

Evaluation of therapeutic effects of vicine against *leishmania donovani*. in vitro

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Abstract: During study isolated vicine from *Hibiscus sabdariffa* and *Helianthus annus*, identified by 13 chemical test, UV and IR spectral analysis techniques beside of determined melting point for it. Also tested purified it by thin layer chromatography and estimated activity as antileishmaniasis by killing promastigote of *Leishmania donovani* in vitro. Prepared five concentrations of vicine as followed : (1 gm / ml, 0.5 gm / ml, 0.25 gm / ml, 0.125 gm / ml, 0.05 gm / ml) the results showed the vicine which extracted from *H.sabdariffa* more activity in killing of promastigote compared with vicine which extracted from *H.annus*. So minimum rat for numbers of living promastigote reached (0) in fourth day at 1gm/ml by effect vicine which extracted from *H.sabdariffa* while maximum rat reached 3025 in first day at 0.05 gm / ml where as the vicine which extracted from *H.annus* appeared minimum rat for numbers of living promastigote 100 at 1 gm / ml while maximum rat reached 5350 in first day at 0.05 gm / ml. Tested cytotoxicity of vicine against red blood cells and the results appeared no toxic against it.

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1- Introduction

During the evolution of humans abroad set of parasites have evolved, that use us a host organism. usually a large group of parasites that use humans as a host them. Usually parasites will not kill the host (at least not immediately) but weakened his health and eventually lead his life to die. Anyway some parasitic infections such as infections caused by a parasite in malaria and leishmaniasis are responsible for 1.2 billion of dangerous infections that lead ultimately to the death of millions of people each year (Wink, 2012).

Leishmaniasis is caused by disease genus of flagellate protozoa named *Leishmania* sp. a factor insect sand fly *Phlebotomus* sp. And which are often transported pathogenic factor by serious injuries and fatal lead human life (Durrani *et al*, 2012)

It was noted in the recent increase in the incidence of the disease in many parts of the world due to the emergence of new strains of chemical drug-resistant parasite used him like Pentamidine, Pentosam And Amphotericine B Or or that chemicals drugs do not work immediately on parasite (Temponeet *al*, 2011).

So used of therapeutic alternatives for the treatment of leishmaniasis particularly glycosides and effectiveness toward parasites including *Leishmania donovani* increased in the recently (Bhutani And Gohil, 2010)

It studies conducted in this field study Jones (2004) Between glycoside vicine Like anaphthalene And 1,4 - nappthoquinone In terms of their work as oxidizing agents affecting the target sites in the cells

of eukaryotic organisms which may assert its influence on statistical leishmaniasis parasite.

While in Pakistan study showed Okokoet *al*, (2013) effect of extract roots for tree *Croton zambesicus* On promastigote of parasite *Leishmania major* in vitro where he showed the effectiveness of high extract because it contains many active compounds alkaloides and glycosides.

So that many promising results of research suggest the possibility of obtaining compounds effective to kill parasites special from medicinal plants therefore present study aimed to isolate and diagnosed compound vicine from *Hibiscus sabdariffa* flowers and *Helianthus annus* seeds and test its ability to kill or inhibit the *Leishmania donovani* in vitro.

2- Materials and methods**Prepare glycosides extract**

Were extracted and isolated vicine glycosides according to the method Gayon (1972).

Chemical tests for vicine glycoside

Made several chemical tests to identify quality chemical compound vicine according to (Adedayo, 2001; Harborn, 1984; Trease and Evans, 1973)

Isolation of vicine

Separate vicine from glycosides extract sunflower seeds and flowers *Hibiscus sabdariffa* Using thin layer chromatography and eleuent system (Butanol: Acetic acid: Water) By ratio (4:1:7). (Harborn, 1984).

Identified vicine

Measured melting point of vicine, Ignition and Solubility. (Antrykin and Keroniz, 1983).

Infrared spectra

Infrared spectra recorded discs technology potassium bromide (KBr discs) In the area between (4000-500) cm^{-1}

Electronic spectra

These spectra recorded in the ultraviolet region. Ethanol used for the measurements in this region and the concentrations used were 0.005 gm / 5 mL solvent. (Schwedt, 1997).

Breeding *L. donovani*

parasite Isolates were obtained *L. donovani* Leishmaniasis Centre at the University of Al-Nahrin and planted on the center NNN-Medium Proportion to Novy-MacNeal-Nicolle Biphasic, and attended the liquid medium (Solution Lock) According to the method Meredith *et al* (1995) He also prepared the solid medium, depending on how Kang And Norman (1970).

Using optical microscope unusual for detecting the presence of the parasite and then added vicine compound from *Hibiscus sabdariffa* L. totals for the (1, 2, 3, 4, 5) different concentrations (0.05, 0.125, 0.25, 0.5, 1) gm/ml, respectively, while the other groups were treated (6, 7, 8, 9, 10) by vicine which extracted from sunflower *Helianthus annuus* different concentrations (0.05, 0.125, 0.25, 0.5, 1) gm / ml respectively with taking control sample contain vicine by 3 replications for each group. And 24 hours after parasite numbers were calculated daily for four days.

Cellular toxicity test for vicine

Red blood cells were used to humans to determine the cytotoxicity of vicine and in accordance with the method of (Xian - guo And Urasella, 1994)

Statistical analysis

Statistical analysis was performed using the variance test results Analysis of Variance test (ANOVA test) And use less significant difference in the rate of (Revised Least Significant Difference) RLSD To compare the averages (Narrator and Khalaf Allah, 2000)

3- Results and discussion

Identified compound vicine

Conducted qualitative tests on the vicine and appointed some initial tests for them such as Ignition detection and solubility, as shown in the table (1).

Observed that the compound Give revealed negative for to flavonides, tannines alkaloids saponin and positive Of phenols and glycosides Table (1) and the advantage of these compound contain hydroxyl group (OH) One or a lot (Gayon, 1972).

Table (1) qualitative and some preliminary tests of vicine compound

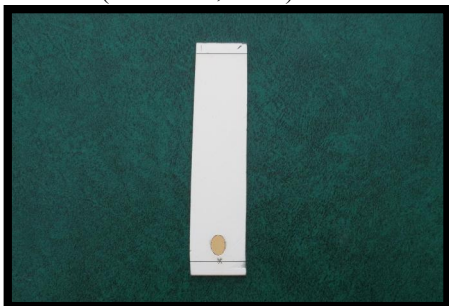
Series.	Qualitative statements	Vicine
1	Detect iodine I2	Yellow
2	Color to the naked eye	Pale yellow
3	Detection of alkaloids	-
4	Detection glycosides	
A	Before decomposition	+
B	After decomposition	+
5	Detection saponins	-
6	Disclosure of flavonoids	-
7	Detection tannins	-
8	Detect Ninhydrine 1%	-
9	Detection of phenols	+
10	Statements unsaturated	
A	Detection of bromine	+
11	Detection of oxygen	+
12	Fusion with sodium	
A	Detection of sulfur	-
B	Detection Nitrogen	+
13	Some preliminary tests	
A	Ignition test	Burning blue flame With generate smoke White and Carbon retardation Black
B	Solubility revealed	Dissolved in Di methyl Sulfoxide, Water, Butanol, Acetic acid, ethanol, methanol and ether

And assigns the group's existence effective detection positive for oxygen, and detect burning of the compound as generate white smoke when burned and backwardness Carbon a black indicates that the

two installation aromatic unsaturated and burn blue flame a glaucoma shows the vicine contain oxygen in organic composition, as well as the confirmed detection of bromine installation unsaturated him (Criddle And Ellis, 1994) As well as can be seen from table dissolved in polar solvents, which confirms that polar compound.

Thin Layer Chromatography (TLC) To test the purity of the compound

Results showed thin layer chromatography that the active compound is a compound one as a result of the emergence of one spot back glycoside (vicine) and a flow rate R_f (Relative flow) Was (0.25) as shown in the picture (1) and was similar to the compound standard glycoside (vicine), which amounted R_f (0.2). This result enables us to initially say that there is a similarity between vicine isolated and standard (Dutta *et al*, 1981).



Picture 1. Thin layer chromatography of isolated vicine

Identified compound vicine

Melting point

Scores ranged melting point vicine isolated from *Helianthus annus* and *Hibiscus sabdariffa* (154-156) C°. The results were compared with the melting point of the composite index vicine, which amounted to (154) C°. Showed vicine isolated behavior similar to that of the standard through chemical tests as explained (Dutta *et al*, 1981). It possessed vicine isolated over thermal melting reached (154-156) C° its similar standard vicine. Also showed solubility in both water, methanol, ethanol, dimethylsulfoxide, and dissolve in the ether.

Infrared spectrum vicine

Record the infrared spectrum of the compound vicine isolated and standard table (2) and Figure (1,2) shows absorption spectra resulting bands and synthetic aggregates belonging (Silverstein *et al*, 1991).

Explain the infrared spectrum of the vicine isolated from *Helianthus annus* and *Hibiscus sabdariffa* As shown in the table (2) (Figure 1). Depending on (Silverstein *et al*, 1991). Noted a similarity in the basic peaks between vicine standard and vicine isolated (Dutta *et al*, 1918) It appeared

clear and distinctive band at wavelength (3413) cm^{-1} - Back to expand vibration of al amine group ((NH₂ And hydroxyl OH, And show band at Site (2931). And (2960) cm^{-1} - Back- expand vibration bond (-CH) Aliphatic, also appeared vibration band expand bond (C = N). When Site (164 5) cm^{-1} -, And (1296) cm^{-1} - bending Vibration CN, And notes the emergence of a band (COC) Alatherah Asymmetric at the site (1409) cm^{-1} -, Also appeared band (O-CH₂ when the wavelength (1116) cm^{-1} -, Band appeared at the site (1089) cm^{-1} - Of the isolated compound and (1070) cm^{-1} - The standard vicine which Due to bending vibration Group OH And ((NH₂ And (COC) Group Alatherah asymmetric.

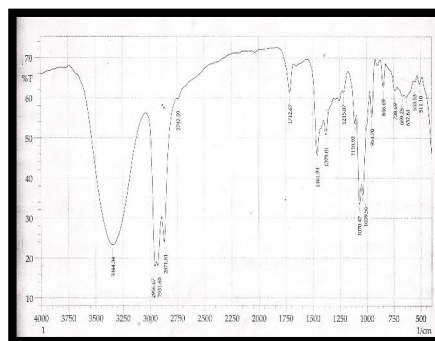


Figure (1): Infrared spectrum of standard vicine (Duta *et al*,1981)

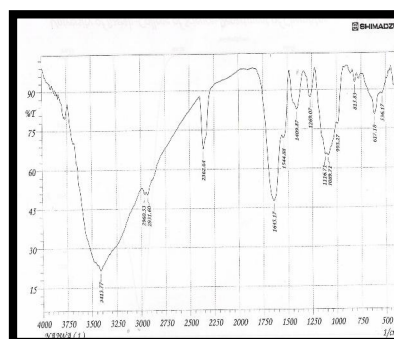
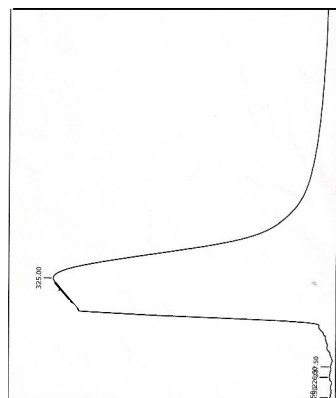


Figure (2): Infrared spectrum of isolated vicine (Duta *et al*,1981)

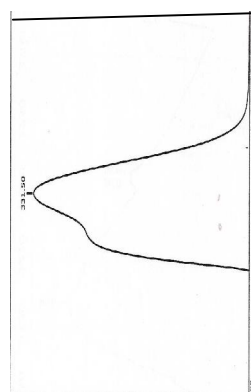
Electronic spectra

Figure (3) Spectrum ultraviolet region vicine isolated from *Hibiscus sabdariffa* and *Helianthus annus* Noting the emergence of absorption band at wavelength 325 nm, Resulting from a type of electronic transitions ($\pi \rightarrow \pi^*$) In the compound and this summit similar to the standard shown by vicine form figure (4) it also noted Lambert *et al* (1987). Existence band absorption wavelength 331 nm belonging to the same type of transitions.



Wave length

Figure(3): Ultra violet spectrum of isolated vicine in ethanol solvent



Wave length

Absorbance

Figure(4): Ultra violet spectrum of standard vicine in ethanol solvent(Dutta et al.1981)

The chemical composition of the conclusion isolate vicine

Depending on the results shown on the basis of chemical and physical tests We conclude that isolate vicine is the same standard vicine *Helianthus annuus* and *Hibiscus sabdariffa* Figure (5).

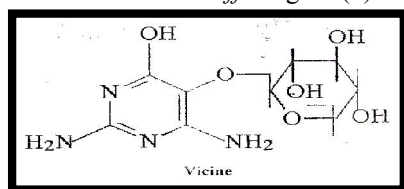


Figure (5): standard vicine

Table (2): Sites most important absorption bands and functional group belonging in infrared spectrum for each of vicine isolated and standard.

Assignment	Band shape and frequency(cm^{-1})	
	Extracted vicine	Standard vicine
O-H Str. vib N-H Str. Vib.	3413 (S)	3344 (S)
C-H Str. Vib.	2960, 2931 (w)	2956, 2931 (W)
C = N Str. Vib	1645 (M)	1 712 (M)
C-N bend. Vib.	1269 (W)	1215 (W)
O-CH ₂ Str. Vib	1116 (M)	1110 (W)
C-O-C Str. Vib	1409 (W)	1461 (M)
C-O-C Bending. Vib. O-H bending. vib. N-H bending. Vib.	1089 (M)	1070 (M)

W = weak m = medium s = strong str = stretching vib = vibration

Test the direct effect of vicine isolated from *Helianthus annuus* and *Hibiscus sabdariffa* On promastigote of *Leishmania donovani* In Vitro

Proved the results of the current study and at the possibility of statistical analysis ($P < 0.05$) The direct impact of vicine of *Hibiscus sabdariffa* Extract On prmastigote *in vitro* and high effectiveness in less number of Promastigote with increasing concentration of the substance as average numbers 1800 at the concentration of 0.05 gm / mL on the fourth day, and decreased numbers of live increase the concentration of up to 100% kill rate concentration at 1 gm / ml for the same period, and vicine was extracted from *Hibiscus sabdariffa* More effective than vicine which extracted from *Helianthus annuus* the elimination of this phase as the average numbers 2350 for the same concentration and the period. (Table 3 and 4).

Unfortunately infections caused by endoparasites Like parasite *Leishmania sp.* Can hardly be prevented by vaccination manufactured chemically because parasites eukaryotic organism so involved often with characteristics biochemical and molecular of its hosts, making is treated very difficult and to find drug against the parasite must be influential on the parasite and nontoxic to humans. (Wink, 2012).

So researchers resorted to medicinal plants as produce secondary metabolites with high diversity with presenting them effectiveness as anti parasites and these drugs often affecting the target sites in the parasite, such as the RNA And DNA Where processes occur alkylation or intercalation with it and affect the integrity of cell membranes, microtubules, transfers nerve signals and the synthesis of proteins. (Wink And Schimmer, 2010).

Table (3) Effect vicine of *Hibiscus sabdariffa* on the number average of promastigote *in vitro*

concentration gm/ ml	vicine of <i>Hibiscus sabdariffa</i>			
	After a day	After two days	After three days	After four days
0.05	3025	2325	1950	1800
0.125	2100	1725	950	725
0.25	1300	975	700	325
0.5	750	525	300	125
1	0	0	0	0
Control	7750	8225	13,775	20,075

Table (4) Effect vicine of *Helianthus annuus* on the number average of promastigote *in vitro*

concentration gm/ ml	vicine of <i>Helianthus annuus</i>			
	After a day	After two days	After three days	After four days
0.05	5350	4075	3300	2350
0.125	4425	3525	2800	1750
0.25	2600	2000	1500	875
0.5	1500	900	500	250
1	350	225	150	100
Control	7750	8225	13,775	20,075

RLSD of vicine: 88.5, RLSD of concentrations: 153.3, RLSD of days: 125.1.

In the current study Caused Treatment promastigote with vicine which extracted from *H. sabdariffa in vitro* decline ratio of Promastigote Living with increasing concentration of the substance as living numbers rats reached to (3025, 2100, 1300, 750, 0) at concentrations (0.05, 0.125, 0.25, 0.5, 0.1) gm/ml for the same concentrations, and was vicine extracted from *H. sabdariffa* More effective than vicine extracted of *H. annuus* in eliminating this phase as rates were prepared live on the first day (5350, 4425, 2600, 1500, 350) and the fourth day (2350, 1750, 875, 250, 100) Same concentrations can explain that to the likelihood that vicine extracted from *H. sabdariffa* Despite appearances spot one in thin layer chromatography but it may contain the isotopes of the same compound as aconovicine and Alcanavicine which can not be detected using only by using Nuclear Magnetic Resonance spectrum, which gives the chemical composition exactly until the chemical isomers which can not be detected by thin layer chromatography, electronic spectra and infrared. (Antrykin and Keroniz, 1983).

And that made it has more effect on the parasite through the phenotypic variations observed on promastigote phase marked by Loss of the whip and change the shape of the spindle to spherical and small size, as well as topical movement vibrational. Could return the reason to vicine link with tubulin heterodimers. And inhibiting an polymerized tubulin Dissuade cellular replication in microtubules. And thus affected the process of doubling DNA, Parasite protein synthesis and leading to cell block fed and growth, small size, and thus to the death of the parasite (Wink, 2007). While the vicine which extracted from

H.annuus less effective and possibly due to contain it only vicine as pointed.

So numbers rate differences in the effect of vicine extracted from both plants during the study for the parasite.

And vicine glycoside compound its part glycane linked to water-hydrophilic and hydrophobic part aglycan and glycane part remains at glycoside surface which facilitates adsorbed and stuck on the surface of the parasite cells and then cause inhibition and the block of the enzyme DNA topoisomerases I, II And which is one of the key enzymes operations doubled DNA Therefore, the cell will not be divided, which makes the cell die programmer or the so-called apoptosis (Medda *et al*, 2012)

Okokon *et al* (2013) noted that some glycosides their ability to form effective oxidizing materials which can result in the generation of kinds of Reactive Oxygen Species (ROS) Such as peroxides free radicals and peroxide And generate ROS Makes the boat has a tendency to break down the cell wall and the installation of the membrane bio-organism through oxidative stress metadata for fat cell membrane of the parasite, leading to loss of integrative membrane and transmission and this results in the loss of ions and vital components intracellular of the organism and this causes the death of the parasite

Or may be caused effect vicine on leishmaniasis parasite because of being aromatic compound can e that interferes with the pairs of nucleotides in particular between pair cytosine and Guanine cause points, deletions and frame shift mutations then death of the parasite because impair the replication process of DNA. (Wink, 2012)

Toxicity testing of vicine has shown no toxicity towards red blood cells Because of the new drug must be more effective and less toxic

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