Purification and Characterization on an Antibacterial Agent from (*Eugenia Caryophllate) Against of Some Pathogenic Bacteria Isolated From Fishes*

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Abstract

Local bacterial cultures could be isolated from 200 *Oreochromis niloticus* and were caught as random samples from ELAbassa, Abou-Hammad, and Sharkia, Egypt. Fish farms with an average body weight 40±5g. Suffered From signs of septicemia as hemorrhages on several parts of the body surface (mouth) base of fins, abdominal part. opercula , and around the anal opening turbidity of the eyes and slight exophthalmia roughness of scales and sometime scale losses, postmortemally showing, hepatomegaly, splenomegaly, congestion of gills, kidney and accumulation, of bloody fluid in abdominal wall to bacterial strains are Gram negative bacteria were isolated from fish. The bacterial isolate taxonomic classification clarified that the bacterial isolates was likely belonging to *Pseudomonas aeruginiosa-I, Aeromonas hydrophila*-2 according to its physiological morphological, and biochemical characters. The active extract using bioactivity-guided technique of aqueous and organic extracts of *(Eugenia caryophllate).* The separation of the active ingredient and its purification was performed using both thin layer chromatography (TLC) and column chromatography techniques. The physico-chemical characteristics of the purified antibacterial agent viz. color, melting point, solubility, elemental analysis and spectroscopic characteristics (GC – mass techniques) have been investigated. This analysis indicates a suggested empirical formula of C_{10} H_{12} O_2. The biological activities i.e. MICs of the Purified antibacterial agent were also determined.

Keywords: Aeromonas hydrophila, MIC, Antibacterial, Fish

Introduction

Antimicrobial substance as one of new widely used for the treatment of bacterial diseases of fish. Fish diseases due, to bacterial infection are considered one of the major problems in aquacultures **(*Okpk Warsill, 1991; Robertson, 2000; Eid et al, 2016*).** The presence of potential danger of many fish pathogens associated with the .stress factors may favor the

occurrence of outbreaks in cultured fishes caused by Pseudomonas considered to be the major bacterial problems facing the aquaculture development causing mass mortalities, reduced production and low quality of aquatic organisms **(*Ghittino, 1976*)** *P. aeruginosa and Aeromonas hydrophila* were incorporated in sever outbreaks among *fish tilapia's* hatcheries ***(Ahmed and Shoreit, 2001; Ali Aberoum, 2010; Yardimci, 2011; Ye, 2013*** and ***Abouelmaatti et al, 2012)*** To treatment fish diseases (bacterial infection) can used antibiotics, but its cause's chinning in environmentals conditions such as ppt the resin and the toxins in fishes and become toxicity in the nutrition to the human or the animals. One of the most recent concepts is replacing the chemical and therapeutic agents with natural components as one of the strategies available and much experimental work is being carried out to assess its commercially applicability **(*Kosar et al.,2005*; *El- Didamoy et al., 2015 and Elfeil et al, 2012)*.** *Eugenia caryophllate* plants belonging to different species and ecotypes (Biotypes) are widely used in in several industries as it has a flavor and cosmetically in pharmaceutical, beverage and food industries. It has also used been as a traditional remedy to treat various ailments such as a spasmodic, antimicrobial, expectorant, carminative and aromatic for whooping and convulsive coughs, digestive disorders and menstrual problems **(*Aligiannis et al., 2001*).** In previous studies, it has been demonstrated that the content of essential oil and extracts of medicinal plants like *eugenia caryophllate* species containing antibacterial activities on many bacteria **(*Sahin et al., 2004* and *Reverter et al., 2014*)** antioxidant and other biological activities may change based on the deference's in cultivation, origin, vegetative stage and growing seasons of the plants **(*Deans et al., 1992*** and ***Milos et al., 2000*).** The chemical compositions of eugenia *caryophllate* are Eugenol & phenol **(*Sahin et al., 2004*)** and **(*Sivasalnkar et al., 2015*).** This study aims to isolate and identify the most common bacterial Fish pathogens and extract and purification of antibacterial bioactive secondary metabolite product of *eugenia caryophllate*.

Material and methods

Isolation and Purification of bacterial pathogen from Fishes:

Total number of 200 *Oreochromis niloticus* showing signs of septicemia collected from central laboratory for aquaculture research and submitted to fall clinical examination postmortem examination and bacteriological examination according to **(*Schapercluas et al., 1992*).** Samples for bacteriological examination taken from affected Fishes from (Skin, fins, muscles, liver, spleen, kidney, intestine,

and gills under aseptic condition loopful from each organ inoculated into nutrient broth, incubated at 28- 30° C For 24 h., then after streaked on plates of nutrient agar and incubated at 28 - 30° C for 24 hrs. It was purified using the streaking plate technique method as recorded by *Williams and Davis (1965)*

Identification of Bacterial isolates:

Morphological characteristics:

Morphological characteristics of colonies colour, Gram reaction. Cell Shape, Spore formation, and Motility and Diffusible pigment were investigated.

Physiological and biochemical characteristics of pathogenic bacterial isolates were conducted according to ***Elwan, et al. (1977*)**, Lipase; ***Ammar, et al. (1991*)**, Protease; ***Ammar, et al. (1995*)**, Pectinase; ***Ammar, et al. (1998*)**, a-amylase On the other hand, Lecithinase was conducted on egg - yolk medium according to the method of ***Nitsh and Kutzner (1969)*** and Catalase Test. Esculine broth has been done. Nitrate reduction was performed. Hydrogen sulphied; poly β- hydroxyl butyrate accumulation. King A & B, Methyl red, Voges-Proskauer, indol production. Urea test, Gelatein liquefaction, Levan formation, Arginine dihydrolase, Malonate utilization, Phenyl alanine deamination. Utilization of KCN, oxidase test and different carbon and nitrogen sources were carried out according to ***Cowan (1974) and Pridham and Gottlieb (1966)*** respectively.

These isolates identified according to **(*Buchanan and Gibsons, 1974; Krieg, 1984 and Hensyl, 1994)***

Antibiogram Sensitivity:-

Antibiogram Sensitivity was performed using different chemotherapeutic agents the test was done according to method described with ***Quinn et al., (1994)*.**

Plant materials:

Shoot system (leaves and stems) of *eugenia caryophllate* wigare were collected from sienna south. Dried shoot system (leaves and stems) of eugenia *caryophllate* at room temperature. Powdered and kept in plastic bags until extraction.

Screening for antibacterial activity:

The antibacterial activity was determined according to ***Kavanagh (1972).***

Extraction of plant materials:

The coarsely powered shoot parts of *eugenia caryophllate* (200 gms) were extracted. Extracted powdered with distilled water, 95 % ethanol and then partitioned using ethyl acetate and chloroform for 6 hours in a Soxhlet, then the extract was filtered using Whatman filter paper No. I after cooling. The excess solvent of crude and partition of aqueous and organic extract removed under vacuum using rotary evaporator. Each extract kept in refrigerator until further biological investigation.

Precipitation. - "The precipitation process of the antibacterial agent was carried out using petroleum ether. The compound precipitate was centrifuged at 5000 rpm for 15 min. The antibacterial agent powder was tested for its antibacterial activity by using cup assay method" **(*Ueno et al, 2002*).**

Separation: "Separation of the antibacterial agent into its individual components has been tried by thin layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v)" **(*Kosar et al, 2004)***

Purification: "The purification of the antibacterial agent was carried out by using Silica Gel Column Chromatography. A column of 2.5 X 50 cm was used for this purpose. Chloroform and Methanol 10:1 (v/v), was used as an eluting solvent. The column was left for over night until the silica gel (BDH - 60- 120 mesh) was completely settled. One-ml crude extract to be fractionated was added on the silica column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml). Antibacterial activities were performed for each separate fraction" **(*Sahin, 2004*).**

Physico-chemical properties of antibacterial agent.'

I- *Eletnental analysis*: The element analysis C, H, O, N, and S was carried out by the regional center for Mycology and Biotechnology Al-Azhar University, Egypt

2- Spectroscopic analysis: The GC-mass techniques was determined at the regional center for Mycology and Biotechnology Al-Azhar University, Egypt.

Biological activity: The minimum inhibitory concentration (MIC) has been determined by cup method assay on the isolated microorganism.

Results

Results of Clinical examination of naturally infected *O. niloticus'*.

A Total Number of 200 Oreochromis niloticus were clinically examined and showed hemorrhages on several parts of the body surface (mouth, base of the fins, abdomen, opercula and around the anal opening), turbidity of the eyes and slight exophalima, roughness of the scales and Sometime scale losses occur – as shown in photo (1) .

Results of postmortem examination of naturally infected *O. niloticus:*

The observed postmortem pictures were almost the same in all examined fish. These changes included congested, gills, hepatomegaly, splenomegaly, and distended gall bladder with bile, congestion of the kidney. Congestion and hemorrhages in intestine and bloody fluid accumulated in abdominal wall as shown in photo (2).

Results of bacteriological examination of naturally infected Orcochromis niloticus'.

The results revealed the presence of different bacterial species which were either specific Fish pathogen including pseudomonas or other bacteria nonspecific fish pathogens including *Pseudomonas* and *Aeromonas*.

Identification of bacterial isolates:
According to the cultured, morphological and biochemical characters; as shown in table-1; it was cleared that all bacterial isolates related to two bacterial genera and 2species (*Pseudomonas aeruginosa-1; Aeromonas hydrophila-2*).

Control of pathogenic bacterial growth using *eugenia caryophllate*:
The antibacterial agent produced by *eugenia caryophllate* exhibited various degrees on pathogenic bacterial growth (Table 2), and (photo 3, 4)

Antibiogram sensitivity test:
The antipiogram sensitivity test revealed that:

1- Pseudomonas aeruginosa is sensitive to Amikan (AK) at a concentration of (30 ug) and resistant to ciprofloxacin (CIP) at a concentration of (5 ug) and Neomycin (N) at a concentration of (30 ug),

2- *Aeromonas hydrophila* is sensitive to ciprofloxacin (CIP) at a concentration of (5 ug) and Amikan (AK) at a concentration of (30 ug) and resistant to Neomycin (N) at a concentration of (30 ug), as shown in Table-3, Table-4, and photo-5

Extraction, Precipitation and Purification of antibacterial activities.
The different filtrates were tested for their antibacterial activity and it the best results obtained with ethyl alcohol extraction one as shown in table-5.

Crude deep brown powder was tested for their antibacterial activities by using cup diffusion method. The obtained results revealed that two band at R_f 0.76; there is one band at R_f 0.76 exhibited obvious inhibitory effects against the growth bacterial strains.

The purification of the antibacterial agent was carried out by using silica gel column chromatography. The active fractions were concentrated. The maximum activity was recorded at fraction No. 9&10 (Table 6).

Physico-Chemical Properties of antibacterial agent.
The physical characteristics of the extracted ingredients showed a specific Physico-chemical properties such as melting point are 133°C. Regarding the solubility the ingredients are soluble in ethanol, water, chloroform, DMSO and methanol but insoluble in petroleum ether, n-Butanol, hexane and benzene.

A-Elemental analysis:
This analysis indicates suggested empirical formula of the ingredient is $C_{10}\ H_{12}O_2$

B- Spectroscopic characteristics: GC- mass techniques (Fig. 1). Area = 88.81% which indicates a suggested name Eugenol (phenol)

(Fig 2).

C- Biological activities of the purified antibacterial Agent: Data of the antibacterial spectrum of antibacterial agent indicated that the antibacterial agent is fairly active against Gram negative bacteria (Table 7)

***Table (1)**: The morphological, physiological and biochemical properties of the bacterial isolates:*

Characteristic	**1**	**2**
Morphological characteristics		
- Gram reaction	Negative	Negative
- motility	+	+
- Cell shape	Short rods	rods
- Spore former	Non – spore former	Non – spore former
- Diffusible pigment	Blue- green	yellow
Physiological characteristics: A-Enzymes activity	**1**	**2**
Protein hydrolysis	+	+
Starch hydrolysis	-	-
Lipid hydrolysis	+	+
Egg – yolk (Lecithin) hydrolysis	-	-
Oxidase test	+	+
Catalase test	+	+
B-Pigment production		
Pyocyanin pigment	+	+
Carotenoid pigment	+	+
Fluorescent pigment	+	+
Biochemical characteristics		
-Degradation of Esculine	+	+
-Gelatin liquefaction	+	+
- H2S production	+	-
- Nitrater reduction	+	+
- Urea test	-	-
- Indole production	-	+
Levan formation from sucrose	-	-
- Arginine dihydrolase	+	+
-poly β–hydroxy butyrate accumulation	-	-
- Utilization on KCN	+	+
- Citrate utilization	+	-
-Phenyl alanine deamination	+	+
- Voges- proskauer test	-	+
-Methyl red test	-	+
Utilization of carbon sources:	**1**	**2**
L- Arabinose	-	+
D-Xylose	-	-
D- Ribose	+	+
D- Mannose	-	-

Characteristic	**1**	**2**
D-Glucose	+	+
D-Fructose	+	+
D-Galactose	-	+
-Mannitol	+	+
-Meso-Inositol	-	-
-Sucrose	-	+
-Maltose	-	+
-Lactose	-	-
-Raffinose	-	-
-Trehalose	-	+
-Melibiose	-	-
-Starch	-	-
Utilization of nitrogen source	**1**	**2**
-Glycine	-	-
L-Alanine	-	-
L-Serine	+	+
L-leucine	-	-
L-valine	+	+
L-lysine	+	+
L-proline	+	+
L-tyrosine	+	+
L-Arginine	+	+
Growth in presence of different NCl Concentrations (%):	**1**	**2**
1	+	+
3	+	+
5	+	-
7	-	-
Growth at different temperature (^{o}C)	**1**	**2**
20-40	+	+
41	+	+

+= Positive, - = negative.

Table (2): *Mean diameters of inhibition zones (mm) caused by 100 µl of the antibacterial activities from eugenia caryophllate in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm).*

Test organism	***Mean diameters of inhibition zones (mm)**
Pseudomonas aeruginosa-1	30
Aeromonas hydrophila - 2	25

Table (3): *Drug sensitivity test on Pseudomonas aerations*

Antibiotics common name	**Concentration in ug**	**Biodise Code**	**Mean diameters of inhibition zones mm**
Amikan	30 ug	Ak	S
ciprofloxacin	5 ug	CIP	R
Neomycin	30 ug	N	R

S= Sensitive R= Resistance

Table (4): *Drug sensitivity test on Aeromonas hydrophila*

Antibiotics common name	Concentration in ug	Biodise Code	Mean diameters of inhibition zones mm
Amikan	30 ug	Ak	S
ciprofloxacin	5 ug	CIP	S
Neomycin	30 ug	N	R

S= Sensitive R= Resistance

Table (5): *Extraction of antibacterial agents of Eugenia caryophllate*

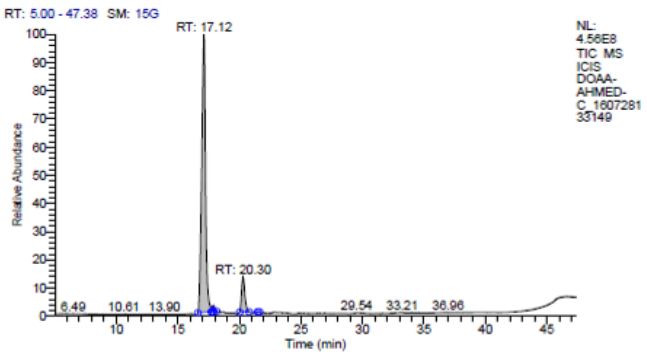
Extract Type	*Mean diameters of inhibition Zones (mm)	
	Pseudomonas aeruginosa-1	***Aeromonas hydrophila -2***
Crude aqueous extract	0.0	0.0
Ethyl acetate	0.0	0.0
Ethyl alcohol	30	25
Acetone	0.0	0.0
Chloroform	0.0	0.0

Table (6): *Isolation, precipitation and purification steps of antibacterial agent from eugenia caryophllate.*

Extract Type	*Mean diameters of inhibition Zones (mm)	
	Pseudomonas aeruginosa-1	***Aeromonas hydrophila -2***
1-Isolation	30 ± 0.15	25 ± 0.22
2-Precipitation	29 ± 0.20	24 ± 0.25
3-Purification by Column chromatography	25 ± 0.23	20 ± 0.17

Table (7): *Antibacterial spectrum of the Purified antibacterial agent by applying the cup method assay.*

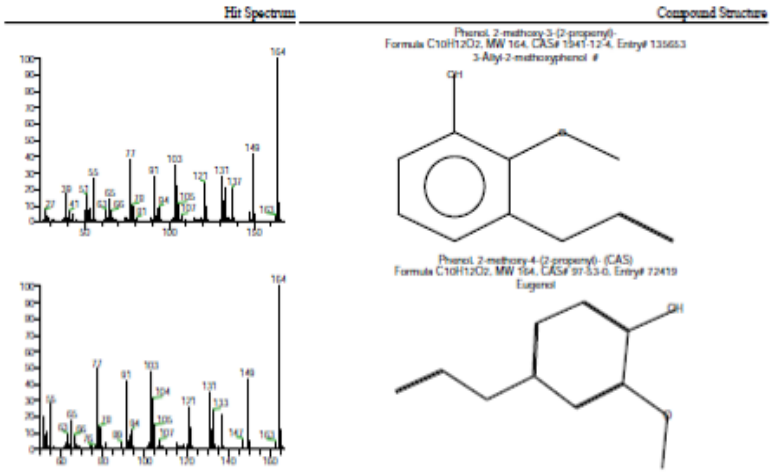
Test organism	MIC (µg/ml) concentration
Pseudomonas aeruginosa-1	83.33
Aeromonas hydrophila -2	83.33



(Fig 1) *GC- mass techniques –Area = 88.81%*

RT	Compound Name	Area %	Area	Molecular Formula	Molecular Weight
17.12	Eugenol	88.81	963301 7916.18	C10H12O2	164
17.12	Eugenol	88.81	963301 7916.18	C10H12O2	164
17.12	Phenol, 2-methoxy-3-(2-propenyl)- (CAS)	88.81	963301 7916.18	C10H12O2	164
17.12	Phenol, 2-methoxy-3-(2-propenyl)-	88.81	963301 7916.18	C10H12O2	164
17.12	Phenol, 2-methoxy-4-(2-propenyl)- (CAS)	88.81	963301 7916.18	C10H12O2	164

Hit Spectrum	Compound Structure
SI 862, RSI 862, nplib, Entry# 23456, CAS# 97-53-0, Eugenol	Eugenol Formula C10H12O2, MW 164, CAS# 97-53-0, Entry# 23456 Phenol, 2-methoxy-4-(2-propenyl)-
SI 849, RSI 849, nplib, Entry# 23453, CAS# 97-53-0, Eugenol	Eugenol Formula C10H12O2, MW 164, CAS# 97-53-0, Entry# 23453 Phenol, 2-methoxy-4-(2-propenyl)-
	Phenol, 2-methoxy-3-(2-propenyl)- (CAS) Formula C10H12O2, MW 164, CAS# 1941-12-4, Entry# 72672 3-Allyl-2-methoxyphenol



***(Fig 2)**: Eugenol, phenol treatment and prevention fresh water fishes from bacterial diseases.*

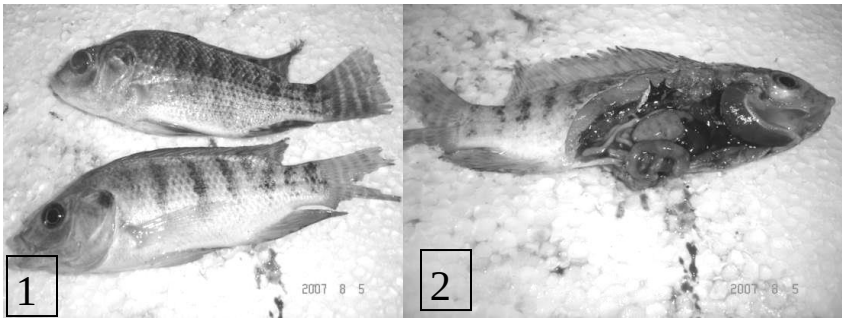


Photo (1) Clinical examination of naturally infected *O. niloticus*
Photo (2) Postmortem examination of naturally infected *O. nilaticus*

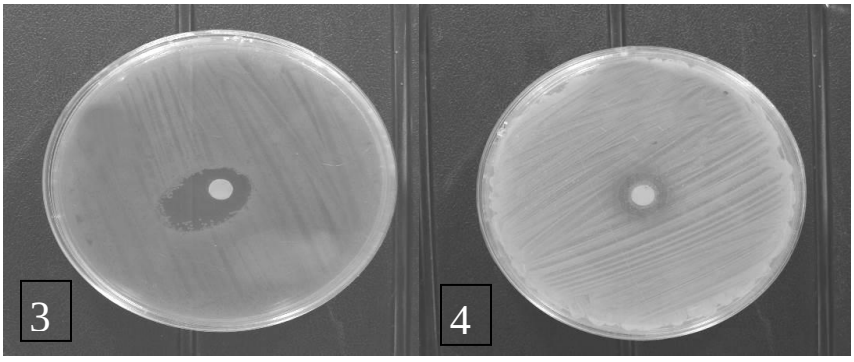


Photo (3) The antibacterial substance produced from *eugenia caryophllate* exhibited various degree of inhibition on *Pseudomonas aeruginosa* growth
Photo (4) The antibacterial substance produced from *eugenia caryophllate* exhibited various degree of inhibition on *A. hydrophila* growth

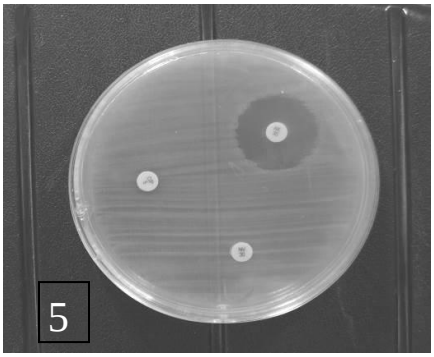


Photo (5) Drug sensitivity test

Discussion

Regarding the examination of naturally infected *O. niloticus hemorrhages* with *Pseudomonas aeruginosia*, and Aeromonas *hydrophila* humiliates all over body surface especially an the mouth, base of the fin, opercula, anal opening and turbidity and exophthalmia of the eyes this results were recorded with ***Marzouk et al., (1989), Badran and Eissa, (1991), Abd El- Rahman et al., (2002) and Abou El-Atta (2003).*** The observed gross lesions of affected fish showed that hepatomegaly, spleenomegaly, congestion of gills, kidney, distended gall bladder, congestion hemorrhage in the intestine and accumulation of bloody fluid is abdominal wall, these results observed by ***Abd EI-Aziz, (1988) , Marzouk, (1989), El-Attar and Moustafa (1996), Abd-Rahman, (1996), Salama, (1999), Abd El- Rahman et al., (2002) & Abou El Atta, (2003), Ali Aberoum,(2010) and Ye, (2013) and El- Didamoy, (2015).*** *Pseudomonas aeruginosia*, and *Aeromonas hydrophila* considered the main isolates from diseased tilapia (*o. niloticus*) this results agree with ***Petrinee et al. (1985), Marzouk et al., (1989), Badran and Eissa (1991), Megahed, (2000), Haenen and Davidse, (2001), Abd El -Rahman el al. (2002) Abou El Atta (2003) and Samal, (2014).*** In last decades *pseudomonas aeruginosa* and hydrophilic are resistant to many antibiotics **(*Stojanov et al ., 2010 and Pannu et al., 2014*)** , also results in table (3,4) showed that regarding to the antibiotic sensitivity of isolated pathogenic bacteria were resistant to Neomycin and were sensitive to Amikan these results are agreement with that finded by ***Abou El-Atta and Wafeek (2005) , Rahman (2010) And Pannu (2014).*** For the purpose of the control of pathogen bacterial isolates, that used *eugenia caryophllate*. The coarsely powdered shoot parts of *eugenia caryophllate* (200 gms) were extracted with 95 % ethanol for 6 hours in a Soxhiet, then the extract was filtered using Whatman filter

paper No. 1. Similar results were recorded by ***Anna Menaker et al.* (2004) and *Sivasankar (2015).*** The excess of crude extract evaporated under vacuum using rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 40-60C) for Precipitation process where only one fraction was obtained in the form of deep brown ppt. Separation of antibacterial agent into individual components has been tried by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v) as developing solvent. The band with an Rf value of 0.76 there is one band at Rf 0.76 exhibited obvious inhibitory effects against the growth bacteria strains. For the purpose of purification process, the antibiotic were allowed to pass through a column chromatography packed with silica gel and eluting solvent was composed of chloroform and methanol (10:1, v/v), fifty fractions were collected and tested for their activities. The maximum activity was recorded at fraction No. 9&10. Similarly, many workers used a column chromatography packed with silica gel and an eluting solvent composed of various ratios of chloroform and methanol, *which match with other finding* ***Akihiko et al., (2000); Masao et al (2000); Naki et al. (2000); Oh-Sung et al., (2000); Toshio et al., (2000), Honda et al., (2001), Kenichi et al., (2001) Yoko et al.. (2001) and Ueno et al. (2002).*** The Physico-chemical characteristics of the purified antibacterial agents revealed that, the melting point are 133°C and soluble in ethanol, water, chloroform, DMSO and methanol but insoluble in, petroleum ether, n-Butanol, hexane and benzene. Similar results were recorded by ***Mitsunobu et al. (2000); Kenichi et al. (2001), Ueno et al. (2002), Anna, et al. (2004), Sahin et al. (2004) and Kosar et al. (2005).*** A study of the elemental analysis of the antibacterial agent lead to an empirical formula of: $C_{10}H_{12}O_2$. The spectroscopic characteristics of antibacterial agent revealed the presence of the maximum absorption peak in GC-mass techniques, area = 88.81% by Sahin *et al., (2004) and Reverter (2014).* The MIC of antibacterial agent under study exhibited various activities against gram negative bacteria. Similar investigations and results were attained by ***Kilbum et al. (2000); Morrissey and George (2000); O'Donnell and Gelone (2000); Oethinger et al. (2000); Okuda et al. (2000); Lomovskaya et al. (2001), Pan et al. (2002) and Atta, et al. (2003) Sahin, et al. (2004) Kosar, et al. (2005) , Sivasanker (2015) and El-Didamoy (2015).***

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