-2-methyl(1H)pyrrolo[2,3-b]	33143	000000-00-0	32
			Benzene, (1-methoxy-2-propenyl)- (	34042	022665-13-0	25

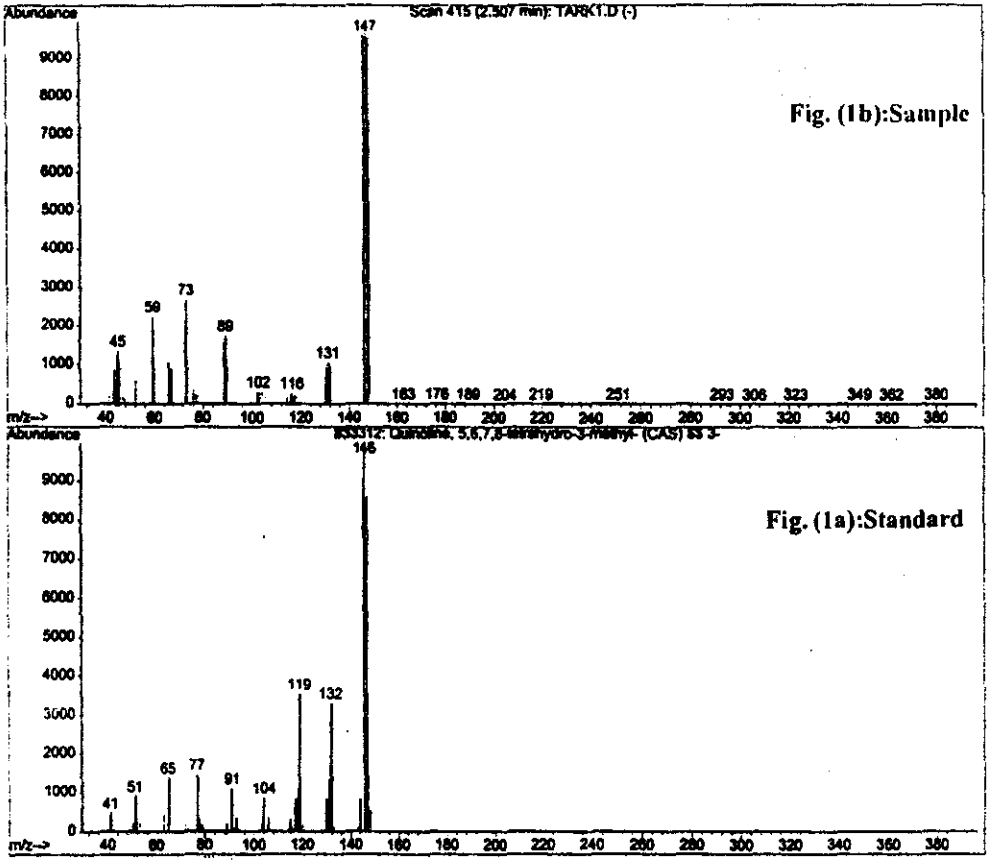

Fig. 1: GC-MS electron mass spectrum for quinoline,5,6,7,8-tetrahydro-3-methyl .

Search Libraries: D:\DATABASE\WILEY275.L Minimum Quality: 0

Unknown Spectrum: Apex minus baseline at 0 minutes
Integration Params: current RTEINT parameters

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
4	5.45	24.20	D:\DATABASE\WILEY275.L			
			Acetamide, 2,2,2-trifluoro- (CAS)	11219	000354-38-1	9
			1h-pyrrole-3,4-dicarbonitrile,5-et	33140	101402-42-0	4
			threo-1,2-dimethyl-1-methylthio-2-	33091	097241-41-3	3

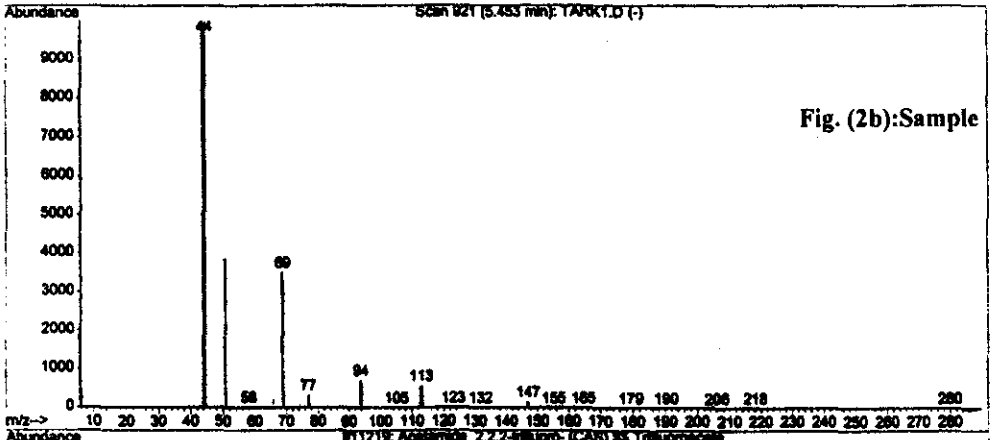

Fig. (2b):Sample

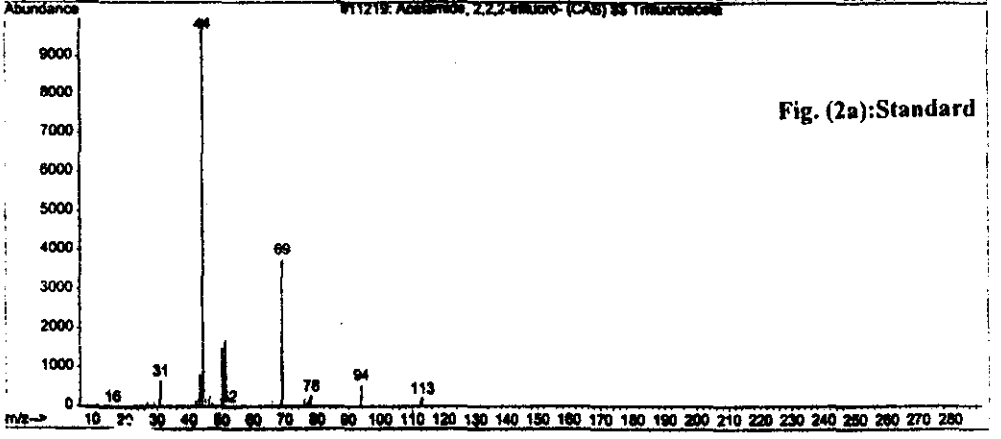

Fig. (2a):Standard

Fig. 2: GC-MS electron mass spectrum for acetamid,2,2,2-trifluoro (trifluoroaceta).

Search Libraries: D:\DATABASE\WILEY275.L Minimum Quality: 0

Unknown Spectrum: Apex minus baseline at 0 minutes
Integration Params: current RTEINT parameters

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	1.88	6.55	D:\DATABASE\WILEY275.L			
			Ethanethioic acid, S-(2-methylprop	21756	002432-37-3	9
			Divinyl sulphone $$ 1,1'-Sulphonyl	13823	000077-77-0	9
			Propanal, 3-ethoxy- (CAS) $$ 3-Eth	7511	002806-85-1	4

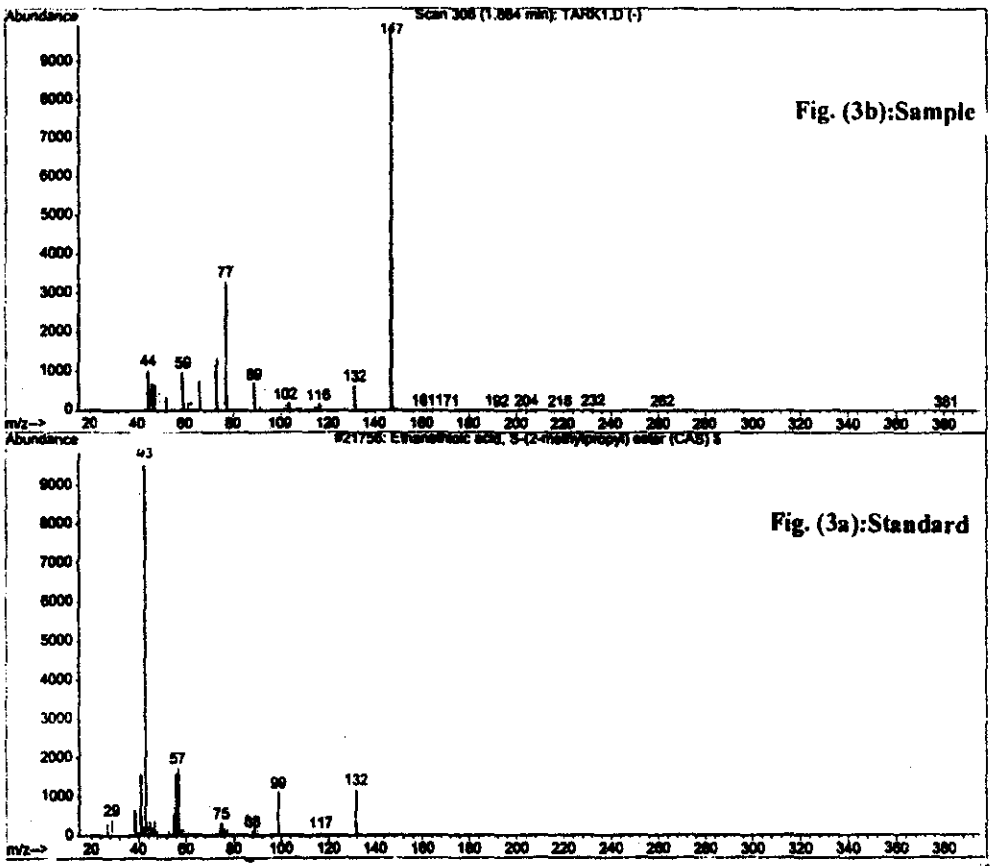

Fig. 3: GC-MS electron mass spectrum for ethanthioic acid, S-(2-methylpropyl)ester.

Search Libraries: D:\DATABASE\WILEY275.L Minimum Quality: 0

Unknown Spectrum: Apex minus baseline at 0 minutes
Integration Params: current RTEINT parameters

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
6	18.57	6.25	D:\DATABASE\WILEY275.L			
			1.BETA.-AMINO-TRANS,TRANS-5,9-CYCL	63594	072193-55-6	55
			Cyclopentadecanone, 2-hydroxy-	126229	004727-18-8	50
			1-cyclopropylideneamino)-2,2,6,6-t	78723	105598-23-0	38

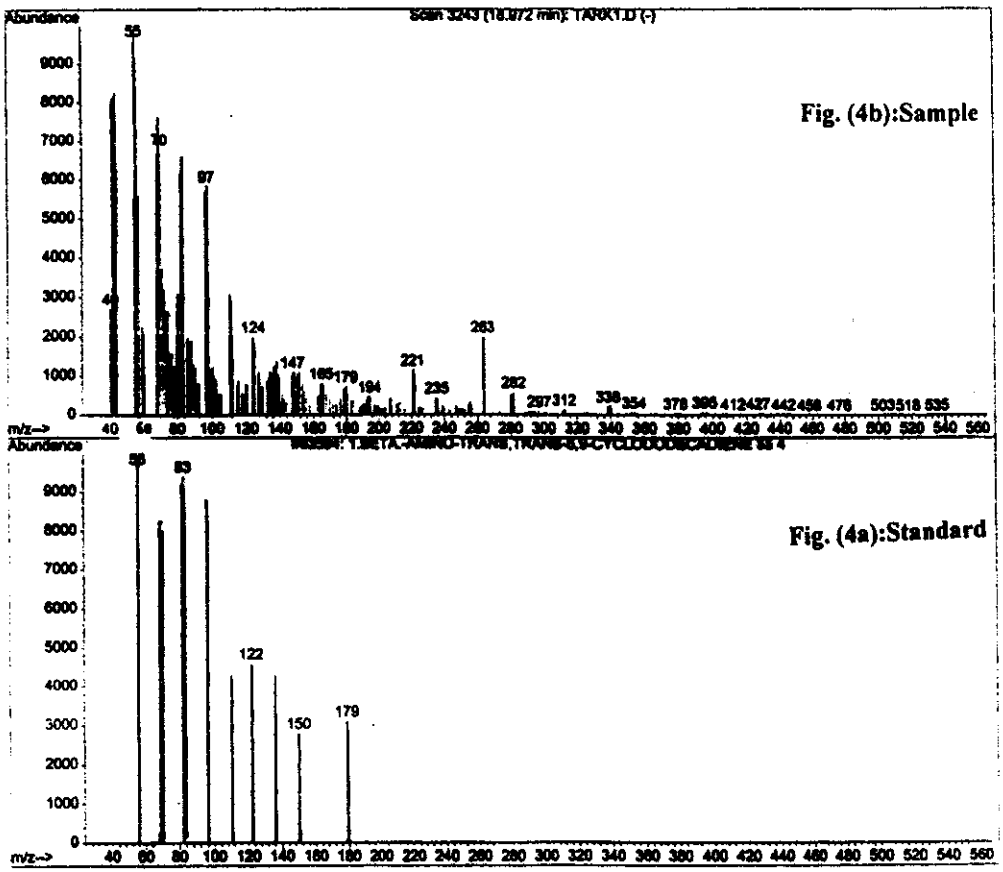

Fig. 4: GC-MS electron mass spectrum for beta-amino-trans,trans-5,9-cyclododecadiene.

Search Libraries: D:\DATABASE\WILEY275.L Minimum Quality: 0

Unknown Spectrum: Apex minus baseline at 0 minutes
Integration Params: current RTEINT parameters

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
7	19.02	1.65	D:\DATABASE\WILEY275.L			
			9-Octadecenoic acid (Z)-, methyl e	175223	000112-62-9	37
			Acetamide, N-methyl-N-[4-[4-BOC-1-	184827	000000-00-0	30
			1-CYCLOHEXYL-2-METHYLAZIRIDINE $$	27214	027159-39-3	27

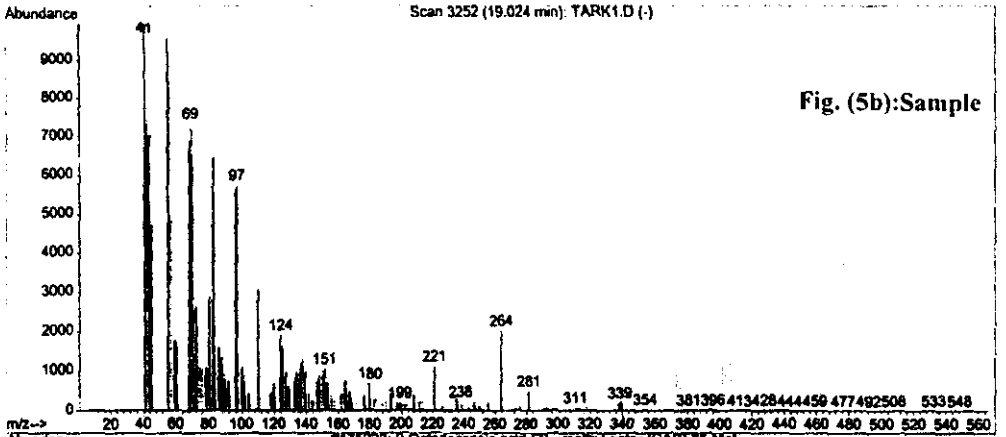

Fig. (5b):Sample

Fig. (5a):Standard

Fig. 5: GC-MS electron mass spectrum for 9-octadecenoic acid (z)-,methyl ester (linoleic).

Data in Table 5 revealed the total viable count in salted sardine fish treatments were little increased when storage period prolonged with fenugreek methanolic extract (3000 ppm), garlic hexane extract (1000and 3000 ppm) and camomile ethanolic extract (1000and3000ppm). On the other hand, during storage periods at 20% with 3000ppm fenugreek methanolic extract, garlic hexane and camomile ethanolic extract, total viable count log (CFU/g) (TVC) were lower than control after 15 days. The different variation in total viable count (TVC) CFU/g may be due to the adding natural extracts type and concentration to brine.

Finally, the total viable count (TVC) log (CFU/g) of samples treated with natural oil extract of camomile, garlic, and fenugreek were lower than total viable count with unfortified brine (control). Treatment with camomile, garlic, and fenugreek oil extract (in combination with brine) lead to an increment reduction of total contamination level in addition their effect against food pathogenic organisms.

6. Organoleptic evaluation of salted sardine fish:

Table 6 shows the organoleptic evaluation for color, odor, taste and overall acceptability of the salted sardine fish treated with different concentration of camomile, garlic, and fenugreek extract. The maximum score for color, odor and taste were 30, 30 and 40 point, respectively. The overall acceptability was calculated as the sum of the score of color, odor and taste and presented as percent of the maximum score. The overall score of the salted sardine fish treated with 1000, 2000, and 3000ppm ethnolic camomile extract were 81.51, 78.15, and 73.74, respectively. The same samples but treated with hexanic garlic extract accumulated overall score of 81.65, 83.72 and 83.36. The samples treated with methanolic fenugreek extract were rated 85.22, 90.52 and 85.14. These data were in agreement with that obtained by Achinewhu and Oboh (2002).

Organolepticaly, the various treatments can be ranked in descending order into two categories:

Superior quality, score more than 80%:

1- Salted sardine fish treated with 1000ppm camomile ethanolic extract.

2- Salted sardine fish treated with 1000ppm garlic hexanic extract.

Table 5: Effect crude oil extracts from different solvents on total viable count (CFU/g) of microorganisms during storage at different concentration.

Type of extract	Treatments No.	Concentration	Storage periods at room temperature (days)** 0		15		30	
			Log of (TVC)					
			CFU/g*	log	CFU/g	log	CFU/g	log
Ethanolic extract of camomile	1	1000 ppm	$1x\ 10^6$	6.000	$1\ x10^6$	6.000	$2\ x10^6$	6.301
	2	2000 ppm	-	-	$2\ x10^6$	6.301	$9\ x10^6$	6.954
	3	3000 ppm	-	-	$3\ x10^6$	6.477	$5\ x10^6$	6.700
Hexane extract of garlic	4	1000 ppm	-	-	$1\ x10^6$	6.000	$5\ x10^6$	6.700
	5	2000 ppm	$5x10^6$	6.700	$13\ x10^6$	7.114	$26\ x10^6$	7.415
	6	3000 ppm	-	-	$2\ x10^6$	6.301	$4\ x10^6$	6.602
Methanolic extract of fenugreek	7	1000 ppm	$3\ x10^6$	6.477	$8\ x10^6$	6.903	$30\ x10^6$	7.477
	8	2000 ppm	$1\ x10^6$	6.000	$2\ x10^6$	6.301	$6\ x10^6$	6.778
	9	3000 ppm	-	-	-	-	$5\ x10^6$	6.700
	Control	-	$2\ x10^6$	6.301	$6\ x10^6$	6.778	$10\ x10^6$	7.000

* (CFU/g) : colony for unit.
**Mean value .
(-) Not detected.

Table 6: Organoleptic evaluation of salted sardine fish prepared with different concentration of natural oil extracts.

Type of extract	Treatments No.	Concentration	Organoleptic evaluation of salted sardine fish (after 15 days storage) Color (30)	Odor (30)	Taste (40)	overall acceptability %	Evaluation
Ethanolic extract of camomile	1	1000 ppm	25.29	25.15	31.07	81.51	Superior quality*
	2	2000 ppm	23.79	25.36	29.00	78.15	Good quality**
	3	3000 ppm	23.80	23.58	26.36	73.74	Good quality
Hexane extract of garlic	4	1000 ppm	26.07	25.65	29.93	81.65	Superior quality
	5	2000 ppm	26.86	26.14	30.72	83.72	Superior quality
	6	3000 ppm	27.79	25.07	30.50	83.36	Superior quality
Methanolic extract of fenugreek	7	1000 ppm	27.43	27.50	30.29	85.22	Superior quality
	8	2000 ppm	25.29	24.36	29.08	78.73	Good quality
	9	3000 ppm	24.00	26.14	28.00	78.14	Good quality
	Control	-	27.43	27.80	37.00	92.23	Superior quality

*Superior quality : score more than 80%.
**Good quality : score between 70 and 79%.

3- Salted sardine fish treated with 2000ppm garlic hexanic extract.

4- Salted sardine fish treated with 3000ppm garlic hexanic extract.

5- Salted sardine fish treated with 1000ppm fenugreek methanolic extract.

Good quality, score between 70 and 79%:

1- Salted sardine fish treated with 2000ppm camomile ethanolic extract.
2- Salted sardine fish treated with 3000ppm camomile ethanolic extract.
3- Salted sardine fish treated with 2000ppm fenugreek methanolic extract.
4- Salted sardine fish treated with 3000ppm fenugreek methanolic extract.

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فصل وتعريف المركبات المضادة للنشاط الميكروبي لمستخلصات بعض الزيوت العطرية وتطبيقها في السمك المملح

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يهدف هذا البحث إلى دراسة التأثير المثبط لمستخلصات الزيوت النباتية المستخلصة من الثوم وأزهار الكلموميل (البابونج) وحبوب الحلبة باستخدام كل من الايثانول و الميثانول والهكسان على بعض الميكروبات الممرضة وغير الممرضة وهي: ***Bacillus subtilis, Listeria monocytogenes, Micrococcus spp, Staphylococcus aureus, Aspergillus orizea, Aspergillus niger, Penecillium sp., and Saccharomyces cerevisiae*** على بيئات متخصصة باستخدام تكنيك الأقراص الورقية. وقد تم استخدام كروماتوجرافيا الطبقة الرقيقة TLC في التعرف على تلك المركبات باستخدام معدل السريان (R_f) واختبارات اللون و التعرف على تلك المركبات النشطة باستخدام جهاز GC-MC .وقد تم تطبيق إضافة تلك المستخلصات في صورتها الخام كمواد مضافة طبيعية بتركيزات ١٠٠٠و٢٠٠٠و٣٠٠٠ جزء في المليون في التمليح الرطب لسمك السردين ، وكانت النتائج كالتالي:-

كانت نسبة الاستخلاص بالميثانول أعلى من الايثانول والهكسان على التوالي، ولكن أظهرت مستخلصات الهكسان كفاءة عالية كمثبطات للنشاط الميكروبي مقارنة بالمستخلصات الأخرى.

أظهرت مستخلصات الهكسان للنباتات الثلاثة، مستخلصات الميثانول والايثانول للثوم والحلبة تأثيرات مثبطة مختلفة على جميع الميكروبات المختبرة، بينما أظهرت جميع مستخلصات الثوم تأثيرات مثبطة لجميع سلالات الفطر و الخميرة المختبرة.

أظهرت النتائج أن العد الحيوي الميكروبي في جميع عينات السردين المملحة والمعاملة بالمستخلصات المختلفة كانت منخفضة مقارنة بالكنترول (غير المعاملة) مما يشير إلى أن هذه المعاملات أدت إلى تقليل مستوى الحمل الميكروبي بالإضافة إلى التأثير المثبط تجاه الميكروبات الممرضة.

أظهرت نتائج جهاز GC-MC وجود مركبات لها تأثير مثبط للنشاط الميكروبي مثل acetamid,2,2,2-trifluoro (trifluoroaceta) و quinoline,5,6,7,8- tetrahydro-3-methyl beta-amino-trans,trans-5,9-cyclododecadiene,ethanthioic acid, S-(2-methylpropyl)ester و "9-octadecenoic acid (z)-,methyl ester linoleic" وتتواجد هذه المركبات بنسبة تناقصية على الترتيب.

كما تم تقدير الخواص الحسية (الطعم ، اللون، الرائحة، درجة القابلية) للمعاملات المختلفة، حيث تراوحت درجة الجودة الحسية للمعاملات من جيدة(أربعة معاملات) إلى ممتازة (خمسة معاملات) وهذا يشير إلى إمكانية حماية المستهلك من الميكروبات الممرضة مع الحفاظ على الجودة المرغوبة.

لذا نوصي باستخدام كل من الثوم و الحلبة و الكلموميل (البابونج) كمصادر لمستخلصات الزيوت العطرية الطبيعية لما لها من تأثير مثبط للنشاط الميكروبي حيث يمكن استخدامها كمواد مضافة طبيعية لخفض الحمل الميكروبي و تحسين الخواص الحسية لبعض الأغذية.