EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 10
45-48
Year: 2016

ACTIVITY OF TWO GREEN ALGAE (*ZYGNEMASTELLINUM* AND *HYDRODICTYON RETICULATUM)* EXTRACTS AGAINST SOME PHYTOPATHOGENIC FUNGI

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Article Received on 02/08/2016 **Article Revised on 22/08/2016** **Article Accepted on 12/09/2016**

ABSTRACT
Two species of the fresh water green algae, namely *Zygnema stellinum* and *Hydrodictyon reticulatum* (belong to class Chlorophyta) have been assayed for antifungal against three of phytopathogen *Alternaria alternate*, *Fusarium solani* and *Rhizoctonita solani*. The results showed that the hot chloroform extracts of *Z.stellinum* was more efficiency than the hot chloroform extracts of *H. reticulatum* ,which showed antifungal activity against the species of fungi at concentrations 50mg/ml as percentage inhibition (68.8,66.63 and 58.18%) and (53.71,45.8 and 37.41%) respectively. Primary detection of active compounds showed the *Z. stellinum* extracts containing Tannins, Terpenoid Flavonoids, Phenols and Alkaloids. While *H. reticulatum* containing Terpenoid Flavonoids and Alkaloids only .In this study the Gas Chromatography-Mass Spectrometry was used to know the compounds which responsible of antifungal activity in *Z. stellinum* extracts which had antifungal activity more than *H. reticulatum*

KEYWORDS: Green algae, antifungal, active compound, extracts.

INTRODUCTION
Algae are now days become a very important component of the aquatic ecosystem. They occur in verity of habitats. In recent years, algae are found to be important sources of useful bioactive substances and there are numerous reports of algae derived compounds that have a broad range of biological activities, such as antibiotic, antiviral, antineoplastic, antifouling, anti-inflammatory, cytotoxic and antimitotic.[1,2,3,4]

In this study ,the main reasons for using algal extract as antifungal agents is their natural origin and low chance of pathogens developing resistance.[5,6] The natural products are considered to be saved and less harmful, mainly because they have higher biodegradability and are often active at lower doses.[7] In addition to that , the scientists are looking for new resources which have biological antimicrobial activities with low coast . So, for these reasons the present study was planned to evaluate the antifungal activity of algae extracts against some Phytopathogenic Fungi.

MATERIALS AND METHODS
Collection and Preparation of Sample
Fresh water green algae (*Z. stellinum* and *H. reticulatum*) were blooms manually collected from AL- Gadreia campus of Baghdad University in season (Spring 2016) when the algae were at their peak biomass (0 – 0.5 cm in depth) .The harvested algae were stored in plastic bags for transportation to laboratory and diagnosed according to[8,9,10] Freshly collected algae samples were washed thoroughly to remove metaphyton matter and an un dissolved portion. Then, samples were carefully cleaned from epiphytes and washed several times with tap and distilled water. After the washing process the sample then dried in shade and powdered.

Preparation of alcoholic extract
The powdered samples were used to prepare extract by using soxhelet extraction according to the methods described [11,12].The dried powders form of algae material extracted by using Chloroform. The concentrated active constituents from algae were kept in sterilized test tubes stored in refrigerator till further use and then solved in distal water to get stock solution.

Isolation of pathogenic fungi
Samples of infected tomato and eggplant were collected from local markets; the samples were taken to the laboratory for isolation within 7 days. The piece of the infect tomato and eggplant tissue were put on Petri dishes with potato dextrose agar (PDA) after sterilized and were incubated at 2 ± 28°C for 2–3 days [13]. When mycelia growth was observed, purification was carried out by cutting a small piece of media with mycelia at the edge of a colony and then transplanted onto new medium plates. The fungi (*Alternaria alternate*, *Fusarium solani*

and Rhizoctonita solani) were identified under the microscope for comparison of fungal morphology with descriptions given.[14]

Antifungal Activity Assay

The crude extracts of the algae were mixed with Potato Dextrose Agar (PDA) medium to get concentrations (50) mg/ml and the fungal mycelia were inoculated to grow. Data on the radial growth were recorded. Potato Dextrose agar plates were inoculated with each fungus by placing a 9 mm diameter disc from an actively growing culture in the centre of each plate.[15] Three replicates were used per treatment. Fungi were also grown on non-ameliorated PDA as a control. All fungi were incubated at 25°C for seven days in the dark. Fungal growth (colony diameter) was measured and percentage of inhibition calculated according to the formula:

Percentage inhibition = (C-T) x 100/C

Where,

C = Colony diameter (mm) of the control.

T = Colony diameter (mm) of the test plate.

Percentage inhibition and analysis of variance for the different treatments were calculated.

GC-MS analysis

The algal samples was performed using GC-MS, a high-temperature column (Inert cap 1MS; 30 m × 0.25 mm id × 0.25µm film thickness) was purchased from Agilent Technologies (SHIMADZU—Japan), by employing a high-temperature column. Derivatization of each sample was eliminated. The injector and detector temperatures were set at 280°C while the initial column temperature was set at 100°C. A 5 µL sample volume was injected into the column and ran using split (1:10) mode After 1 min, the oven temperature was raised to 225°C at a ramp rate of 12.5°C/min (hold time 4 min). The oven temperature was then raised to 300°C at a ramp rate of 7.5°C/min (hold time 5 min). The helium carrier gas was programmed to maintain a constant flow rate of 17.5 mL/min and the mass spectra were acquired and processed using both Agilent GC-Mass. Solution (SHIMADZU—Japan) and postrun software. The compounds were identified by comparison of their mass with NIST library search and authentic standards.

Qualitative estimation of active compounds from the (*Z. stellinum* and *H.reticulatum*) specimens

The presence of active compounds of the studied algae as the hot alcoholic extract were determined by adopting standard protocols as following:

1- Terpenoids indicators:

A – Foam test:

This reagent was used for the detection of saponines, bottle contained aqueous or ethanol extract was shacked and then the appearance of big foam for a long time as a result of stirring. The aqueous of macro-algae in test tube indicated Saponins existence.[16]

B – HgCl2 reagent:

This reagent was used for the detection of Saponines by adding 1-2 ml from $HgCl_2$ 1% to 5 ml of aqueous extract then white precipitate was appeared which indicates that the test is positive.[16]

C – Acetic anhydride reagent:

According to[16] 1 ml of the extract was added to 1-2 drops of chloroform, then 1 drop of anhydride acetic acid and then 1 drop of concentrated H2SO4. The appearance of brown colour means the presence of terpens and the appearance after a period of time of black-blue means the presence of steroids.

2 - Alkaloids indicators:

A – Mayer reagent:

This reagent was used for the detection of alkaloids .The stock solution (1) was prepared by dissolving 13.5g HgCl2 in 60 ml water, stock solution (2) was prepared by dissolving 5g KI in 10 ml water, then combined with stock (1) and (2) and diluted with water to 100 ml, then added 1-2 ml of Mayer reagent to 5 ml of aqueous, or ethanol extract was added .A creamy or white precipitate indicates the presence of alkaloids.[17]

B – Tannic acid reagent:

This acid was used to precipitate alkaloids.[16] 1% tannic acid was prepared, then 1-2ml of reagent was added to 5ml of the extract. The white turbidity was appeared.

3 – Phenolic indicators:

A – Lead acetate reagent:

It's aqueous or ethanol solution of lead acetate 1%. Amount of reagent was added to an equal amount of aqueous or ethanol extract then white precipitate will appear which indicates the presence of phenols.[16]

B – Ferric chloride and Potassium ferric cyanide reagent:

It was used for the detection of general phenols. Prepared by 2 equal amounts of aqueous solution of ferric chloride 1% and Potassium ferric cyanide 1% Blue- green colour appeared indicating that the test is positive.[16]

RESULTS AND DISCUSSION

The current study carried out only with the hot chloroform extracts. In present study *R. solani* show the minimum inhibitory effect by using hot chloroform extracts of *H.reticulatuum* (37.41%) while, *A. alternate* show the maximum inhibitory effect by using hot chloroform extracts *of Z. stellinum*a (68.8%) table(1). In generally the hot chloroform extracts of *Z.stellinum* was more efficiency than the hot chloroform extracts of *H. reticulatum*, which showed antifungal activity against the species of fungi at concentrations 50mg/ml as percentage inhibition (68.8,66.63 and 58.18%) and (53.71,45.8 and 37.41%) respectively table (1).

The antifungal activity of *Z. stellinuma* and *H.reticulatuum* crude chloroform are shown in the Table (1).

From above, may be conclude that's hot chloroform extracts of *Z. stellinum* in general have more effect on the activity and growth of fungi comparing to *H.reticulatum* that maybe due to present of different active compound in *Z. stellinum* [18 ,19,20].

Table (1): Mean Percentage inhibition of different fungi at 50 mg/ml concentrations of *Z. stellinuma* and *H.reticulatum* extracts.

	Conc.of algae extract. 50 mg/ml		
Fungi	***Z. stellinum*a**	***H.reticulatuum***	**Control**
A. alternate	**68.8%**	**53.71%**	**0.00**
F. solani	**66.63%**	**45.8%**	**0.00**
R. solani	**58.18%**	**37.41%**	**0.00**

The current results indicate that the primary detection of active compounds showed the *Z. stellinum* extracts containing Tannins, Terpenoid Flavonoids, Phenols and Alkaloids. While (macro-algae *H. reticulatum*) containing Terpenoid Flavonoids and Alkaloids only table(2). This result supports the findings of many authors[21,22] that the phytochemical analysis of the hexane extract of *H.reticulatum* led to the isolation of the known sesquiterpenes.[23]

Table (2): Presence or absence of active compounds in *Z. stellinum* and *H.reticulatum* extracts.

Active compounds	*Z.stellinum*	*H.reticulatum*
Glycosides	-	-
Tannins	+	-
Terpenoid	+	+
Flavonoids	+	+
Phenols	+	-
Saponins	-	-
Alkaloids	+	+

Algae compose a natural source of a variety of drugs for pharmaceutical, food and cosmetic applications including carotenoids, terpenoids, steroids, amino acids, phlorotannins, phenolic compounds, halogenated ketones, alkanes and cyclic polysulphides.[21,22] Therefore, algae have been used in traditional medicine for a long time.[23]

Gas Chromatography-Mass Spectrometry was used to know the volatile compounds which responsible of antifungal activity in *Z.stellinum* extracts .The resulted identification of 8 compounds. Salicylic acid is a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. This colourless crystalline organic acid is widely used in organic synthesis and functions as a plant hormone.[24] It is derived from the metabolism of salicin which found to be a major compound in area (44.7%) from the analysis by GC-Mass in table(3).

Table (3): The major identified compounds of hot crud chloroform extract of *Z.stellinum* by using GC-Mass spectrophotometer

Rt	Compound	Area%
12.98	**Octadecane**	**10.8**
13.78	**Eicosane**	**19.2**
14.11	**Salsalic acid**	**44.7**
16.61	**Tetradecanedihydroxyl**	**4.2**
16.75	**Nonadecane**	**8.3**
18.94	**Hexadecane 2-hydroxyl**	**4.1**
21.77	**Hexadecane**	**2.6**
22.87	**Pentadecane**	**1.8**

Followed by Eicosane (14.22%) which are responsible for antifungal activity because it is a fatty acid., Octadecane (10.8%), Nonadecane (8.3%). In addition to Tetradecanedihydroxyl and Hexadecane 2-hydroxyl (4.2 and 4.1%) respectively. Finally Hexadecane and Pentadecane (2.6 and 1.8%) respectively.

The active compounds contain multi free hydroxilic groups have ability to formation hydrogenic bounds with carbohydrates and proteins which found at cell wall and its inside living cells, or to these phenolic compounds may be linked with active sites of enzymes and change its nature and precipitate the enzymes as complex mixture that's causes inhibition of essential metabolism reactions for reproduction and growth of fungi.[25]

Present of Octadecene, Pentadecane and Hexadecane which found in algae show anticancer, antioxidant and antimicrobial activity.[26,27] Antimicrobially active fatty acids is present in a high concentration in this algae. It was hypothesized by[28] that lipids kill microorganisms by leading to disruption of the cellular membrane as well as bacteria, fungi and yeasts because they can penetrate the extensive meshwork of peptidoglycan in the cell wall without visible changes and reach the bacterial membrane leading to its disintegration. Present investigations is contradictory with the results of other studies[19,20,21,29] may be due to the production of bioactive compounds related to the seasons, method, organic solvents used for extraction of bioactive compounds. Biological activities of this algal could be attributed to chemicals such as 1-Pentadecene and 1-Tetradecene which were present in smaller amounts.[29]

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