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Biochemical studies on certain biologically active nitrogenous compound

H.G. Abdel Aziz, S.M. Abdel Kader, M.M.El Sayed, E.A.El Malt and E.S.Shaker

Biochem. Dept. Fac. Agric. Minia Univ.

ABSTRACT

Certain biologically active nitrogenous compounds such as alkaloids are widely distributed in many wild and medicinal plants such as *peganum harmala* L. (Phycophyllaceae). However, less literature cited on the natural compounds was extracted from the aerial parts of this plant, therefore this study was conducted on harmal leaves using several solvents. Data indicated that methanol extract was the most inhibitory effect against some pathogenic bacteria, particularly Streptococcus pyogenus. Chromatographic separation illustrated that presence of four compounds, the most active one was the third compound (3). Elementary analysis (C, H, N) revealed that the primary chemical structure of the active antibacterial compound (C3) was: C17 H21 N3 O7 S with molecular weight 411. Spectroscopic analysis proved that coninical structure was = 1- thioformyl, 8β- D glucoperanoside- Bis- 2,3 dihydroisopyridino pyrrol. This new compound is represented as a noval β- carboline alkaloid compound.

Key words: Nitrogenous compounds, Alkaloids, Antibacterial agent, Inhibitory effect, Bioassay against of some pathogenic bacteria.

INTRODUCTION

Wild plants have proved to be an important resources of natural biological compounds useful in medicine and plant protection (Towers,et al., 1989). Antimicrobial activity of plant extract has long been recognized in human medicine (Kang, et al., 1992). Harmal is one of wild plant grows spontaneously in semiarid areas in North Africa and Middle East that is considerd a rich sources of natural medicinal substances (El – Bahri and Chemli, 1991). These substances are found in a major amount in the harmal seeds and a minor amount in the aerial parts of plant. Alkaloids are presented in four β- carboline nucleus (harmine, harmal, harmaline and harmalol) as reported by (Wu, et al.,1999 and Ma, et al.,2000).

In addition, phenolic compounds (simple phenols, phenyl prepanoids, flavonoids glucosides and tannins) *(* Sharaf, et al.,1997 and Lambert,et al.,2005). Triterpenoids (saponins in free form and glucosides) as reported by (Melek, et al., 1995 and Xu, et al.,1998). Steroidal glucosides compounds are also found in harmal seeds (Wang, et al., 1997).

Other investigations have been reported by (Al Sharma, et al., 1981; Adday, et al., 1998; Sokmen, et al., 2000; El Sayed, et al., 2003 and Lambert, et al., 2005) for the natural products extracted from harmal,ariularly in the seeds.

However, little investigations are discussed in the harmal leaves, therefore the objective of this work to evaluate the natural compounds that are extracted from the leaves

p.harmal L. and bioassay experiments are carried out against some pathogenic bacteria to evaluate the role of antibacterial activity particularly aganst *S.pyogenous* that infects human respiratory system. The active compound was also examined to illustrate its chemical structure.

MATERIALS AND METHODS

The green plant was collected from Matrouh Governorate, Egypt, the fresh leaves was washed, then air dried leaves were ground to become flour and kept in a closed container at $4^{O}C$ until used.

Extraction:

Adjustly 100g of fine powder was extracted in Soxhelt apparatus with n-hexane, followed by CCL4 and methyl alcohol, siquincelly. The three crude extracts were bioassayed against three strains of bacteria, individually (*Bacillus subtlus, Shegilla sonei* and *Streptococcus pyogenous*).methanolic extract was the most active against the three tested bacteria, particularly S. pyogenus.

Fractionation:

Methanolic extract was fractionated into 5 fractions using a separator funnel according to method was described by **Al Kofahi, et al., (1996).** Bioassay test proved that first fraction (F_1) has the most inhibitory effect against the tested bacteria. Chromatographic separation using TLC was carried out for F_1 fraction using an optimal solvent system (chloroform/methanol/water, 9:1:1) according to the method of **Stahl (1972).**Fourcompounds are appeared on TLC. Each compound was bioassay, the major and most effective one was the third compound (C_3). Purification and determination was carried out for the active compound. Inhibitory effect of different concentrations (50, 100,150,and 200 Ug/ml) was carried out against the same tested bacteria using width zone method that that described by **Jain and Kar (1971)**and compared to artificial antibiotic (cursafe) to evaluate the relative percent of antibacterial activity, particularly against *S.pyogenous*.

Characterization and identification of the active compound:

The chemical structure of the purified active (C3)compound was studied by elementary analysis (C,H,N) and spectroscopic analysis (UV,IR,MS and H1-NMR). These measurements were done in the central laboratory, Faculty of science, Cairo University.

Statistical analysis:

It was conducted according to the procedure outline by **Steel and Torrie (1981)** by using Duncan's rang test as described by **Gomez and Comes (1984).**

RESULTS AND DISCUSSION

Harmal (*piganum harmala* L.) is represented a medicinal plant because it's content a large scale of alkaloids and phenolic substances that have highly antibacterial activity against large scale of pathogenic microorganisms **(Al Sharma, et al., 1981; Abaza and Asar, 2003; Sanchiata, et al., 2004 and Splettoesser, et al., 2005).**

Most investigations have been carried out on harmal seeds, little investigations have been reported on the natural substances that located in harmal leaves.

However, extraction of natural products from harmal leaves by using several solvents indicated that crude methanolic extract was the major amount (2.61%) as dry weight bases and the most effective against the tested bacteria particularly S.pyogenous (16.0± 0mm).

FRACTIONATION OF METHANOLIC EXTRACT :

Fractionation of the active methanolic extract was conducted by the method was mentioned previously indicated the presence of 5 fractions. Bioassay against the tested bacteria (table 1) showed the presence of four compound (C_1, C_2, C_3 and C_4), bioassay test proved that the major and most active one was the third compound (C_3) that has RF value (0.55) against all tested bacteria, particularly S.pyogenous (18±2 mm). Therefore, this compound was eluted and purified. Different doses from the purified active compound (3) are used 50, 100, 150and 200 Ug/ ml and bioassayed against the three tested bacteria, individually with comparing to cursafe as artificial antibiotic in dose (200 Ug/ ml). Relative percentages of the inhibitory effect (Table 3) showed that the third and last dose (150 and 200 Ug) were the most active and its relative percentages 72.8 and 73.8 % against S.pyogenous, respectively (Table 3).

Table (1): Inhibitory effect of methanolic fractions againstthe tested bacteria.

Fractions	Width zone (mm)					
	B.subtilus mm I		*S.sonnei* mm I		*S.pyogenus* mm I	
Control	---	---	---	---	---	---
F1	12±2	++	9±2	+	18±2	+++
F2	---	---	---	---	---	--
F3	---	---	---	---	---	--
F4	7±1	+	8±1	+	11±1	++
F5	---	---	---	---	7±0	+

All measurement were done in triplicates

I = Inhibition zone,
(+)= less than 10mm,
(++)= from 10 to 15mm,
(+++)= more than 15mm,
(---)= No active

Table(2): Inhibitory effect of the purified compoundsseparated from (F_1) against the tested microorganisms.

Compounds	***R_f***	Width zone (mm)					
		B.subtilus mm I		*S.sonnei* mm I		*S.pyogenus* mm I	
Control	---	---	---	---	---	---	---
C1	0.23	---	---	---	---	7±1	+
C2	0.36	8±1	+	7±1	+	11±2	++
C3	0.55	13±2	++	10±1	++	20±1	+++
C4	0.62	---	---	----	---	---	---

All measurement were done in triplicates at dose of 50µg/ml
I = Inhibition,
(+) = less than 10mm,
(++) =from 10 to 15mm,
(+++) = more than 15mm,
(---) = no active

Table (5): Means of relative percentages of antibacterial activity at different doses for purified active compound (C_3) compared to cursafe as standard against the tested Bacteria.

Doses (µg/ml)	**B.subtilus**		**S.sonnei**		*S.pyogenus*	
	Mean/ mm R.%		**Mean/ mm**	**R.%**	**Mean/mm.**	**R.%**
Control	-----	-----	-----	----	-----	-----
50	12.667 I	42.72	10.000 J	34.48	20.000 DE	58.25
100	15.333 H	51.69	12.000 I J	41.38	22.000 D	64.07
150	18.333 EFG	61.80	16.000 J H	55.17	25.000 C	72.81
200	19.000 EF	64.06	16.667FGH	57.45	25.333 C	73.79
Cursafe(200)	29.667 B	100	29.000 B	100	34.333 A	100

1- Cursafe = artificial antibiotic.

2- R.% = Relative percentages compared with cursafe inhibition.

3- L.S.D 5% for concentration within columns. (A) = 1.3

4- L.S.D5%for bacteria within raws(B) = 1.041

5- L.S.D(AB) = 2.

However, similar results have been reported by Siddiqui, et al., (1987); Wang, et al., (1997), El-Sayed, et al., (2003) and Monsef, et al., (2004). It is interest to indicate that some natural compounds extracted from harmal leaves has potent antibacterial activity and this inhibition increased with the increasing of doses (Nychas,1995).

Chemical structure of the active compound:

1- Elemental analysis:

The results showed that C, H, N, O, S atoms are 16.7 , 20.7 , 3.12 , 6.5 and 1.0, respectively. Thus the empirical formula is C17 H21 N3 O7S for the active compound. The active compound contains a number of 9 unsaturated centers (4.5 Π bond) with colorless fine needle crystals, mp95^{O}C and (α) d^{t} = -39.3 CC, ..82, $CHCl_3$ – MeOH (1:1), Rf = 0,46 in solvent system (benzene / ethylacetate – 1:7) and MW = 411.

2- **Spectroscopic analysis :**

UV spectrum :

Data showed that the presence 4 absorption maximum bands = at 232nm, characteristic of dihydropyridinopyrrol ring A / B or D / B; at 262 nm. Characteristic of (C-S) bond; 267 nm. Characteristic of (C = 0) bond (Scott, 1964).

IR spectrum :

The active compound showed absorption bands at 3416;3024 and 1610 cm^{-1} corresponding to amino group (NH) as secondary amine, another absorption at 3507 cm^{-1}(O – H) ; 2585 cm^{-1} (C-S-C) and 1685 cm^{-1} (6 membered cyclic & lactone). Absorption bands at 2680 and 1757 cm^{-1} corresponding to the presence of β- glycosides linkage.

H_1 – NMR spectrum :

Data of the active compound showed 21 protons in ratio (4 : 3 : 2 : 1 : 1 : 1 : 4 : 1 : 1)eleven chemical shift groups. It shows three protons doublet of doublet (dd) at 4.67 ppm for three secondary amino groups (N_2, N_7 and N_9) respectively it also show four – protons multiplet at 3.84 ppm which could be attributed to two methylene groups (-CH_2-) as a doublet signal indicated the presence of two nitrogen functions on vicinal C_1 / C_3 and C_6 / C_8. the down field shift triplets resonating at 5.38 ppm . assignments to the C_4 and C_5 methene protons (-C-H) vicinal to C_3 and C_6, respectively.

Atta – Ur - Rahman, (1986), spectrum shows another signals indicated the presence of 7 – azo – 2 , 3 , 6 , 7 tetrahydro-β- carboline nucleuos moity **.**

Mass spectrum :

(MS) showed a molecular peak (M^{+}) at m/z 412.47 (m/z 411.3) as determined by high resolution electron impact mass spectrum (HREI – MS). Corresponding to the molecular formula C_{17} H_{21} N_3 O_7 S indicating 9 degrees of unsaturation centers (with 4.5 π bonds).

Eight for these were eventually accounted by the tricyclic alkaloid skelton (A/B ring or D/B ring in β- carboline), and one for aldehydic carbonyl group at C_1 (ring as – S – C), fig (1).

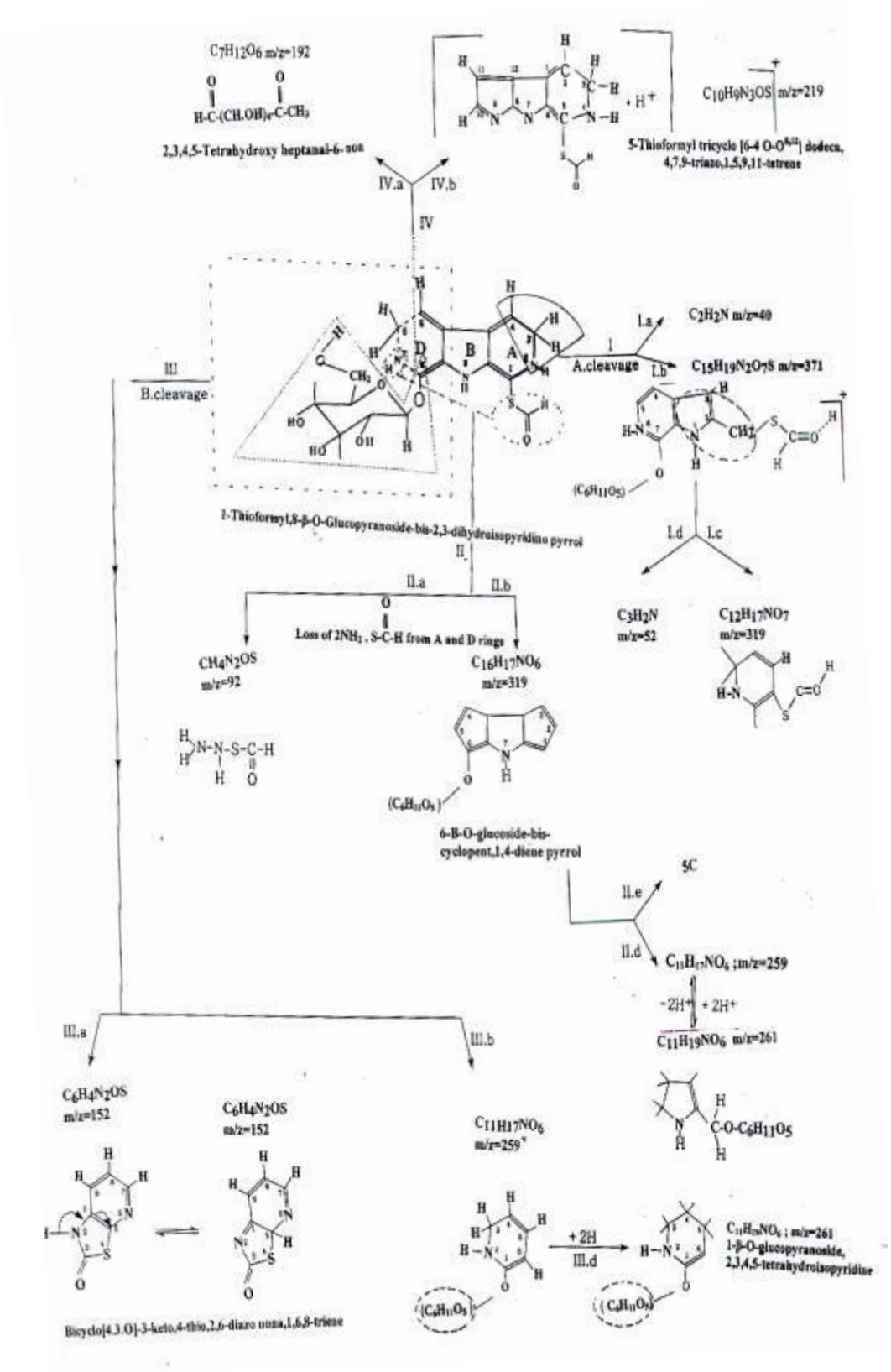
The fragments at m/z (371) $C_{15}H_{19}N_2O_7S$;(319) by loosing of a C_3H_2N; (261) $C_{11}H_{19}NO_6$ after loosing 5C atoms; (192)$C_7H_{12}O_6$ are shown in scheme (1) and(2) indicated of one O and one S and three N functions in rings A,B and D of the β – carboline – 7 azo **(Mckenna and Towers, 1981).** These results indicated the presence of hexose moity was attach to D- ring at position (6) in isopyridine ring or at position (8).

Further evidences for the presence of two dihydroisopyridine rings linkages between C_2/C_3 and C_4/C_5 at the pyrol ring was adduced by the analysis of MS of the active compound. Peaks at m/z 371 (Ib); 319 (I.C); 261 (II.d); 219 (IV.b) and 152 (III.a) in the MS (Scheme I and Table 4) showed the presence of 3 N functions in the rings A,B and D(Bis-2,3-dihydroisopyridino or β- carboline – 7 azo). Moreover, mass fragments at m/z 371, 319 and 219 regulation from the cleavages of rings A and D, respectively was indicative the presence of pyrrol ring. Unambiguously determined the positions of 3 N functions at position 2, 7 and 9 in the tricyclic alkaloids skeleton (Bis- 2, 3-dihydroisopyridinopyrol). The overall mass fragments of this compound (Fig.2) and scheme (1) and (2) was characteristic of the active compound (Siddiqui, et al., 1987

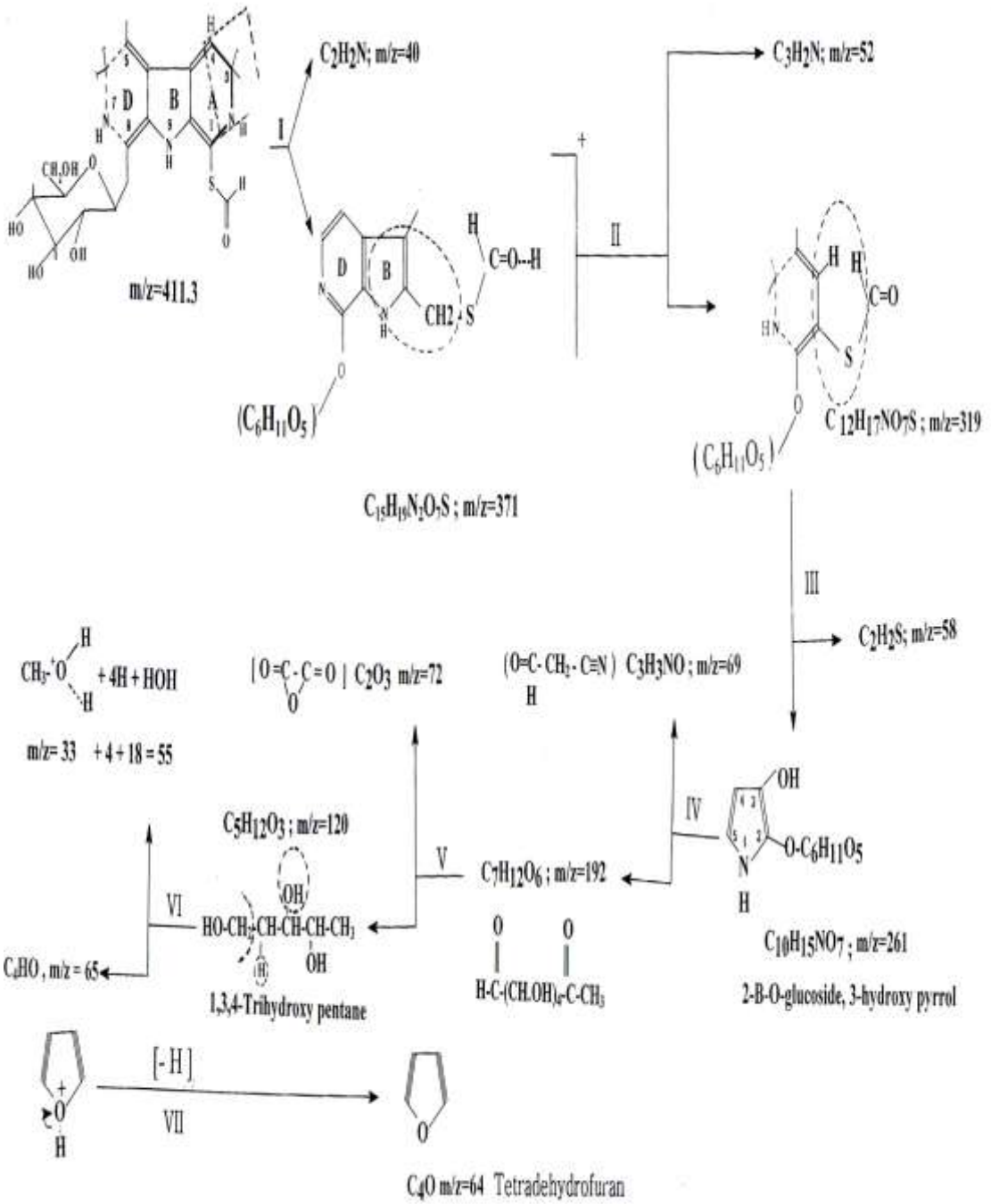
Table (4) **Table (8): The Mass ions of the sequential fragmentation of the antimicrobial active compound**

Mass	Int(%)	Mass	Int(%)	Mass	Int(%)	Mass	Int(%)
50	12.0	51	16.5	52	11.3	53	16.8
54	7.8	55	31.2	56	13.7	57	4.3
58	5.0	59	6.5	60	14.0	61	3.7
62	8.1	63	10.0	64	10.4	65	37.2
66	10.4	67	8.9	68	6.7	69	3.9
70	12.2	71	10.9	72	9.1	73	12.8
74	5.0	75	4.1	76	4.1	77	17.2
78	7.6	79	7.4	80	5.7	81	7.4
82	7.6	84	3.9	85	9.4	86	6.3
87	9.4	88	1.8	89	6.7	90	5.7
91	7.8	92	7.8	93	15.5	94	8.7
95	8.5	96	4.6	98	4.6	99	5.4
100	8.5	101	4.4	102	7.0	103	3.0
105	7.9	106	12.9	107	22.0	108	8.9
109	2.0	110	2.6	111	4.8	112	7.4
113	7.4	114	10.4	115	5.7	116	12.0
117	4.4	118	8.9	119	11.5	120	100.0
121	81.7	122	27.2	123	9.4	124	5.9
125	4.1	126	3.1	127	9.8	128	4.1
129	16.5	130	7.6	131	10.4	132	4.4
133	5.5	134	25.7	135	9.4	137	1.7
138	6.5	139	5.7	141	9.1	142	7.4
143	6.7	144	4.3	145	8.1	146	8.9
147	5.9	148	21.4	149	6.1	150	2.2
151	2.0	152	4.4	156	8.7	158	7.2
159	7.2	160	3.3	161	4.4	162	12.2
163	14.8	164	13.5	165	3.7	170	20.3
171	12.8	172	4.4	173	5.7	174	4.3
177	20.5	178	4.6	179	3.0	190	2.8
186	8.3	187	3.9	188	3.3	189	6.1
190	8.1	191	13.1	192	48.2	193	8.5
196	4.3	202	8.1	203	3.9	204	12.4
205	14.6	206	3.9	207	1.8	212	2.8
213	12.9	214	3.7	215	6.3	217	3.1
218	9.1	228	4.4	229	1.1	232	19.2
233	15.2	234	7.0	235	3.1	256	6.3
257	19.8	259	4.1	260	11.8	261	41.4
262	10.5	263	4.6	264	3.1	276	5.9
277	4.4	285	5.4	304	8.3	316	5.5
317	6.5	319	90.8	320	19.4	321	8.5
371	10.4	372	5.0				

Scheme (1): Proposed pathways of the fragmentation of the antimicrobial active compound.



Scheme (2): Proposed pathways of the sequentialfragmentation of the antibacterial active compound



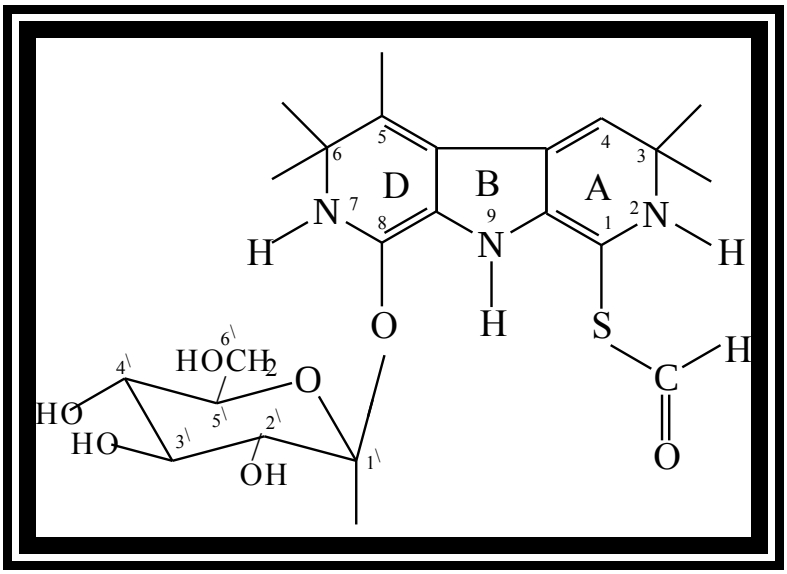


Fig (2): The suggested structure of the antimicrobial activecompound

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دراسات كيميائية حيوية على بعض المركبات النتروجينية ذات النشاط الحيوي

هشام جمعة ، صلاح عبد القادر ، محمد مصطفى السيد ، عصام الملط ، عماد شاكر

قسم الكيمياء الحيوية – كلية الزراعة – جامعة المنيا.

بعض المركبات النتروجينية الطبيعية ذات النشاط الحيوي مثل القلويدات منتشرة في عديد من النباتات البرية والطبية واحد هذه النباتات هو الحرمل البري الذي ينمو بكثرة بالساحل الشمالي بمحافظة مطروح . عموما معظم البحوث السابقة تشمل المركبات المستخلصة من الاجزاء الهوائية للنبات (الاوراق) لذلك كان الغرض هو فصل مركبات طبيعية ذات نشاط مضاد لبعض الميكروبات المرضية من اوراق الحرمل.

تم استخلاص مركبات طبيعية بمذيبات عضوية متدرجة القطبية كل على حدى وكان اكثر المستخلصات كمضاد بكتيري هو المستخلص الميثانولي. وامكن تفريده بالفصل الكروماتوجرافي على TLC باستخدام انظمة مذيبات وكان افضلها (كلوروفورم / ميثانول / ماء 9/1/1) وامكن الحصول على 4 مركبات تمت دراسة حيوية على الميكروبات المختلفة كل على حدى وكان المركب الثالث (C3) هو اشدها نشاط مضاد للميكروبات خاصة ميكروب S.pyogenous لذلك تم تنقيته ودراسة تركيبيه الكيميائي.

اثبتت دراسة التحليل العنصري (C, H, N) ان التركيب الاولي للمركب C17H21N3O7S واثبتت دراسة التحليل الطيفي بوسائل (UV, IR, MS, H1-NMR) ان التركيب الكيميائي المقترح هو

1- Thioformyl,8-B-O-Glucopyranoside-bis dihydroisopyridinopyrrol / or 1-Thioformyl,8-B-O-D- glucopyranoside - carboline-7-azo.

وهو مركب القلويدي مشتق من البيتاكربولين وهو مركب جديد تماما وممكن استخدامه كمضاد حيوي طبيعي .