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# Evaluation of Some Biological Activities of *Phialosimplex asmahalo*

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## ABSTRACT

*Phialosimplex asmahalo* is an obligate halophilic fungus that requires at least 5 % (w/v) sodium chloride for growth. It was previously isolated and identified. The ethyl acetate extract of *Phialosimplex asmahalo* was fractionated and three compounds 1, 3, and 6 were purified. In this study pure compounds of *Phialosimplex asmahalo* were evaluated for their anti-diabetes, anti-inflammatory, antiviral and hepatoprotective activities. Compounds 3 and 6 have anti-diabetic activities with $IC_{50}$ 47.03 and 82.3 respectively; whereas compound 1 has no anti-diabetic activity. Compounds 3 and 6 have anti-inflammatory activities with $IC_{50}$ 125 and 294 respectively; whereas compound 1 has no anti-inflammatory activity. Compound 3 has antiviral activity against Hepatitis A virus and Herpes Simplex type 1 virus in a percentage of 24.7 and 10.2 respectively. Compound 6 has antiviral activity against Hepatitis A only with a percentage of 11.3. Compound 1 has no antiviral activity against Hepatitis A virus and Herpes Simplex type 1 virus. Compounds 1 and 3 have hepatoprotective activities percentage 7.1 and 23.6 respectively; whereas compound 6 has no hepatoprotective activity.

## INTRODUCTION

Halophiles are organisms represented by archaea, bacteria, and eukarya for which the main characteristic is their salinity requirement, halophilic "salt-loving". Halophilic microorganisms constitute the natural microbial communities of hypersaline ecosystems, which are widely distributed around the world (Oren, 2008)

The general features of halophilic microorganisms are the low nutritional requirements and resistance to high concentrations of salt with the capacity to balance the osmotic pressure of the environment (Ventosa *et al*., 1998).

The discovery of new compounds from halophilic and halotolerant microorganisms has become increasingly important in natural products research (Quadri *et al*., 2016; Chen *et al*., 2010; Mazguene *et al.*, 2018; Chen *et al*., 2019; Tang *et al*., 2019) as these microorganisms represent reservoirs of novel bioactive metabolites with diverse groups of chemical structures (Karthikeyan *et al*., 2018).

Halophiles are the best source of producing bioactive compounds as low water activity induces the production of secondary metabolites (Sepcic *et al*., 2010).

Halophilic microorganisms, in particular, are being seen as a source of novel bioactive compounds (Waditee-Sirisattha *et al.,* 2016; Díaz-Cárdenas *et al.,* 2017).

Bioactive secondary metabolites are part of the natural products defined as "small chemical (carbon) compounds isolated from different living organisms" (Berdy, 2005).

Bioactive compounds are produced by almost all living forms, including prokaryotes (bacteria and cyanobacteria) and eukaryotes (fungi, plants, and animals), and possess different activities such as antimicrobial, cytotoxic, anti-inflammatory, antioxidant, and anti-allergic. The bioactive compounds bioprospecting from natural sources could provide a solution or lead to the development and discovery of new drugs. As the second diverse organisms on Earth, fungi are believed to exceed the terrestrial plants in order of magnitude (3–5 million species) (Blackwell, 2011).

The vast majority of secondary metabolites produced by microbes that have been developed for controlling microbial infections are directed against bacterial and fungal infections but not against viral infections. To date, antiviral agents have been isolated from natural products that are limited, and the studies in this field are few (Raveh *et al.*, 2013). The antiviral agents available commercially in pharmacological markets are over 40 compounds, including those being tested as promising antiviral agents or alternative antiviral medicines (Yasuhara-Bell and Lu, 2010).

## MATERIALS AND METHODS

**Organism:**

***Phialosimplex asmahalo*** was previously isolated, identified and its ethyl acetate extract was fractionated to obtain purified active compounds.

**Compound 1**: the expected molecular formula for the compound is $C_{18}H_{21}N_5O$ and the suggested chemical name is (Dimethylamino) benzylidenehydrazino-4-methoxymethyl-6-methyl-3-pyridiecarbonitrile. (Hydrazine derivatives)

**Compound 3**: the expected molecular formula for the compound (3) is $C_{13}H_{16}N_2O_5$ and the suggested chemical name is methyl[N-(salicyl)-3-amidopropanoyl]-aminoacetate. = Phenolics.

**Compound 6**: the expected molecular formula is $C_{34}H_{31}ClN_2O_3$ and named as proposed: 9'-[9H]xanthen]-3-one, 2'-chloro-3' (cyclohexylmethylamino) -6'-methyl-7'-(phenylamino)- Spiro[isobenzofuran-1(3H) = Spiroisobenzofuran Group (Ragab *et al.,* 2015).

**Medium Used:**

**Malt Extract Liquid (ME) Medium:**

This medium consists of (g/L); malt extract, 20; peptone, 1; glucose, 20; distilled water1L. pH was adjusted at 7.4 before autoclaving at 121°C. for 20 minutes (Smith and Onions, 1983). The medium was supplemented with 20% NaCl concentration for the optimal growth of fungal isolate. This medium was used for fungal secondary metabolites production.

**Production of Secondary Metabolites:**

For the production of secondary metabolites, MEA liquid medium was used. The medium was distributed into 250-mL Erlenmeyer flasks each containing 100 ml of the medium supplemented with optimal % of salt level supporting growth for each fungus.

The medium was then autoclaved at 121°C for 15 minutes. The medium (100 ml) was inoculated with 1ml spore suspension of the studied fungus and incubated at 28°C for 21 days in static conditions (Elaassar, 2011).

**Extraction of Extracellular Secondary Metabolites:**

For each fungal isolate, after the end of the incubation period, the whole broth (5 L) was filtered through Whattman No.1

filter paper to separate culture filtrate and mycelia. The culture filtrates were mixed with n-hexane in a separating funnel, shaken vigorously well, and left to settle down for at least six hours until complete separation. The hexane layer was separated. The culture filtrate was combined with an equal volume of ethyl acetate in a separating funnel. The aqueous and ethyl acetate phases were separated. The samples were extracted three successive times with ethyl acetate then concentrated by using a rotary evaporator (Buchi RV 4) to dryness and stored at 5°C till tested (Sherif *et al*., 2013).

**Separation and Purification of The Active Metabolites:**

The active extracts among the previously prepared extracellular metabolites extracts were subjected to separation using silica column chromatography. The column (1.5 cm diameter and 50 cm long) was packed with 20 gm silica gel (G100; mesh 63-200 µm) after insertion of the stopper in the tapering lower end of the column. The silica gel was previously activated at 60ºC for 3 h. The extract obtained was fractionated by open column silica chromatography using elution solvents (10-ml volume each) started with ethyl acetate (100%), followed by gradient volume from mixture of ethyl acetate-methanol (90:10 v/v; 80:20 v/v; 70:30 v/v; 60:40 v/v; 50:50 v/v; 40:60 v/v; 30:70 v/v; 20:80 v/v & 10:90 v/v) ended by 100% methanol.

In order to stabilize and equilibrate the bed, the void volume was passed through the column, and then 5ml fractions were collected separately. All fractions were stored at 0-4ºC. Bioactivity was used as a guide for purification hence all fractions were tested for their antimicrobial, antitumor, and antioxidant activities to determine the active ones.

Each active fraction was analyzed by thin-layer chromatography (TLC) to check the purity and then the fractions in which the active compounds were found were collected and evaporated to the dryness then characterized biologically or chemically for structure determination and nomenclature. Some of the collected fractions were combined based on similarities in TLC properties. TLC was performed on aluminum sheet plates precoated with silica gel G-60 ($GF_{254}$, layer thickness 0.2 mm, Merck, Darmstadt, Germany). Fractions having high activity were automatically spotted on TLC plates using CAMMAG LINOMAT 5 application system and the developing processes were carried out with two solvent systems consisting of Chloroform: Methanol (9:1; v/v), Chloroform: Methanol (8:2; v/v), Toluene: Ethyl acetate: Formic acid (TEF 5: 4: 1; v/v/v), 1-Butanol: 1-Propanol: Acetic acid: Water (BPAW 3:1:1:1, v/v/v/v), 1-Propanol: Ethyl acetate (3:1, v/v) and Chloroform: Methanol: Acetonitryl (CMA7: 2:1; v/v) (Younis *et al.,* 2014).

**Evaluation of Anti-Diabetic Activity:**

**In-vitro Antidiabetic Assay: *(α-glucoidase inhibitory activity*)**

α-glucosidase (*Saccharomyces cerevisiae*) and 3, 5, di-nitro salicylic acid (DNS) were purchased from Sigma-Aldrich, Bangalore. P-nitro-phenyl-α-D-glucopyranoside (p-NPG), sodium carbonate ($Na_2CO_3$), sodium dihydrogen phosphate, di-sodium hydrogen phosphate were purchased from Hi-Media

α-glucosidase inhibitory activity of *Berberis vulgaris* subspecies *cicla* L. var. *flavescens* leaves different extracts and fractions was carried out according to the standard method with minor modification (**Shai *et al.,* 2011**). In a 96-well plate, the reaction mixture containing 50 µl phosphate buffers (100 mM, pH = 6. 8), 10 µl alpha-glucosidase (1 U/ml), and 20 µl of varying concentrations of extracts and fractions (1000 to 7.81 µg/mL) was preincubated at 37°C for 15 min. Then, 20 µl P-NPG (5 mM) was added as a substrate and incubated further at 37°C for 20 min. The reaction was stopped by adding 50 µl $Na_2CO_3$ (0.1 M). The absorbance of the released p-nitrophenol was measured at 405

nm using Multiplate Reader. Acarbose at various concentrations 1000 to 7.81 µg/mL) was included as a standard. Without a test, the substance was set up in parallel as control and each experiment was performed in triplicates. The results were expressed as percentage inhibition, which was calculated using the formula, Inhibitory activity (%) = (1 − As/Ac) ×100, where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

The $IC_{50}$ value was defined as the concentration of alpha-glucosidase inhibitor to inhibit 50% of its activity under the assay conditions.

**Evaluation of Anti-Inflammatory Activity (Membrane stabilization%)**

Preparation of erythrocyte suspension: Whole blood was obtained with heparinized syringes from rats through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged for 10 minutes at 3000 g .

Hypotonic solution-induced erythrocyte haemolysis: Membrane stabilizing activity of the samples was assessed using hypotonic solution-induced erythrocyte haemolysis (Shinde *et al*., 1999). The test sample consisted of stock erythrocyte (RBCs) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate-buffered saline (pH 7.4) containing the extract (1000-7.81 µg/ml) or indomethacin. The control sample consisted of 0.5 ml of RBC mixed with hypotonic-buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g. In 96 well plates, the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated according to the modified method described by Shinde *et al*. (1999).

%Inhibition of heamolysis (membrane stabilization%) = 100 x {OD1-OD2/OD1} Where: OD1 = Optical density of hypotonic-buffered saline solution alone OD2 = Optical density of test sample in hypotonic solution.

The IC50 value was defined as the concentration of the sample to inhibit 50% RBCs haemolysis under the assay conditions.

**Evaluation of Anti-Viral Activity:**

The antiviral screening was performed using a cytopathic effect inhibition assay at the Regional Center for Mycology and Biotechnology (RCMB, Al-Azhar University, Cairo, Egypt). This assay was selected to show specific inhibition of a biological function, that is, a cytopathic effect in susceptible mammalian cells (Hu and Hsiung, 1989; Al-Salahi *et al*., 2015). In brief, monolayers of ($2x10^5$ cells/ml) Vero cells adhering at the bottom of the wells in a 96-well microtiter plate were incubated for 24 h at 37 °C in a humidified incubator with 5% CO2. The plates were washed with fresh DMEM and challenged with 104 doses of herpes simplex 1 or 2 viruses, and then the cultures were simultaneously treated with two-fold serial dilutions of the tested compound, starting from 500 µg/mL and going up to about 2 µg/mL (500, 250, 125, 62.5, …, 1.95 µg/mL) in a fresh maintenance medium; following this, they were incubated at 37 °C for 48 h. Infection controls, as well as an untreated Vero cell control, were made in the absence of tested compounds. Six wells were used for each concentration of the tested compound. Antiviral activity was determined by the inhibition of the cytopathic effect compared to a control, that is, the protection offered by the tested compound to the cells was calculated. Three independent experiments were assessed, each containing four replicates per treatment. Amantadine was used as a positive control in this assay system. After the incubation period, the viability of the cells was determined by MTT assay as described before in the cytotoxicity section (Mosman, 1983).

The viral inhibition rate was calculated as follows:

$$[(A - B)/ (C - B)] \times 100\%$$

where A, B, and C indicate the absorbance of the tested compounds with virus-infected cells, the absorbance of the virus control, and the absorbance of the cell control, respectively.

**Evaluation of Hepatoprotective Activity:**

**Principle:**

HepG2 Cell lines are suitable for *in-vitro* model system for the study of polarized human hepatocytes. HepG2 cell line with proper culture conditions displays robust morphological and functional differentiation with a controlled formation of apical and basolateral cell surface domain in models. The HepG2 cells were exposed to toxicant containing 1% CCl4 along with /without a tested sample of different concentrations or the medium alone is considered as control. At the end of the period, cytotoxicity was assessed by estimating the viability of the HepG2 cells by the MTT reduction assay.

**Methods:**

**Cell Line and Growth Media:**

The HepG2 cells of the human liver cell line were cultured in DMEM (Dulbecco's modified eagle's medium) contains 10% fetal calf serum, penicillin (100 U), and streptomycin (100µg).

**The Hepatoprotective Effect in HepG2 Cell Line Estimated by MTT Assay:**

The monolayer cell culture was trypsinated and the cell count was adjusted to 1.0 $x10^5$ cells/mL using a medium containing 10% newborn calf serum. To each well of the 96-well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once, and 100µL samples with various drug concentrations were added to cells in wells of the microtitre plate. The plate was then incubated at 37°C in 5% CO2 atmosphere for 24 h.

**Experimental Design:**

Human liver HepG2 cells were exposed to a medium containing CCl4 (1%) with/without various concentrations from the tested compounds (6.25, 12.5, 25, 50,100, and 200 µg/mL). Then, cytotoxicity was assessed by estimating the viability of HepG2 cells by MTT reduction assay. The experimental groups were as follows:

Group 1: Control, untreated HepG2 cell line;

Group 2: HepG2 cells with 1% $CCl_4$

Group 3: HepG2 cells with 1% CCl4 and tested compound

Group 4: HepG2 cells with 1% CCl4 and silymarin standard drug

Each treatment was repeated four times (i.e. 4 wells for each treatment).

**MTT Assay:**

Following treatment with the abovementioned methods, after 24 h incubation, the medium was removed and 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml; Sigma, St. Louis, CA, USA) was added to each well. The plates were gently shaken then incubated in the dark at 37˚C for an additional 4 h in 5% CO2 atmosphere. The reaction was stopped by the addition of 150 µl DMSO (Sigma) and the absorbance of samples at 570 nm was measured with a microplate reader (SunRise, Tecan, USA).

The tetrazolium salt (3-(4,5–dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide, MTT) is introduced into cells and reduced in a mitochondria-dependent reaction to yield a blue-colored formazan product. The product accumulates within the cell due to the fact that it cannot pass through the plasma membrane. On solubilization of the cells, the product is liberated and can be readily detected and quantified by a simple colorimetric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity, which in turn may be interpreted as a measure of viability and/or the number of cells. The assay has therefore been adopted for use with cultures of exponentially growing cells. Determination of the cell's ability to reduce MTT to the formazan derivative after exposure to test compounds

shows a hepatoprotective effect. The optical density of the formazan formed in the control cells was taken as 100%. The viability of HepG2 cells in other groups was presented as a percentage of the control cells.

**Data Analysis:**

The results were expressed as:

**The percentage of viability** was calculated as **[(ODt/ODc)] x 100%** where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells.

**Hepatoprotective Percent =** % Viability of treatment group – % Viability of negative control**.**

## RESULTS AND DISCUSSION

*Phialosimplex asmahalo* is an obligate halophilic fungus that requires at least 5 % (w/v) sodium chloride for growth. In this study pure compounds of *Phialosimplex asmahalo* were evaluated for their anti-diabetes, anti-inflammatory, antiviral and hepatoprotective activities.

**Evaluation of Anti-Diabetic Activity:**

Three compounds had been submitted for evaluation of their Anti-diabetic activity. As shown in Figure (1) compounds 3 and 6 have anti-diabetic activities with *IC* 50 47.03 and 82.3 respectively. Compound 1 has no anti-diabetic activity.

In contrast, Pandey *et al.* (2013) found that bacteria associated with the marine sponge, *Aka coralliphaga*, produced a large number of glucosidase inhibitors. Also, Imada (2005) reported bioactive compounds from marine actinomycetes *Streptomyces corchorusii* subsp. rhodomarinus with interesting α-amylase inhibition, While, Pavithra *et al*. (2014) concluded that *Stemphylium globuliferumis* one of the best endophytic fungi having both α-amylase inhibition and α-glucosidase inhibition activity.

Kumar *et al*. (2017) concluded that the endophytic extract of, *Penicillium* Sp. has shown potent antidiabetic activity by *in silico* assay.

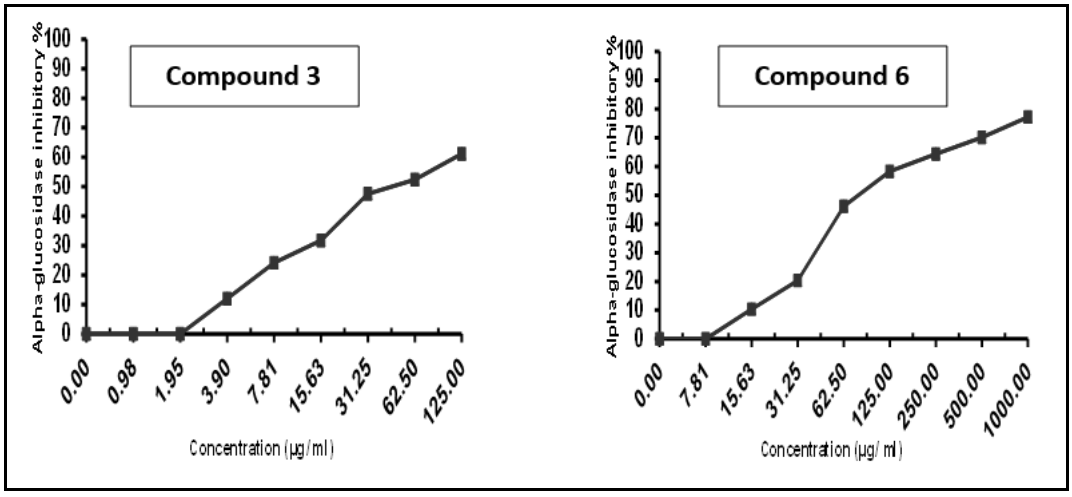


**Fig. 1:** Anti-diabetic activity of *Phialosimplex asmahalo* pure compounds**.**

**Evaluation of Anti-inflammatory Activity:**

Three compounds had been submitted for evaluation of their Anti-inflammatory activity**.** Figure 2 illustrates that compounds 3 and 6 have anti-inflammatory activities with $IC_{50}$ 125 and 294 respectively. While compound 1 has no anti-inflammatory activity.

Maskey *et al.* (2004) found that cyclomarin A is a novel anti-inflammatory agent isolated from halophilic *Streptomyces* sp. It is a cyclic heptapeptide and possesses potent anti-inflammatory activity in both in vivo and in vitro assays.Also,Niu *et al*. (2018) extracted three new guaianes named graphostromanes D, F, and I together with

known ((1R,4S,5S,7S,9R,10S,11R)-guaiane-9,10,11,12-tetraol) (137–140) compounds having anti-inflammatory activity were isolated from deep-sea *Graphostroma* sp. MCCC 3A00421 obtained from the Atlantic Ocean. The compound 139 displayed potent anti-inflammatory activity with an IC50 value of 14.2 μM in LPS-induced RAW264.7macrophages cells, indicating its potential therapeutic effect.

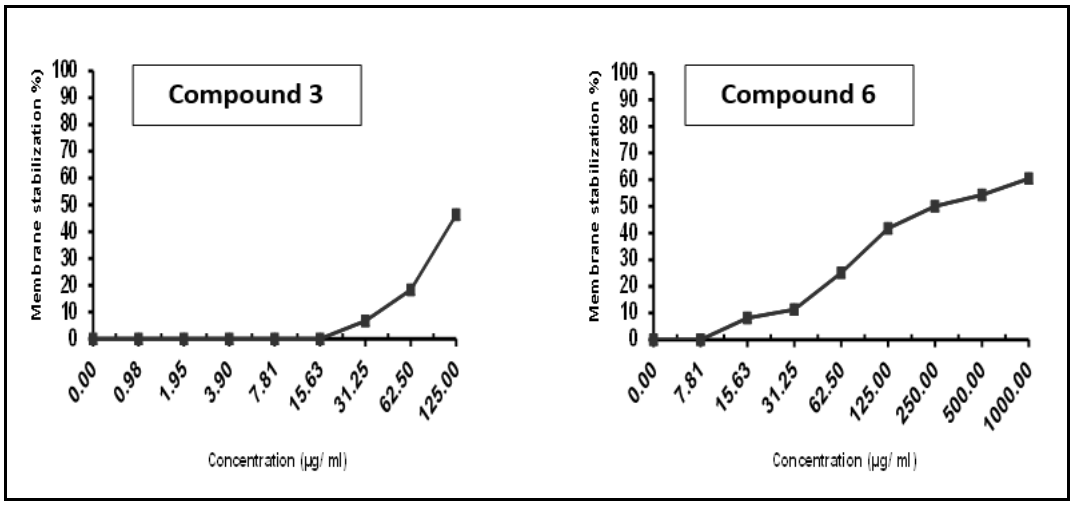


**Fig. 2:** Anti-inflammatory activity of *Phialosimplex asmahalo* pure compounds**.**

**Evaluation of Anti-viral Activity:**

Three compounds had been submitted for evaluation of their antiviral activities against Hepatitis A virus and Herpes Simplex type 1 virus.

Table (1) illustrates the antiviral effects of the tested compounds on HAV and HSV-1 viruses when tested at maximum noncytotoxic conc.

Compound 3 has antiviral activity against Hepatitis A virus and Herpes Simplex type 1 virus in a percentage of 24.7 and 10.2 respectively.

Compound 6 has antiviral activity against Hepatitis A only with a percentage of 11.3.

Compound 1 has no antiviral activity against Hepatitis A virus and Herpes Simplex type 1 virus.

Boyd *et al*. (1997) reported that cyanovirin-N was obtained from the halophilic cyanobacteria *Nostoc ellipsosporum.* Cyanovirin-N has antiviral activity against HIV-1 and HIV-2 by targeting the replication. While, Lee *et al*. (2007) revealed that benzastatin is a 3-chloro-tetrahydroquinolone alkaloid extracted from the halophilic actinomycete *Streptomyces nitrosporeus*). This compound exhibited antiviral activity against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and vesicular stomatitis virus (VSV), respectively, in a dose-dependent manner with $IC_{50}$ values of 1.92, 0.53, and 1.99 µg/mL.

**Table 1:** Antiviral activities of *Phialosimplex asmahalo* pure compounds.

| Sample Code | MNCC (µg/ml) | Antiviral effect (%) | |
|---|---|---|---|
| | | HAV | HSV-1 |
| 1 | 84.2 | 0 | 0 |
| 3 | 60.8 | 24.7 | 10.2 |
| 6 | 127.6 | 11.3 | 0 |

**Evaluation of Hepatoprotective Activity:**

The hepatoprotective activities were tested against HepG-2 intoxicated with carbon tetrachloride. The samples were tested at noncytoxic conc. (200 µg/ml) and the results in table (2) show that compound 1 and 3 have hepatoprotective activities percentage 7.1 and 23.6 respectively. While compound 6 has no hepatoprotective activity.

In contrast, Elaasser *et al*. (2019) revealed that the extract of marine-derived fungus isolated from the Egyptian marine environment exhibited hepatoprotective activity when tested in vitro against intoxicated HepG2 cells.Also, Puri *et al*. (2018) found that A4EA and A4nB of leaf endophytic fungus, *Aspergillus tubingensis strain Cs/7/2* isolated from *Andrographis paniculata* exhibited antioxidant and hepatoprotective activity in $CCl_4$ induced hepatotoxicity**.**

**Table 2:** Hepatoprotective activities of *Phialosimplex asmahalo* pure compounds.

| Sample code | Hepatoprotective effect% |
|---|---|
| 1 | 7.1± 1.5 |
| 3 | 23.6±1.8 |
| 6 | 0 |
| Silymarin (standard drug) | 86.8±2.6 |

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## ARABIC SUMMARY

**تقييم بعض الأنشطة البيولوجية لفطر *فيالوسيمبليكس أسماهالو***

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فيالوسيمبليكس أسماهالو من الفطريات المحبة للملوحة والذي يتطلب على الأقل 5٪ (وزن/حجم) من كلوريد الصوديوم للنمو. تم عزله وتعريفه في دراسة سابقة. تمت تنقية ثلاث مركبات 1 و3 و6 بعد تجزئة راشح الخلايا بواسطة اسيتات الإيثيل. اختبرت هذه المركبات كمضادات لأنشطة كلاً من السكري، الألتهاب، الفيروسات الكبدية. أظهر المركبان 3 و6 أنشطة مضاده للسكري بتركيز مثبط 50 47.03 و82.3 على التوالي، بينما المركب 1 لم يظهر نشاط مضاد لمرض السكري. المركبان 3 و6 كان لديهم أنشطة مضادة للإلتهاب بتركيز مثبط 50 125و 294 على التوالي، بينما المركب 1 ليس له نشاط مضاد للالتهاب. سجل المركب 3 نشاط مضاد لفيروس الالتهاب الكبدي أ وفيروس هيربس سيمبليكس من النوع 1 بنسبة 24.7 و10.2 على التوالي، بينما سجل المركب 6 نشاط مضاد لفيروس الالتهاب الكبد أ فقط بنسبة 11.3. المركب 1 ليس له نشاط مضاد لفيروس الالتهاب الكبدي أ وفيروس هيربس سيمبليكس من النوع 1. هناك أنشطة وقائية للكبد للمركبين 1 و3 بنسبة 7.1 و23.6 على التوالي، بينما المركب 6 لم يظهر هذا النشاط.