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IMPACT OF *Allium sativum* AGAINST *Enterobacter sp.* AS WATER BORNE PATHGENIC BACTERIA ISOLATED FROM RIVER NILE

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ABSTRACT

To explore the antibacterial activities of *Allium sativium* (garlic) extract was tested against two waterborne pathogenic strains isolated from River Nile, **to** mitigate the increase of bacterial resistance to conventional antibiotics. The two isolates were identified as *Enterobacter cloacae* DSM 3264 BRB & *Enterobacter cloacae* MB11506_1CHB by MALDI-Tof-MS. Aqueous, methanolic and oil extraction of garlic were tested for their inhibitory activity against the selected strains using well diffusion method. *Enterobacter* sp. were more sensitive towards oil extract with inhibition zone 2.3 cm rather than aqueous and methanolic extractions with was 50%. Analysis of garlic essential oil by GC-MS dedicated six sulfur compounds represented 25% of total detected compounds in the oil.

Keywords: *Enterobacter cloacae*, Garlic oil, MALDI-Tof-MS, GC-MS spectrometry. *Allium sativum*, Well diffusion method

INTRODUCTION

Garlic plant (*Allium sativum)* is mainly classified as a species member of the family Alliaceae (onions), subfamily Allioideae, and the *Allium* genus. Garlic plant has many biological activities such as anti-fungal, anti-septic and antihistamine. It has been used as traditional remedy for cold, cough and asthma treatment **(Gebreselema and Gebreyohannes 2013)**. As described by **(Palani et al 2014),** *Allium sativum* has a chemical composition of many components; sulfur compounds: allicin, alliin, allylpropyl disulfide, diallyl trisulfide (DATS), S-allylmercaptocysteine, S-allylcysteine (SAC), several enzymes, such as (allinase, myrosinase, catalases, superoxide dismutases, arginases and lipases), amino acids (arginine, glutamic acid, aspartic acid, methionine and threonine), proteins (glutamyl peptides), vitamins (B1, B2, B6, C, Biotin and nicotinic acid), minerals (Selenium, Germanium, Tellurium and other trace minerals), elements, lipids, prostaglandins, fructan, pectin and adenosine.

Early Egyptians used garlic to treat diarrhea and its medical power was drawn on the walls of ancient temples and on papyrus dating to 1500 BC **(Gebreselema and Gebreyohannes 2013)**. The Old Greeks, Hippocrates and Galen Garlic tried garlic to cure intestinal and extra-intestinal disease infections. Ancient Chinese and Japanese used it as headache, flu, sore throat and fever remedies. In Africa, especially in Nigeria, garlic is traditionally used to heal the abdominal infections, diarrhea and respiratory tract infections **[Jaber and Al-Mossawi, 2007 and Gebreselema and Gebreyohannes, 2013].** European and Indian people used it in folk remedies to recover high fever, asthma and common colds. Garlic has a byname as Russian penicillin due tops its large scale utilities as intrinsic antimicrobial agent; it is commonly used in many cultures as an excitement and reputation of healing power **(Timbo et al 2006).** As noted by **Palani et al (2014)** garlic oil extracts had a high antibacterial potential against

both of Gram positive and Gram negative bacteria as well as species of *Escherichia, Klebsiella, Salmonella*, *Proteus*, *Staphylococcus, Streptococcus, Bacillus*, and *Clostridium.* According to **Cellini et al (1996)** and **Palani et al (2014)**, aqueous garlic extract at concentrations of 2–5 mg/ml inhibited the growth of *Helicobacter pylori* clinical isolates from patients suffered from chronic gastritis or duodenal ulcer. **Rubin et al (2012)**, reported that 0.1 ml of 10% (w/v) extract of garlic inhibited the growth of *Staphylococcus aureus* with diameter zone of inhibition ranged from 19.68 – 20.75 mm with mean of 20.22 mm diameter of inhibition zone. **Jan et al (2014)** showed that the mode of action of garlic oils is reverted to the existence of allicin which causes a total inhibition of RNA and DNA synthesis. While **Brij et al (2016)** found that thiosulfinates and other, glutamyl peptides, Scordinins, Steroids, terpenoids, flavonoids and other phenols, have the most inhibitory effect on protein synthesis**.**

MATERIALS AND METHODS

Microorganisms: Two isolates were isolated from River Nile in Egypt namely *Enterobacter sp. (19)* and *Enterobacter sp. (37)* in a previous work.
Media used: all medium components were prepared as decribed by **(APHA, 1992)** MacConkey agar No.3 (g/l) peptone 20.0 , lactose 10.0, bile salts, sodium chloride 5.0 , neutral red 0.03, crystal violet 0.001, agar 15.0 with pH adjusted to 7.1 ± 0.2 at 25°C. It is used for *Enterobacter* sp. Nutrient agar medium (g/l) with composition of meat extract 3, peptone 5, pH 7. Tryptone glucose yeast extract broth (TGY) (g/l) Casein enzymic hydrolysate 10.0, yeast extract 1.0, glucose 5, dipotassium phosphate 1.25. It was used for the maintenance of the isolate. Muller and Hinton agar (g/l) beef, dehydrated infusion 30.0, casein hydrolysate 17, Starch 1.5, agar 17.0 with pH adjusted to 7.3 ± 0.1 at 25°C. It was used for testing the inhibitory activity tests.

Preparation of garlic lobes extracts

a) Aqueous Extraction: Ten Gram of dried and crushed garlic lobes were soaked in 100 ml of distilled water for 6 h. at 50 °C. Every two hour it was filtered through eight layered muslin cloth and centrifuged at 9000 rpm for 10 min. The supernatant was collected and concentrated by evaporating at 40 °C to the final volume. Strilization of aquoues extracts was carried out by filtration then, stored at 4°C in air tight bottles for further studies. **(Parekh et al 2005).**

b) Solvent extraction: Ten grams of dried and crushed garlic lobes was added to 100 ml of methanol 90 % (v/v) then, kept on a rotatory shaker 120 rpm/24◦C (Lab-Line Orbital Shaker- USA Lab Equipment) for 24 h. Thereafter, it was filtered through eight layered Muslin cloth followed by filtration using Whatman No. 1 filter paper and centrifuged at 9000 rpm for 10 min. the supernatant was collected and concentrated by evaporating at 40 °C to the final volume (10%). The extraction was stored at 4 °C in air tight bottles till demand, as recommended by **Parekh et al (2005).**

c) Essential oils of garlic

The garlic essential oil was prepared by extraction of aromatic oil unit in National Research Center (NRC).

Standard inoculum

Standard inoculum was prepared by picking up 3-5 single colonies then inoculated into 5 ml (saline solution), incubated at 37°C for 24 h. After that, Optical density (O.D.) of grown culture was adjusted on spectrophotometer at 625 nm for reading 0.06-0.8 which is equivalent to ($14x10^6$ CFU/ml).

Inhibitory effect of *Allium sativum* against *Enterobacter sp.* using well diffusion method

Antibacterial activity of garlic extracts was tested separately using well diffusion method as described by **(NCCLS, 1993).** Muller Hinton agar medium was poured into petri-dishes and inoculated with 1 ml of *Enterobacter sp* ($14X10^6$ CFU/ml). Agar wells were made using a 7 mm corkborer. Each well was filled by 100 µl of the tested garlic essential oil. Inoculated plates were incubated at 37 °C for 24 h. All experiments were carried out in triplicate and the inhibitory activity was expressed as inhibition zone diameter's mean **(NCCLS, 1993)**.

Effect of different concentration of garlic essential oil against *Enterobacter sp.* by well diffusion method

Serial concentrations of garlic oil were prepared by emulsifying (30- 40 – 50 – 60 – 70 – 80 % v/v) in 2 % of tween 80. these concentrations of garlic oil were tested for their inhibitory activity against *Enterobacter sp*. Inoculum was prepared by growing *Enterobacter sp.* in nutrient broth for 24

h. at 37°C. Muller Hinton agar medium **(APHA, 1992)** was poured into petri-dishes. Poured dishes were inoculated with 1 ml of *Enterobacter sp* ($14x10^6$ CFU/ml) Agar wells were made using a 7 mm corkborer . Each well was filled by 100 µl of the tested concentrations separately. All inoculated Petri-dishes were incubated at 37 °C for 24 h. All experiments were carried out in triplicate and the inhibitory activity was expressed as inhibition zone diameter's mean. **(Mahdi et al 2013).**

GC-MS analysis of garlic essential oil: A Hewlett Packard Gas Chromatographer (HP6890) with controlled electronic pressure was used for Gas Chromatography (GC) analysis. This apparatus system is equipped with an HP-5MS (30 m x 0.25 mm, film thickness 0.1 µm) capillary column. A detector was set at temperature range from 50 °C for 5 minutes to 280°C. as over temperature increasing was programmed at 40°C/ 5 minutes and held for 5 minutes. 50°C each minute using a H2/Air mixture and split - split less injector set at 280°C .A sample of 1 µl was injected by split mode. Nitrogen gas was used as a carrier gas with a flow rate of 1.5 m/mm-1. The essential oil compounds were done according to their retention indices identified by reference to a homologus series of (C4-C28) as described by **(Adams, 2017).**

Identification of pathogenic waterborne isolates by MALDI-TOF MS

MALDI-TOF-MS was used to analzye bacterial species as follows: single colony of a pure culture was transferred directly directly using wood backs on MALDI target plate at room temperature untill drying and over laid after over laying of samples , 1µl Bruker HCCA solution was added to all samples then allow the samples to dry . After that measure of MALDI-TOF-MS was carried out as illustrated by **(Bruker, 2014).**

Statistical analysis

Determination coefficient (R^2) was calculate according to (Microsoft office Excel 2007 package)

RESULTS AND DISCUSSION

Isolation and identification of *Enterobacter* isolates by MALDI-TOF MS

MacConkey agar was used to isolate *Enterobacter* isolates from River Nile (Giza) in Egypt, which represent 49% of the bacterial water borne microorganisms. Medium was inoculated with 1 ml of water sample then incubated at 37 °C for 24 h. Single colonies with lactose-fermenting appearance (pink and mucoid colonies) were picked up and subcultured into nutrient agar. Table (1) shows that the two isolates has a high score ranged from 2.17 and 2.32 identified as *Enterobacter cloacae* 3264 & 11506, respectively.

Table 1. MALDI-TOF-MS identification of the waterborne pathogenic isolates

Isolation NO.	Isolate source	Score value	Suggest strains
19	River water	2.17	*Enterobacter cloacae DSM 3264 BRB*
37	River water	2.32	*Enterobacter cloacae MB11506_1 CHB*

Inhibitory effect of *Allium sativum* against both strains of *E. cloacae*

Different extracts of garlic were prepared using aqueous, methanolic and oil extraction methods. These extractions were tested for their inhibitory activity against *Enterobacter* strains using well diffusion method. **Fig. (1)** shows that *E. cloacae DSM 3264 BRB* was highly sensitive towards oil extract with inhibition zone 2.3 cm and *E. cloacae MB 11506_1 CHB* 1.9 cm rather than aqueous and methanolic oil extraction.

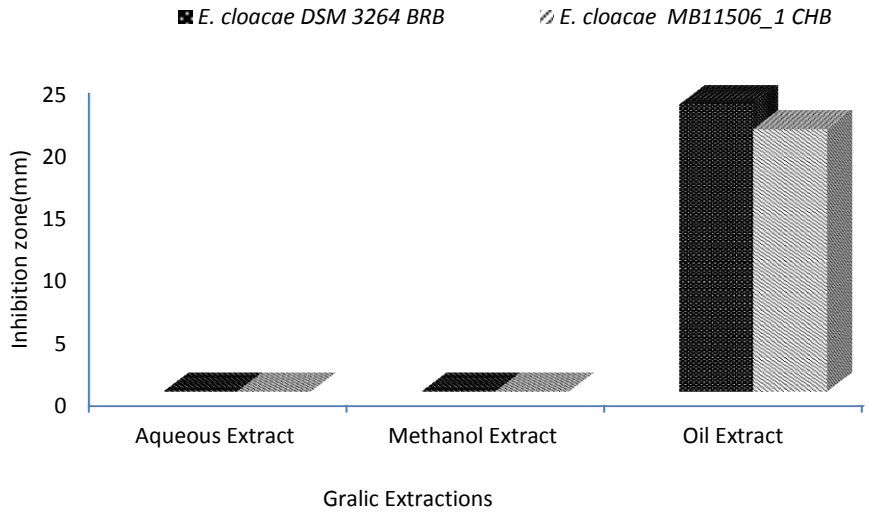

Fig. 1. Antimicrobial activity of different garlic extracts against *Enterobacter cloacae* 3264 & 11506 strains

Effect of different concentration of garlic essential oil against *Enterobacter cloacae* 3264 & 11506

Different concentrations of garlic oil extracts were prepared from 30-80% (v/v) and tested against *Enterobacter cloacae* 3264&11506 **Fig. (2)** shows that oil concentration 80% had the highest inhibitory activity followed by 70% and 60%, respectively. These results referred to the presence of Allicin which exhibits different antimicrobial activity in the high concentration as reported by **Ankri and Mirelman (1999).** Determination coefficient (R^2 of 0.94) confirmed that the oil extract affected the growth significantly. These results were in agreement with previous work of garlic essential oil against *Staphylococcus aureus* found by **(Asmaa et al 2017).** Also, **Goncagul, (2010)** and **Brij et al (2016)**, who stated that *Allium sativum* showed weaker antimicrobial activity against Gram negative bacteria than tested Gram positive bacteria.

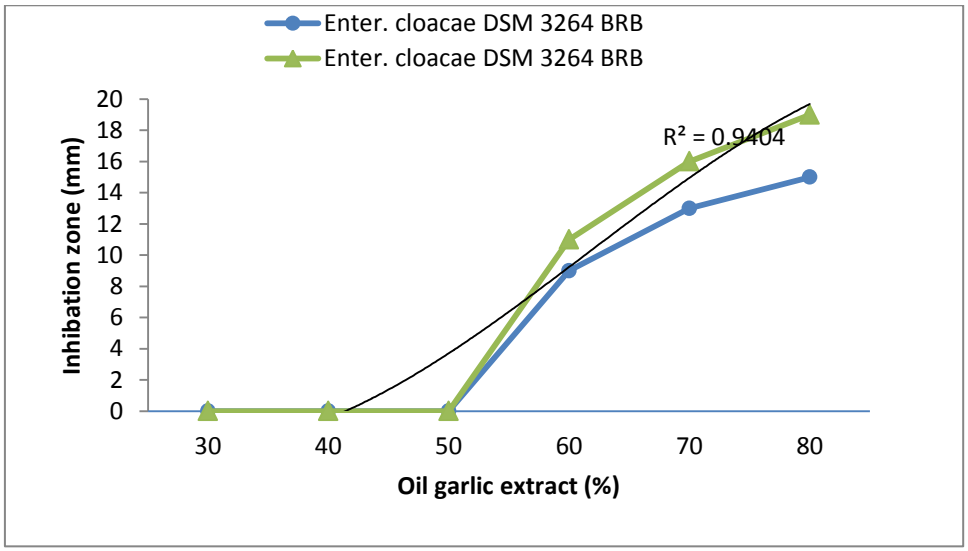

Fig. 2. Effect of oil garlic extract concentrations on *Enterobacter cloacae* 3264 & 11506 strains

Chemical constituent of garlic essential oil extract

Chemical composition of garlic oil was illustrated using GC mass technique. **Table (2)** shows the G.C report that identifies the chemical composition of *Allium sativum*. *Allium sativum* is chemically composed of diallyl disulphide 28.17%, Trisulfide, di-2-propenyl 27.12%, 3-(Methylthio) penta-2, 4-dione 12.9% and Diallyl sulfide 9.5%. These results were in similarly with **Dima *et al* (2014)** who found that diallyl disulfide components have the highest percentage in the all *Allium sativum* composition.

Table 2. Chemical constituents (%) of garlic oil detected by GC-MS

	Retention	%	Compound name
1	4.9377	9.5559	Diallyl sulfide
2	7.0441	28.1784	Diallyl disulphide
3	8.5238	27.128	Trisulfide, di-2-propenyl
4	8.6434	0.3752	(Z)-1-Allyl-3-(prop-1-en-1-yl) trisulfane
5	9.8387	12.9281	3-(Methylthio)penta-2,4-dione
6	11.0918	3.2227	1-Allyl-3-(2-(allylthio)propyl)trisulfane

CONCLUSION

The inhibitory activity of different garlic extracts show a high sensitivity on *Enterobacter* sp. isolated from river nile where oil extract has the most inhibitory effect followed by water and methanol extractions.

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تأثير الثوم *Allium sativium* على الأنتيروباكتر كبكتريا مرضيه تنتقل عن طريق المياه ومعزوله من نهر النيل

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الموجـــــــز

تم إستخدام الثوم بهدف إعاقة إزدياد أعداد البكتريا المقاومه للمضادات الحيوية التقليدية ولإستكشاف تأثير الثوم المضاد لبكتريا الإنتيروباكتر المسببه للامراض المعوية والمعزولة من مياه النيل والتى تمثل 49% من الميكروبات المعزوله، تم تعريفها بجهاز مالدى–توف– وأظهرت النتائج أنها تتبع *Enterobacter cloacae* *DSM* 3264 BRB & *Enterobacter cloacae* MB11506_1 CHB. وبإختبار مدى كفاءة المستخلص المائى والميثانولى وزيت الثوم على السلالتين ثبت كفاءة زيت الثوم حيث تم تثبيط نمو السلالتين *Enterobacter cloacae DSM* 3264 BRB & *Enterobacter cloacae* MB11506_1 CHB بقطر 2.3 و 1.9 سم، بينما أعطت المستخلصات الأخرى اقل من ذلك بكثير. وبدراسة ستة تركيزات مختلفة من زيت الثوم والتى تراوحت مابين 30 إلى 80 % ثبت أن أقل تركيز مثبط كان عند 40/50%. وبتحليل الثوم بطرق التحليل الكروماتوجرافى GC-MS ثبت إحتوائه على ستة مركبات كبريتيه تمثل 25% من المركبات الكلية (24 مركب كلي) التى تم تقديرها فى الزيت .

الكلمات الدالة: *Enterobacter cloacae*، زيت الثوم، MALDI-Tof-MS, GC-MS spectrometry, *Allium sativum*، طريقة الانتشار بالآجار

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