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TREATMENT PETROLEUM OIL CONTAMINATED SOIL BY INOCULATION OF DIFFERENT BACTERIAL STRAINS

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ABSTRACT

Petroleum oil contains a large number of poly cyclic hydrocarbons (PAH's) that are toxic to living beings. The complete degradation of petroleum oil required a population of microorganisms in the soil. In the present investigation petroleum oil contaminated soil samples were incubated with four bacterial strains (Mycobacterium sp., Pseudomonas aeruginosa, Alcaligenes faecalis and Enterobacter cloacae) to study the bioremediation efficacy. The soil samples were analyzed for soil reaction (pH), soil moisture content, soil organic carbon (SOC), available phosphorus (P), total petroleum hydrocarbon content (TPH), total bacterial count (TBC) and total petroleum degrading bacteria at the interval of 0 days (initial), 2 weeks, 4 weeks, 6 weeks and 8 weeks prior and after treatment by bacteria. Values obtained reveals that there was a clear modulating effect of bacteria on above determinations. Maximum decrements in TPH (86%), soil pH (18.2%) and SOC (40%) were recorded in Pseudomonas aeruginosa inoculated samples..

Keywords: *Pseudomonas aeruginosa*, pH, Total Bacterial Count (TBC), Soil Organic Carbon (SOC), Total Petroleum Hydrocarbon (TPH) content

INTRODUCTION

The world depends on petroleum oil. Vast amount is used, transported, processed and stored around the world. In 2016, the total world consumption of petroleum is over 98.21 million barrels per day (**US Energy Information Administration, 2017**). India is the World's 23rd largest producer of crude oil (36.942 MMT in 2015-16). The total number of refineries is 21 and has second rank after China in Asia and in 2016-17 total refining capacity is 230.066 MMTPA (**Ministry of Petroleum and Natural Gas, 2017**). With such a high consumption, oil spills are inevitable. Environmental pollution with petroleum and petrochemical products (diesel, petrol, kerosene etc.) has been recognized as one of the most important serious current problem (**Lu et al., 2015; Pant et al., 2016; Cachada et al., 2018**). Since the introduction of oil exploration, the environment has been heavily contaminated with hydrocarbon pollutants, which enters in the environment through several routes.

Exxon Valdez, known as the most notable oil spills at sea which spilled thousands of tones oil (**Paine et. al., 1996; Albaiges et. al., 2006**). These oil spills can cause severe damage to sea and shoreline organisms (**Whitfield, 2003**). Other most responsible for the contamination of soil and water are service stations, garages, scrap yards, waste treatment plants, sawmills, wood impregnation plants etc. Mechanic workshops generating thousand million gallons of waste engine oil annually and discharged carelessly into the environment (**Adegodroye, 1997; Faboya, 1997**), out of which only one liter is enough to contaminate one million gallons of freshwater (**USEPA, 1996**). Thereafter, several researches have been examined the fate of petroleum in various ecosystem (**Boehm *et al.*, 1995; Whittaker *et al.*, 1999**). These petroleum pollutants in the terrestrial and aquatic environments cause public health and socio-economic hazards (**Adelowo and Oloke, 2002; Okerentugba and Ezeronye, 2003; Edewor *et al.*, 2004**).

The release of oil into the environment causes environmental anxiety and attracts the public awareness (**Roling *et al.*, 2002**). Accidentally or deliberately releasing of oil into the environment, leads serious pollution problems (**Thousand *et al.*, 1999**). Even small release of petroleum oil into aquifers can lead to concentrations of dissolved hydrocarbons far in excess of regulatory limits (**Spence *et al.*, 2005**). Huge disturbances of both the biotic and abiotic components of the ecosystem are created by such these pollutants (**Mueller *et al.*, 1992**) and carcinogenicity, immunotoxicity and neurotoxicity has proved by the some of these hydrocarbons (**Hallier-Soulier *et al.*, 1999; Das and Chandran, 2011**).

Microbial biodegradation of pollutants has intensified in recent years as human strives to find sustainable ways to clean up contaminated environments (***Diaz, 2008)***. Biodegradation of petroleum hydrocarbons by natural populations of microorganisms (bacteria, fungi algae etc.) represent one of the primary mechanism by which petroleum and other hydrocarbon pollutants are eliminated from the environment. The effects of environmental parameters viz.- temperature, pH, concentration of hydrocarbon, bioavailability etc. on microbial degradation of hydrocarbons, the elucidation of metabolic pathways, genetic basis for hydrocarbon dissimilation by microorganisms and the effects of hydrocarbon contamination on microbial communities have been the areas of intense interest and the subjects of several reviews (**Atlas, 1981; 1994**). If there is no contamination in the area, the percentage of hydrocarbon degraders of all the microbial strain in the soil or water is quite low (<1%), but after an oil spill, their level may increase 1000 fold (**Atlas, 1981; Prince, 2005**). These microorganisms can degrade a wide range of target constituents present in oily sludge (**Eriksson *et al.*, 1999; Barathi and Vasudevan, 2001; Mishra *et al.*, 2001**). So, the biodegradation of oil pollutants has been intensively studied either in controlled condition (**Chaillan *et al.*, 2006**) or in open field experiments (**Gogoi *et al.*, 2003; Chaineau, *et al.*, 2005**).

Biodegradation of oil contaminated soils (which exploits the ability of microorganisms to degrade and/or detoxify organic contamination) has been established as one efficient, economic, versatile and environmentally sound treatment (Margesin and Schinner, 1997). Biodegradation of petroleum hydrocarbon pollutants and petrochemicals by bacteria have been extensively investigated (**Obire and Nwaubeta, 2001; Ijah and Akpera, 2002; Okoh, 2002; Nweke and Okpowasili, 2003; Okerentugba and Ezeronye, 2003; Sanni and Ajisebutu, 2003; Oboirien *et al.*, 2005; Ojumu *et al.*, 2005**).

A large number of bacteria for example *Pseudomonas* spp. (**Kiyohara *et al.*, 1992; Johnson *et al.*, 1996; Pathak *et al.*, 2008**), *Yokenella* spp., *Stenotrophomonas* spp., *Alcaligens* spp., *Roseomonas* spp., *Flavobacter* spp., *Corynebacterium* spp., *Streptococcus* spp., *Providencia* spp., *Sphingobacterium* spp., *Capnocytophaga* spp., *Moraxella* spp., *Bacillus* spp. (**Rusansky *et al.*, 1987; Antai, 1990; Bhattacharya *et al.*, 2003**). *Enterobacter* spp., *Escherichia* spp. and *Hafnia* spp. ((**Grant, 1967; Ijah., 1998; Diaz *et al.*, 2001; Jain *et al.*, 2010**), *Acinetobacter baumannii* KSS1060 (**Shiri et. al., 2015**), *Burkholderia xenovorans* (**Bhattacharya and Khare, 2017**), *Rhodobacter sphaeroides* (Peng et al., 2018), *Bacillus salmalaya* strain 139SI (**Dadrasnia et. al., 2016**) have been reported in the literature for hydrocarbon degradation.

In the present study, four bacterial strains namely *Mycobacterium* sp., *Pseudomonas aeruginosa*, *Entrobacter cloacae* and *Alcaligenes faecalis* were studied for bioremediation of petroleum contaminated site.

MATERIALS AND METHODS

Sample collection and preparation

Sub surface oil contaminated soil samples were collected after removal of surface litter to a depth of about 5 cm in an approximately 2 m^2 area from automobile workshops and near petrol pump stations of Mussorrie, India. These sites have a history of oil contamination of over 30 years. Soil samples were processed immediately upon arrival at laboratory. Dried sludge was passed

through a 2 mm sieve and stored at 10ºC for physico-chemical and microbiological characterization.
These soil samples were analyzed by soil reaction (pH) (**ISO 10390. 1994**), soil moisture content (**ISO 11465. 1993**), soil organic carbon (SOC) (**ISO 10694. 1995**), available phosphorus (P) (**Olsen and Cole, 1954**), total petroleum hydrocarbon content (TPH), total bacterial count (TBC) and total petroleum degrading bacteria prior (0 days) and after 2 weeks, 4 weeks, 6 weeks and 8 weeks with bacterial strains of *Mycobacterium* sp., *Pseudomonas aeruginosa*, *Alcaligenes faecalis* and *Enterobacter cloacae*.

Enumeration of total bacterial count (TBC)

The mean total aerobic bacteria present in the samples at the beginning of the experiment (day 0) and at weekly intervals for each of the treatment options were estimated using spread plate method with nutrient agar as medium. A ten-fold dilution using physiological saline was prepared and 0.1 ml of appropriate dilution was plated in duplicates and incubated for 18-24 h at 37°C temperature after which the colonies were counted.

Enumeration of total petroleum degrading bacteria

Aliquots (0.1 ml) of appropriate dilutions of soil samples were plated on to modified mineral salts medium containing the following in $g.L^{-1}$: NaCl, 10.0; $MgSO_4.7H_2O$, 0.42; KCl, 0.29; KH_2PO_4, 0.53; NH_4NO_4, 0.42; agar, 15.0 and distilled water (Mills *et al.*, 1978). The vapor phase transfer technique (**Okpokwasili, 1988**) was adopted, which employs the use of sterile filter paper soaked in crude oil, which served as the carbon and energy source. The soaked sterile filter papers were then aseptically placed onto covers of the inoculated inverted plates and incubated for 5 to 7 days at 37°C temperature. Average mean counts of colonies from duplicate plates were recorded and used for the calculation of colony forming units per gram (cfu/g) of soil.

Measurement of crude oil utilization

Residual crude oil was extracted from the soil samples using a modified method of **Abu and Ogiji, 1996**. Quantitative determination of the crude oil extracts was carried out as described by **Udeme and Antai, 1988**. A standard curve of absorbance (A_{520} nm) against varying concentrations of engine oil (1 to 5%) in chloroform was drawn after taking readings from a Thermo Fischer scientific UV/VS spectrophotometer. The hydrocarbon concentrations were calculated from the standard curve after multiplying by the appropriate dilution factor.

RESULTS AND DISCUSSION

The contaminated soil samples were incubated with various strains of bacteria to study the bioremediation efficacy. The soil samples prior and after incubation were analyzed for soil reaction (pH), soil moisture content, soil organic carbon (SOC), available phosphorus (P), total petroleum hydrocarbon content (TPH), total bacterial count (TBC) and total petroleum degrading bacteria. Gradual changes in pH, soil moisture content, SOC, available P, TPH, TBC and total petroleum degrading bacteria in soil samples at different time intervals recorded.
Table 1 represents the soil composition, physico-chemical properties and microbiological analysis of petroleum contaminated soil samples at 0 days. It indicates high TPH and SOC in soil samples. Total bacterial count (TBC) and total petroleum degrading bacteria were close to 10^7 cells per gram dry soil and marginal difference was recorded between total bacterial count and total petroleum degrading bacteria.

A considerable decrement in soil pH value was recorded in all the treated soil samples after 8 weeks of incubation period. The maximum decline in pH was recorded in soil samples inoculated with bacterial strain of *Pseudomonas aeruginosa* (figure1). This decrement may be attributed to CO_2 evolution as a result of microbial respiration and other enzymatic reactions. Similar reports have been put forward by **Daylan *et al.*, 1990**. pH values major parameter to be taken in the account during major industrial bioremediation practices like land farming (USEPA, 2006). Besides neutral, pH 6-8 has also been reported to be acceptable (**USEPA, 2006**). High pH values have been reported to be inhibitory to the majority of the microbial degradation processes. The optimal biodegradation activity at pH 7.4 and a considerable inhibition at pH 8.5 have been reported by **Verstraete *et al.*, 1975**.

Table 1 Soil composition at the time of sample collection (0 week)

S. No.	Soil parameters	Unit	Quantity
1.	Sand	%	38.07 ± 2.2
2.	Slit	%	37.02 ± 1.8
3.	Clay	%	25.05 ±1.2
4.	pH	-	7.68±0.18
5.	Moisture	%	18.86±1.1
6.	Organic carbon	%	5.79±0.08
7.	Total Nitrogen	%	0.05±0.02
8.	C/N Ratio	-	115.8
9.	Available P	mg/kg	1.8±0.1
10.	Total petroleum hydrocarbon (TPH)	mg/kg	13826±135
11.	Total bacterial count (TBC)	CFU/g	4.5 x 10^7
12.	Total Petroleum degrading bacteria	CFU/g	1.3 x 10^7

Value represent mean ± SEM (n=3).

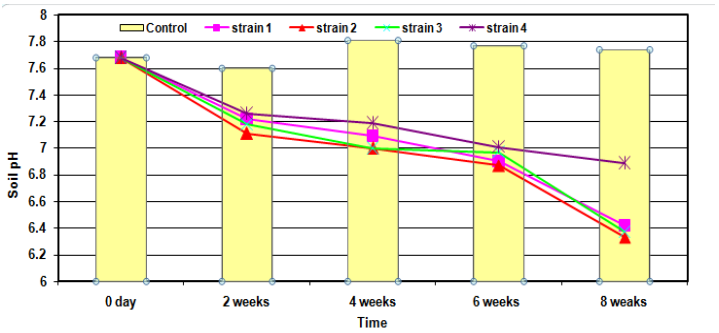

Figure 1 Soil reaction (pH) in soil sample inoculated with different strains of hydrocarbon degrading bacteria

Variable soil moisture content was recorded in soil samples after 8-weeks incubation which was relatively higher in treated soil samples. In the present investigation, during remediation moisture content was dropped in control due to soil drying and evaporation of water. As the soil samples remediate through the introduction of microbes, moisture content was increased. The maximum moisture content was found in bacterial strain *Pseudomonas aeruginosa* inoculated soil sample where it was least in soil sample incubated with bacterial strain *Alcaligenes faecalis*. This might be due to the reduction of hydrophobic component of oil (**Ghulam *et al.*, 2008**).
A significant reduction in soil organic carbon (SOC) content was recorded in soil samples inoculated with different bacterial strains. These data indicate that the organic carbon reduced with the time due to consumption of carbon. The maximum reduction was observed in soil samples inoculated with bacterial strain *Pseudomonas aeruginosa* (figure 2).

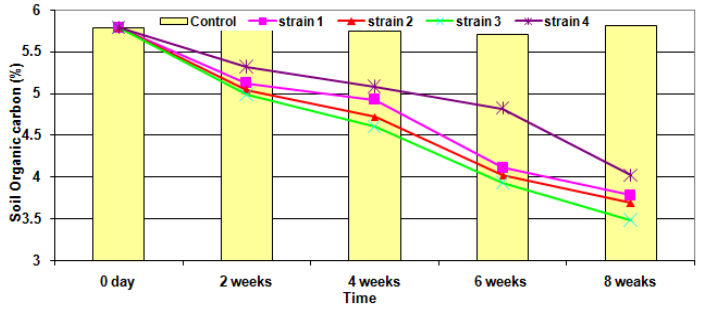

Figure 2 Soil organic carbon (SOC) in soil samples inoculated with different strains of hydrocarbon degrading bacteria

During biodegradations unlike organic carbon the available phosphorus (P) levels were also modulated during the bioremediation (figure 3). The available P increased from considerably during soil remediation. This increment in available phosphorous can be attributed to solubilization of soil phosphorus through microbial reactions. The increased partial pressure of CO_2 generated from microbial respiration and other biological activities may be an important factor for solubilization of phosphorous. Therefore, increased level of available phosphorous was recorded.

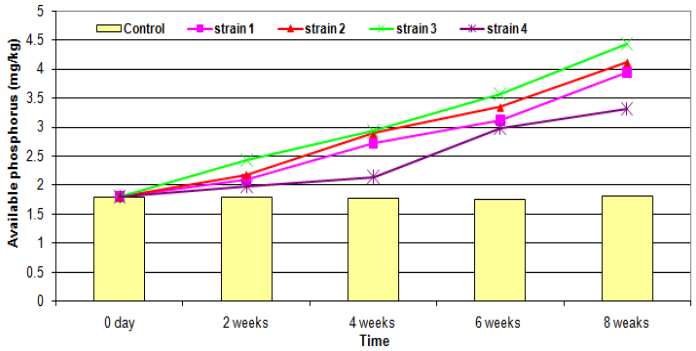

Figure 3 Available phosphorous in soil samples inoculated with different strains of hydrocarbon degrading bacteria

All the strains tested were capable to degrade total petroleum hydrocarbon (TPH). After eight weeks of incubation, the maximum TPH concentration was retained in soil samples inoculated with bacterial strain *Alcaligenes faecalis*. Data suggested that *Enterobacter cloacae* and *Pseudomonas aeruginosa* was most efficient and *Alcaligenes faecalis* was the slowest in the petroleum hydrocarbon degradation (figure 4). This was also paralleled by increment in number of total bacterial count (figure.5) and total petroleum degrading bacteria.

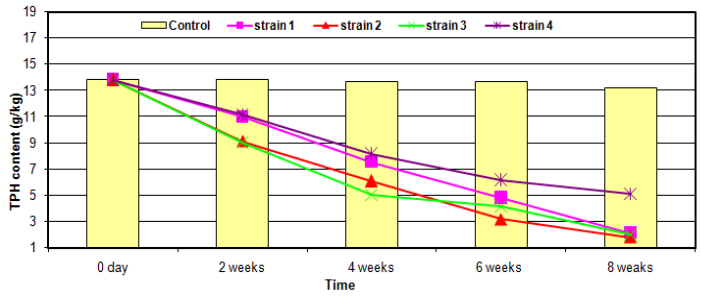

Figure 4 Total petroleum hydrocarbon content (TPH) in soil samples inoculated with different strains of hydrocarbon degrading bacteria

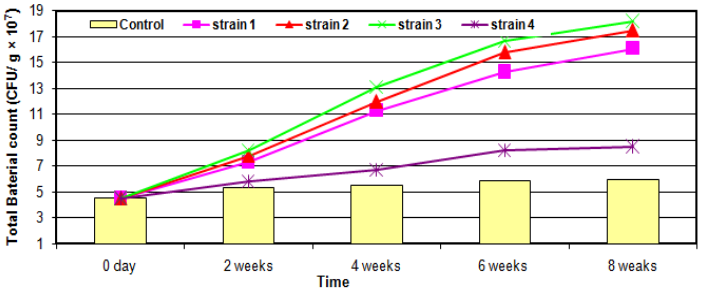

Figure 4 Total Bacterial Count (TBC) in soil samples inoculated with different strains of hydrocarbon degrading bacteria

In the present investigation, one soil sample inoculated with *Mycobacterium*. Degrading capacity of this bacterium has been widely supported in various reports (**Cheung and Kinkle, 2001; Plohl *et al*., 2002**). This bacterium showed 83% decrement of the TPH after 8 weeks incubation. Such kinetics for degradation of TPH has been intensively described by **Panikov *et al*., 2007**. Similar bacterium was also isolated by **Toledo *et al*., 2006** from waste oil. These data suggest that this bacterium can be utilized to remove the loads of contaminants from petroleum polluted sites.

The other soil sample was inoculated with *Pseudomonas aeruginosa*. This potential degrader of petroleum hydrocarbons has also been reported for its efficiency in bioremediation by several researchers (**Emtiazi *et al*., 2005; Toledo *et al*., 2006; Adelowo *et al*., 2006; Madri and Lin, 2007; Pathak *et al*., 2008; Shokrollahzadeh *et al*., 2008; Tao Cheng et. al., 2017**). The soil samples incubated with *Pseudomonas aeruginosa* established that this bacterium was more efficient in degradation of TPH as compared to other strains studied here. TPH degradation up to 86% was recorded in 8-weeks incubation period clearly establishes the potential of this strain. The similar results were also put forward by **Madri and Lin 2007**. However, they achieved 90% degradation of oil component. This difference in percentage degradation achieved by them and percentage degradation recorded in the present investigation may be attributed to the pH and temperature conditions.

In the present investigation, other soil sample inoculated with *Enterobacter cloacae*. This bacterium belongs to Enterobacteriaceae family and mainly regarded as inhabitant of animal gut (**Diaz *et al*., 2001**). The ability of this group to degrade high molecular weight PAH compounds appear to be an unusual feature, as this phenomenon is associated with typical soil bacteria. However, few reports indicated the utilization of aromatic compounds by *Enterobacter* (Ijah, 1998). *Enterobacter* inoculated sample exhibited that after 8 weeks 84% TPH contents were degraded. The similar results were observed **Katsivela *et al*., 2003**. They isolated two bacterial strains viz. *Enterobacter* sp. EK 3.1 and *Enterobacter* sp. EK 4 and demonstrated novel degradation capabilities for mixture of petroleum hydrocarbons. The *Enterobacter* sp. was observed to possess the unique capability of metabolizing PAH as well as the recalcitrant branched alkanes. Although there are several reports on bioremediation of high molecular weight PAH, but research pertaining on biodegradation of these substances by Enteric bacteria are relatively rare (**Kanaly and Harayama, 2000; Diaz *et al*., 2001**).

One soil sample was inoculated with *Alcaligenes faecalis.* This bacterium has capacity to degrade 61% TPH after 8 weeks incubation period. *Alcaligenes* sp. described as *Alcaligenes faecalis* 212-2 was also isolated from waste crude oil by **Toledo *et al*., 2006**. They observed that fluorothene and phenantherene were effectively utilized by *Alcaligenes faecalis* 212-2, whereas, naphthalene was utilized by *Bacillus subtilis*, *Alcaligenes faecalis* 212-2 and *Enterobacter* species.

During the initial phase of remediation, low values of total bacterial count and total petroleum hydrocarbon degrading bacteria were observed. The possible reason was that the high concentration of petroleum hydrocarbons present in soil renders the toxicity which in turn may be detrimental to the soil microbes. The microbial population is directly affected by this lethal concentration. Similar results for bacterial counts have also been reported by **Atlas (1981), Wang (1984), Bauda and Block (1985), Pettibone and Cooney (1988)** and **Odokuma and Dickson (2003)**. Further increase in duration of incubation period for hydrocarbon degradation resulted in reduction in number of bacteria in all the inoculated soil samples which may be attributed to reduction in the amount of available nutrients required for bacterial growth and altered physico-chemical conditions of soil.

The disappearance of TPH may be due to biodegradation by indigenous microorganism in soil, but also by physiochemical process like volatilization, photo-oxidation, absorption or by leaching (**Northcott and Jones, 2000).** However, due to absence of the light during these experiments, photo-oxidation cannot explain the loss of TPH. In contrast, biodegradation and volatilization certainly played a predominant role in the fate of low molecular weight TPH. The high degree of degradation observed in unfertilized mesocosms can be explained on the basis of these physico-chemical processes (**Park *et al*., 1990**).

The alkane fraction of oil was degraded faster than the aromatic fraction. The degree of degradation of n-alkanes shorter chains was higher than the n-alkanes longer chains. This was independent to initial level of contamination. These results are in agreement with the observation of **Seklemova *et al*., 2001**. The degree of degradation of TPH was inversely proportional to the number of rings in the PAH (poly cyclic hydrocarbon) molecule. This observation is in consistency with previous studies on PAH degradation by **Leblond *et al*., 2001**.

Hydrocarbons loss through natural processes decreased with time. However, biodegradation rates are low or negligible at concentration below a critical level (**Allard and Neilson, 1997; Paton *et al*., 2003**). This critical level may depend on the soil structure and on the composition of the contaminant. It has been suggested that bioremediation rate is not likely to be effective with extensively degraded oil (**Bragg *et al*., 1994**). Aged hydrocarbon residues were not bio-available to metabolically competent microorganisms capable of degrading hydrocarbon (**De Jonge *et al*., 1997; Eriksson *et al*., 2001; Paton *et al*., 2003**).

Present study suggests that these strains possess optimal growth capacity for removing of hydrocarbons under the experimental conditions. However, utilization of TPH seems to be under the control of inducible systems and obviously a variety of factors can affect the ability of these microbes to degrade hydrocarbons in natural environment.

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SCICELL

REVIEW

HARMFUL CYANOBACTERIAL BLOOMS AND DEVELOPED CYANOPHAGES AS A BIOLOGICAL SOLUTION

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ABSTRACT

Cyanobacterial Harmful Algal blooms (CHABs) cause devastating impacts to fisheries, tourism, public health and ecosystem around the world, and have increased in frequency. Cyanobacterial blooms occur in fresh water and marine environments, producing a variety of toxins, and poisoning risks to humans and animals. Chemicals can be used to kill cyanobacteria. Unfortunately, many of these chemicals are toxic to other forms of life, including fish and organisms they eat. The use of chemicals in natural lakes could create more problems than they solve, is not permitted. Cyanophage is a double-stranded DNA virus that infects cyanobacteria and is detected in both freshwater and marine environments as a biological solution developed Cyanophages can use for long term treatment options.

Keywords: Cyanobacterial Harmful Algal blooms, Cyanophage, DNA Viruses

INTRODUCTION

Cyanobacteria are unicellular organisms that live in marine waters, freshwater and brackish water **(Carmichael, 2001)**.When these bloom become harmful it known as the cyanobacterial harmful algal blooms (CHABs) **(Schmidt *et al*., 2014)**. CHABs naturally occurring in aquatic environment according to the pH level, water temperature, law water flows, light level, nutrition level **(Sellner *et al*., 2003)**. Many types of algae have an optimal growth temperature between 12°C and 15°C. However, the optimal temperature for most cyanobacteria growth is 25°C. When water is warmer than 25°C cyanobacteria can grow faster than other types of algae (diatoms, green algae). Some algal bloom and green plants growing fast when the high concentration of N and P present in aquatic environment and others die. The dead organic matter will be decomposed by bacteria. The bacteria increased in number and use up devolved oxygen in the water. According to that condition many fish and other living animals in water cannot survive results in dead aquatic environment **(O'Neil *et al*., 2012)**. Algal bloom colors can be green, yellowish-brown, or red, Bright green blooms in freshwater caused by different types of cynobacteria or green blue algae and can have different appearances. Not all cyanobacteria species caused CHABs **(Carmichael, 2001)**.

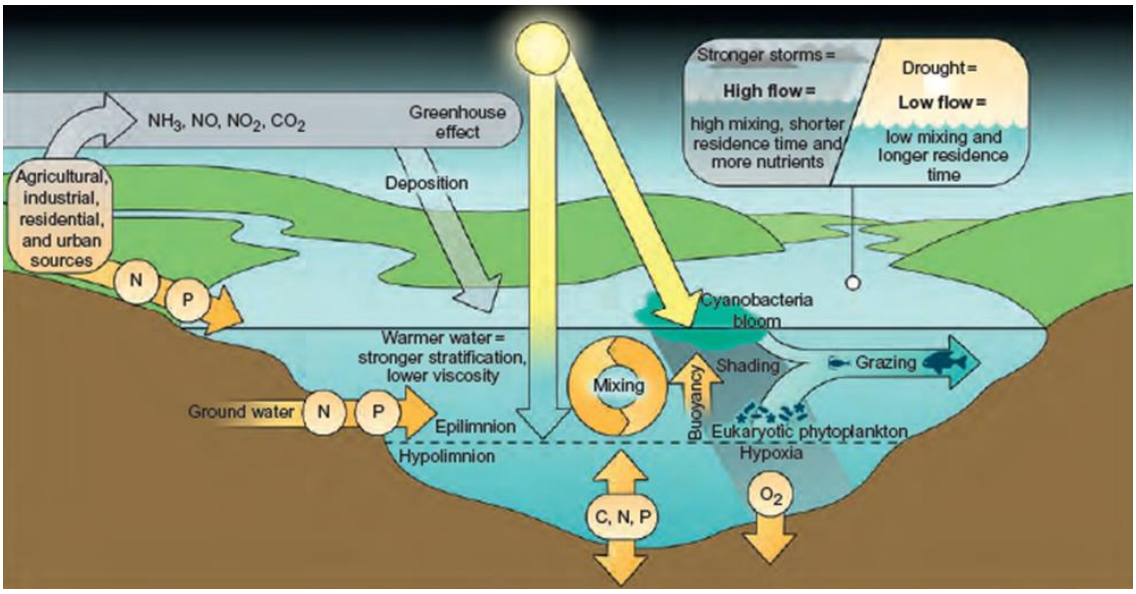

Figure 1 External and internal factors controlling growth of cHABs **(Source: Watson et al., 2015)**

Some blue green algae produce toxins (Cyanotoxins) which is toxic for humans, animals, aquatic environment. Examples for cyanotoxins are Hepatotoxins, Neurotoxins, Endotoxins, Dermatoxins **(Zanchett and Oliveira-Filho, 2013)**.

The current treatment options available for Cyanobacterial Harmful Algal Blooms (CHABs) are mechanical, physical/chemical, and biological control. Mechanical control are the use of filters, pumps and barriers to remove or exclude CHAB cells or other materials related to the proliferation of contaminated water **(Nienhuis and Gulati, 2002)**. Chemical control are the use of chemical compounds to kill, inhibit, or remove CHAB cells. Biological control involves the use of organisms or pathogens (e.g.: viruses, bacteria, parasites, zooplankton, crustaceans) that can kill, dissolve or remove CHAB cells. These options are short term treatment **(MacKay *et al*., 2014)**.The idea of developed Cyanophages introduce as a long term biological treatment option for the Cyanobacterial Harmful Algal blooms **(Watson *et al*., 2015)**.

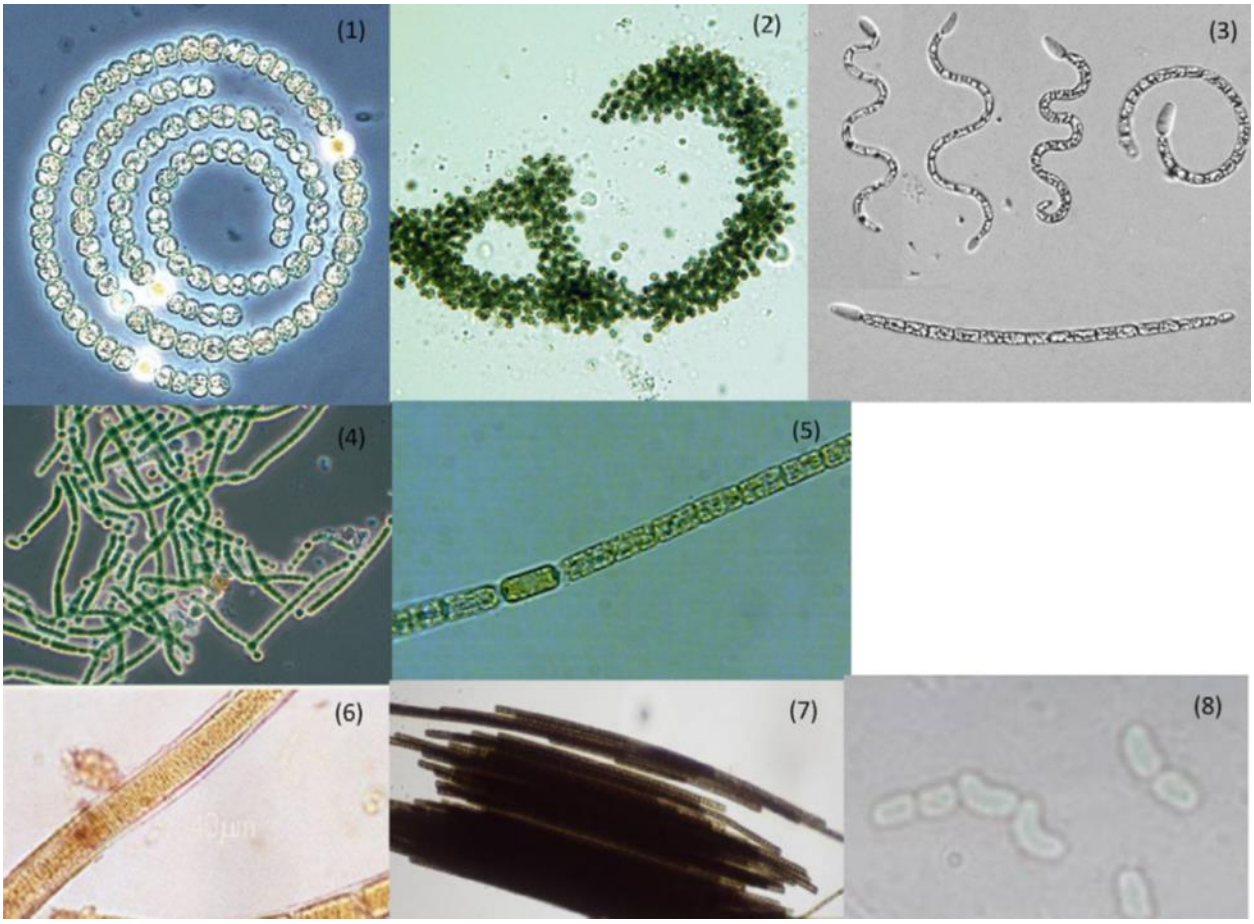

Figure 2 Major types of cyanobacterial genera (1) Anabaena (2) Microcystis (3) Cylindrospermopsis (4) Nodularia (5) Aphanizomena (6) Lynbya (7) Trichodesmium (8) Synechococcus **(Source: O'Neil et al., 2012)**

Errors of chemical control

Photosynthetic organisms, including algae and cyanobacteria (blue-green algae), produce new particulate organic matter in the water system. When these organisms come into contact with water treatment chemicals in either the reservoir or the treatment plant, damage to the cells can result in the release of cell contents as dissolved organic matter **(Codd *et al*., 1989)**. The release of dissolved organic material from cyanobacteria is particular concern. Cyanobacteria, in many cases, dominate freshwater phytoplankton in surface water of eutrophic systems, is the major producer of toxins **(Kenefick *et al*., 1992)**.

Two of these compounds, geosmin and methylisoborneol, have been identified as major agonists of soil musty odour common in aqueous systems **(Kenefick *et al*., 1992)**. These odorants are saturated cyclic tertiary alcohols which are resistant to oxidation by conventional water treatment chemicals **(Izaguirre *et al*., 1982)**.The production of these odorous compounds was the main reason for inclusion of granular activated carbon (GAC) reactors in water treatment plants. Cyanobacteria also produce several other organic compounds that can react with chemical disinfectants to form byproducts of interest. Dissolved organics of cyanobacterial origin produce chloroform per unit mass of carbon, like humic acid and fulvic acid when in contact with chlorine. Release of organic compounds by phytoplankton occurs spontaneously during active growth and decay of cells **(Baines and Pace, 1991)**. Deterioration of physiological condition is associated with geosmin release **(Rosen *et al*., 1992)**.

However, chemically induced cellular damage can cause sudden release of organic compounds within the cell. Prechlorination in a water treatment plant leads to the release of geosmin to the water **(Ashitani *et al*., 1988)**. Water treatment with copper sulphate can leads to release of toxin, microcystin-LR **(Kenefick *et al*., 1993)**.

The release of dissolved organic compounds by cyanobacteria presents the risks to consider before using chemical plants or groundwater reservoirs. To avoid this risk, it is important to note chemicals that cause this reaction in cyanobacteria **(Matsumoto and Tsuchiya, 1988)**.

Cyanophages

Cyanophages are double-stranded DNA viruses. They can infect cyanobacteria and able to detected in freshwater and marine water. They have a complex pattern of host range and play an important role in controlling the cyanobacteria population. It is divided into three families, Myoviridae, Siphoviridae, and Podoviridae (Table.1). Major types are LPP−1, N−1, AS−1, and SM−1 (Fig.2) **(Xia *et al*., 2013)**.

Table 1 Types of freshwater Cyanophages **(Source: Xia *et al*., 2013)**

Family	Morphology	Phage species	Host	References
Myoviridae	Contractile tail	AS-1	*Aancystis nidulans*, *Synechococcus cedrorum*	Safferman R S, et al., 1972
		N-1	*Nostoc muscorum*	Adolph K W, et al., 1971
		Ma-LMM01	*Microcystis aeruginosa*	Yoshida T, et al., 2006
	Long, non-contractile tail	SM-2	*Synechococcus elongates*,*Microcystis aeruginosa*	Fox J A, et al., 1976
Siphoviridae		S-2L	*Synechococcus sp. 698*	Khudyakov I Y, et al., 1978
		S-4L	*Synechococcus. elongatus*	Khudyakov I Y, et al., 1982
		LPP-1	*Lyngbya*, *Plectonema*, *Phormidium*	Sherman L A, et al, 1970
Podoviridae	Short tail	SM-1	*Synechococcus elongatus*	Safferman R S, et al., 1969
		Ma-LBP	*Microcystis aeruginosa*	Tucker S, et al., 2005
Unassigned	Tailless	PaV-LD	*Planktothrix agardhii*	Gao E B, et al., 2009

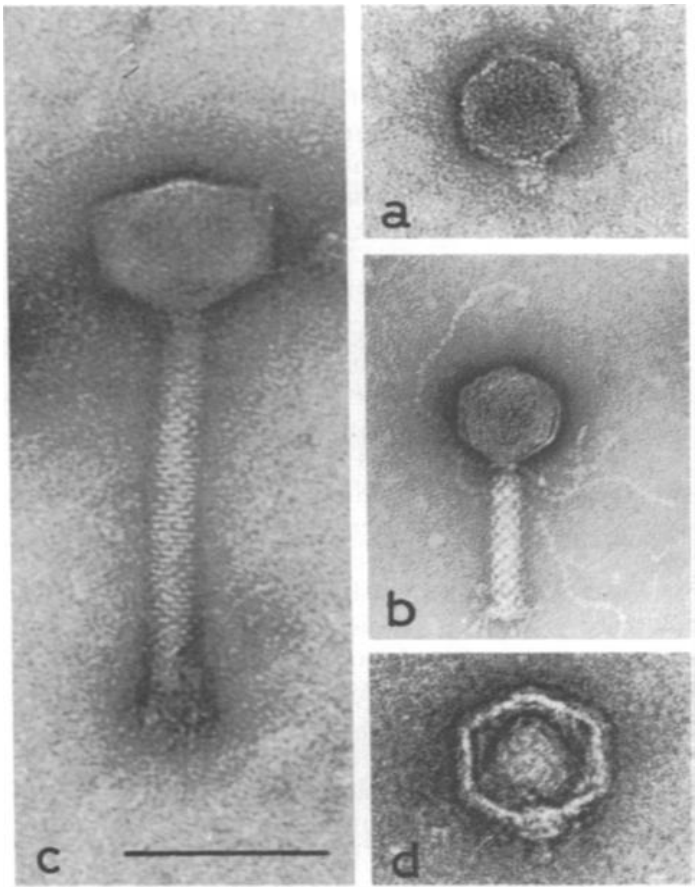

Figure 3 Types of Cyanophages (a) LPP−1 species of cyanophage (b) N−1 (c) AS−1 (d) SM−1 **(Source: Etana and Moshe., 1973)**

Cyanophage-Cyanobacterial interaction

Cyanophage nucleic acids contain only a few genes necessary for the synthesis of new viruses, their structural components such as capsid proteins, and enzymes used in the phage life cycle. The synthesized enzyme is involved in the complete replication or processing of the nucleic acid and functions only when the phage is in the host cell **(Sandaa, 2009)**.

In order to synthesize phage, proteins and enzymes, ribosomes, transfer ribonucleic acid (t-RNA) and energy production are supplied by host cells **(Hyman and Abedon, 2009)**.

Their existence in the host cells after viral infection and their electronic tomographic analysis for molecular degradation are useful for understanding viral infection mechanisms and therefore for studying the development of cyanophage or other phage within host cells it turned out to be. The two main modes of phage propagation are the lysis cycle and the lysogen cycle **(Hanlon, 2007; Peduzzi and Luef, 2009; Hyman and Abedon, 2009)**.

Cyanophage is remarkably similar to T phage in terms of morphological attributes and life cycle patterns **(Herskowitz and Hagen, 1980)**. The lysis cycle is terminated by lysis and death of the host cells. The lytic cycle is highly dependent on the activity and efficacy of the enzyme lysozyme produced by the cyanophage itself In the case of non-production of lysozyme, it has been found that respiration and metabolism of the host are affected and may be stopped, but until induction of the dissolution process takes place in both cases It is certain that there is nothing **(Herskowitz and Hagen, 1980)**.

Role of Cyanophages

Cyanophage plays an important role in the evolution of cyanobacteria. It controls the abundance of cyanobacteria, the dynamics of populations, and the structure of nature. Cyanophage is a global reservoir of genetic information. They transfer genes, confer cyanobacteria with novel properties, and act as vectors that affect the rate and direction of the evolution process **(Xia *et al*., 2013)**.

Lysogeny makes an important contribution to the maintenance of the cyanobacterial gene pool and ecological adaptation. The incorporation of many cyanobacterial genes into the cyanophage genome indicates that genetic transmission occurs between the host and the phage. Such gene transfer plays a driving function in adaptive microscopic evolution **(Xia *et al*., 2013)**.

Unlike bacteriophages, which usually have a host-specific host range, cyanophage generally has a host in more than one genus **(Singh1 *et al*., 2012)**. Recently, the role phage plays in the ecological dynamics of poisonous bloom-forming Pseudomonas aeruginosa has been studied **(Yoshida *et al*., 2008)**.

CONCLUSION

Reports on various systems in which gene recombination or gene exchange are performed between phage and cyanobacteria are regularly conducted. The structure need to be further evaluated, focusing on the use of molecular biology.

An important role can be played in the field of new evolving molecular evolution for Cyanophage in different ecosystems like lakes, ponds, rivers, marine environment.

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SCICELL

REGULAR ARTICLE

MOLECULAR DIAGNOSIS OF RICKETTSIAE INFECTING CAMELS AND IXODID TICKS IN EGYPT

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ABSTRACT

Rickettsioses including their pathogens, vectors, and hosts have an epidemiological importance and zoonotic importance. The objective of the present article was to define the prevalence and genotypic properties of *Rickettsia* in camels and their ticks in Egypt. Sixty one blood samples and 99 adult ticks were taken from camel hosts from Cairo, Giza and Sinai, during a period extending from 2013 to 2014. Based on the morphological identification of both male and female tick specimens, 91.9 % of the collected ticks were *Hyalomma dromedarii*. The prevalence of *Rickettsia* in camels using Gimenez staining technique and PCR was 0 and 41 %, respectively. The rickettsiae infection in ticks recorded 10.1 and 1.01 %, by Gimenez stain and PCR, respectively. Further, the phylogenetic analysis was conducted based on the sequences of *OmpA* and *glt*Agenes and three intergenic spacers (*mppA, dksA* and *rpmE*) of *Rickettsia* species. The phylogenetic analyses revealed a novel strain of *Rickettsia africae* in *Hyalomma marginatum* that was collected from camel in Sinai province. In addition, the phylogenetic analysis based on Clustal omega suggested that *Rickettsia* sequences which detected in camels were *R. africae*. Moreover, the highest Rickettsial infection rate was recorded in age groups of 17 to 19 years (80.0 %), Abady camel breeds (56.8 %) and ticks-infested camels (42.8 %). Concerning hematological changes, macrocytic anemia and leucopenia were recorded in camels with rickettsioses. The molecular characterization of *Rickettsia* detected in camels and their tick vectors will help in a better understanding of the epidemiological approach of rickettsioses in Egypt.

Keywords: Camels, *Hyalomma* species, rickettsioses, Multi-genes typing

INTRODUCTION

Rickettsioses are considered emerging and re-emerging zoonotic vector-borne diseases (**Parola and Raoult, 2001; Dantas-Torres *et al*., 2012; Kernif *et al*., 2012b; Parola *et al*., 2013**). Rickettsioses in general have high morbidity and low mortality except some *Rickettsia* spp. such as *Rickettsia rickettsia*, which showed high mortality in both dogs and human (**Raoult and Roux, 1997; Parola *et al*., 2005; 2013**).

The order Rickettsiales are simply known obligatory intracellular gram negative bacilli, cocci or thread-like bacteria that retained basic fuchsin when stained by Gimenez stain (**Gimenez, 1964; Fournier and Raoult, 2007; Kang *et al*., 2014**). The taxonomy of Rickettsiae has undergone extensive reorganization **(Raoult and Roux, 1997; Hechemy *et al*., 2003**). The order Rickettsiales includes Anaplasmataceae and Rickettsiaceae families. The 16S rRNA, *gltA*, *ompA*, *ompB*, and *sac 4* genes were suggested for rickettsial taxonomy (**Dumler *et al*., 2001; Fournier *et al*., 2003**). The rickettsiae are divided into four groups; Spotted Fever (SFG), Typhus (TG), *R. belli* and *R. candensis* group (**Fournier and Raoult, 2007; Merhej and Raoult, 2011**).

Ticks are considered secondary to mosquitoes in their ability to transmit diseases (**Hillyard, 1996**). They are the main vectors and reservoirs of *Rickettsia* spp.; especially SFG Rickettsiae that were transmitted transstadially through the developmental stages and transovarial (**Raoult and Roux, 1997; Anderson and Magnorelli, 2008; Socolovschi *et al*., 2009b**). Ixodid ticks (hard ticks) transmit the microorganisms to vertebrates through tick bites via their salivary secretions, or through feces and blood transfusion (**Socolovschi *et al*., 2009b**).

In Egypt, *Hyalomma* species are the most dominant ticks on camels, especially *H. dromedarii*, *H. marginatum*, *H. excavatum*, and *H. impeltatum* (**Abdel-Shafy, 2000; El-Kammah *et al*., 2001; Abdel-Shafy *et al*., 2012**).

Tick-borne rickettsioses have been diagnosed serologically in animals and human (**Botros *et al*., 1989; Soliman *et al*., 1989; Corwin *et al*., 1992; 1993; Reynolds, 2004**). In previous studies, SFG were detected in *Rhipicephalus sanguineus* and *Hyalomma* spp. from Sinai (**Lange *et al*., 1992; Loftis *et al*., 2006ab**). Socolovschi and his colleagues detected *R. sibirica mongolitimonae* in a traveler returned from Egypt to France (**Socolovschi *et al*., 2010**). Moreover, *R. africae* was recorded for the first time in *Hyalomma* spp. in Egypt by **Abdel-Shafy *et al*. (2012)**. In addition, *R. aeschlimannii* alsohas been reported in *Hyalomma* spp. by **Loftis *et al*. (2006ab)** and **Abdel-Shafy *et al*. (2012)**.

The diagnosis of rickettsioses is still considered a challengedue to the non-specific clinical signs, laboratory abnormalities and/or subclinical infection (**Gasser *et al*., 2001; Parola *et al*., 2005; 2013**). Molecular techniques were applied targeting accurate and rapid detection and identification of *Rickettsia* spp. PCR followed by sequencing improved the sensitivity of diagnosis and specificity of taxonomy (**Parola *et al*., 2013; Guillemi *et al*., 2015**). Primers amplifying the *OmpA* and *gltA* genes were less conserved genes, so it had a higher discriminating power between genomes of SPG *Rickettsia* spp. (**Roux *et al*., 1997; Fournier *et al*., 1998**). Moreover, intergenic spacers (*mppA, dksA* and *rpmE*) were more variable than genes and conserved spacers (**Fournier *et al*., 2004**). Therefore, they had highly variable sequences.

Few previous studies aimed to detect rickettsioses in camel ticks. However, to the best of our knowledge, this is the first study to detect camels' rickettsioses in Egypt. Camels have been used in meat and milk production, security purposes in desert and border areas, also in racing as a traditional sport. Therefore, the objectives of this study were the determination of the prevalence of tick-borne rickettsioses in camels and their ixodid tick vectors at different provinces in Egypt, in addition the molecular characterization of novel genotypes of *Rickettsia* compared to the previously published genotypes. The genotypic relationship between these *Rickettsia* species and previously recorded worldwide is targeted by *OmpA*, *gltA*, *mppA*, *dksA* and *rpmE* sequences alignment with GenBank related records.

MATERIALS AND METHODS

Sampling sites and collections (Animals and Ticks)

Sixty one camels were examined for the presence of ticks at Cairo, Giza and Sinai from Mar. 2013 - Oct. 2014. AnEDTA-whole blood (5 ml each) was collected from jugular vein of each animal. The blood samples were used for hematological studies, preparing blood smears for Gimenez staining techniques (**Gimenez, 1964**). An amount of blood per animal was stored at -20 °C for molecular studies. Other blood samples were collected without anticoagulants for serum separation. Sera were used for further biochemical parameters investigations. The animals were checked for tick infestations through their whole body (**Abdullah *et al*., 2016b**). Ninety nine adult ticks were collected from animals. Ticks were removed from animals by forceps and put into plastic tubes. The tubes were transferred to the lab for further processing.

Ticks Identification

Ticks were identified according to the taxonomic keys of **Hoogstraal and Kaiser (1958)**, and **Estrada-Peña *et al.* (2004)**. Further, hemolymph staining technique was performed for all collected ticks according to **Gimenez (1964)**. Then, the ticks were stored at -20 °C until DNA was extracted for molecular studies.

DNA extraction

The DNA was extracted from blood samples using GF-1 Tissue Blood Combi DNA Extraction Kit (SNF, Vivantis, Malaysia) according to the manufacturer's instructions. The DNA of adult ticks was extracted after dividing the tick body into quarters. The DNA was extracted by high salt concentration protocol (**Zilberman *et al*., 2006**). The purity and concentration of DNA were measured by nanodrop 2000c (Thermo Scientific) and stored at -20°C.

Primers Design

The primers of *OmpA* and *gltA* genes were designed according to **Fournier *et al.* (1998)**, **Roux *et al.* (1997)** and **Mediannikov *et al*. (2004)** (Table 1). *Rickettsia* positive sample further characterized using primers targeting intergenic spacers (*mppA*, *dksA* and *rpmE*) (Fournier et al. 2004) (Table 1).

Table 1 Primers utilized in amplification and sequencing of genes

DNA Marker	5'- Primers Sequences-3'	Amplified Fragments	References
OmpA gene 190.70-F 190.701-R	 5'-ATGGCGAATATTTCTCCAAAA-3' 5'-GTTCCGTTAATGGCAGCATCT-3'	590-634 bp	Fournier et al. (1998)
gltA gene CS2d-F CSEnd-R	 5'-ATGACCAATGAAAATAATAAT-3' 5'-CTTATACTCTCTATGTACA-3'	852-1265 bp	Roux et al. (1997) Mediannikov et al. (2004)
Intergenic spacers:			Fournier et al. (2004)
mppA-purC-F mppA-purC-R	5'-GCAATTATCGGTCCGAATG-3' 5'-TTTCATTTATTTGTCTCAAAATTCA-3'	155-197 bp	
dksA-xerC-F dksA-xerC-R	5'-TCCCATAGGTAATTTAGGTGTTTC-3' 5'-TACTACCGCATATCCAATTAAAAA-3'	164-292 bp	
rmpE-tRNA-F rmpE-tRNA-R	5'-TCAGGTTATGAGCCTGACGA-3' 5'-TTCCGGAAATGTAGTAAATCAATC-3'	297-417 bp	

PCR amplification of target sequences

The PCR amplifications were performed in a PTC-100™ thermal cycler according the protocol described by **Abdel-Shafy *et al.* (2012)** and **Abdullah *et al*. (2016a)**. PCR products were electrophoresed in 1.5 % agarose gels. The gels were stained with ethidium bromide. A 100 bp ladder was used with each gel. The Gel photos were analyzed by Lab Image software (BioRad).

Sequencing of PCR products

The PCR products were purified by ExoSAP-IT PCR Product Cleanup Kit according to manufacturer's recommendation. Sequencing reactions were performed in an MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM®BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme; Applied Biosystems), following the protocols supplied by the manufacturer. The sequencing was performed in Macrogen Center, Seoul, South Korea. Each sequencing reaction was repeated at least three times.

Data submission in GenBank

The sequences of *OmpA*, *gltA* and intergenic spacers were aligned, assembled and corrected using ChromasPro 1.49 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia), then the corrected *Rickettsia* sequences were submitted in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) to record each sequence with accession number.

Phylogenetic relationship and Multigene typing

Amplified sequences of each fragment were aligned using Blastn program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence homology searches against *Rickettsia* spp. GenBank database. Multiple sequences alignments for evolutionary relationships between new Egyptian records and other reference isolates were inferred using the ClustalW 1.8® program (**Dessen *et al.*, 1990**) after modification of sequences length by BioEdit sequence alignment editor (v. 7.0.9.0). In addition, the percent of the identity matrix were constructed by using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Using the MEGA4 software, two phylogenetic trees were constructed with the neighbor joining method (NJ) (**Saitou and Nei, 1987; Tamura *et al*., 2007**), and the unweighted pair group method with arithmetic mean (UPGMA) (**Dawyndt *et al*., 2006**). The evolutionary distances were calculated by the maximum composite likelihood method (**Tamura *et al*., 2004**). Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (**Felsenstein, 1985, 2004**).

Hematological and Biochemical profiles

Haematological parameters including total erythrocytic count (RBCs), total leucocytic count (WBCs), differential leucocytic count (DLC), haemoglobin (Hb) and packed cell volume (PCV) were done as described by **Schalm *et al.* (1986)**. All biochemical prameters were determined spectrophotometerically. Serum total protein (Biuret method) was determined according to **Gornal *et al.* (1949)**. Serum albumin was determined according to **Doumas *et al.* (1971)**. Serum globulin was determined by subtraction of serum albumin from serum total protein according to **Doumas *et al.* (1971)**. A/G ratio was estimated by dividing the albumin content on globulin value. Serum AST and ALT were determined according to **Reitman and Frankel (1957)**. Serum ALP was determined according to **Belfield and Goldberg (1971)**. Serum Urea was determined according to **Fawcett and Soctt (1960)**. Serum Creatinine was determined according to **Schirmeister (1964)**.

Statistical analysis

Statistical analysis for hematological and biochemical parameters was performed using Student's *t* test (SPSS 14.0 for Windows Evaluation Version). Probability values (P-value) < 0.05 were considered of statistical significant and < 0.001 were considered of high statistical significant.

RESULTS

During sampling, the main clinical signs were observed in the 61 tested camels ranged between apparently healthy (n = 49) to number of camels with fever (n = 9), anorexia (n = 6), Lethargy (n = 5), anemia (n = 8), enlargement of superficial lymph nodes (n = 2), and emaciation (n = 2). In addition, the five tick species found in Cairo, Giza, and Sinai were identified into *Hyalomma* species. Where, the camel tick *H. dromedarii* was the most dominant that recorded 91.9 % (Table 2). *H. marginatum*, *H. impeltatum*, *H. excavatum* and *H. rufipes* recorded low infestation (Table 2).

Table 2 The prevalence of *Rickettsia* spp. in camels and their tick by Gimenez stain and PCR

Camels and Ticks species	No.	Prevalence of Tick species (%)	No. of positives with *Rickettsia* spp.			
			Gimenez staining technique		PCR using *OmpA*&*gltA* genes	
			No.	%	No.	%
Camels	61	_	0	0	25	41
*Hayalomma*spp.	99	100	10/99	10.1	1/99	1.01
H. dromedarii	91/99	91.9	8/91	8.79	0	0
H. marginatum	5/99	5.05	1/5	20	1/5	20
H. excavatum	1/99	1.01	0	0	0	0
H. impeltatum	1/99	1.01	1/1	100	0	0
H. rufipes	1/99	1.01	0	0	0	0

Prevalence of tick-borne rickettsioses in camels and their tick vectors

The prevalence of *Rickettsia*spp. in camels and their tick species using Gimenez staining technique was 0 % and 10.1 %, respectively (Table 2). Moreover, all blood camels' specimens and their ticks were screened by PCR amplifying fragments of *OmpA* and *gltA* genes with the products sizes 500 to 600 bp and 1000 to 1200 bp, respectively (Fig.1). For more discrimination, the *Rickettsia* positive samples were further screened by intergenic spacers amplification (*mppA*, *dksA* and *rpmE*), where the obtained fragment sizes were 146, 229 and 344 bp, respectively. The total of 25/61 camel samples (4 *OmpA* and 23 *gltA*) and 1/99 ticks' samples (*H. marginatum* from Sinai province) were confirmed positive. Therefore, the prevalence of rickettsioses using PCR was 41.0 % in camels and 1.01 % in their tick species (Table 2).

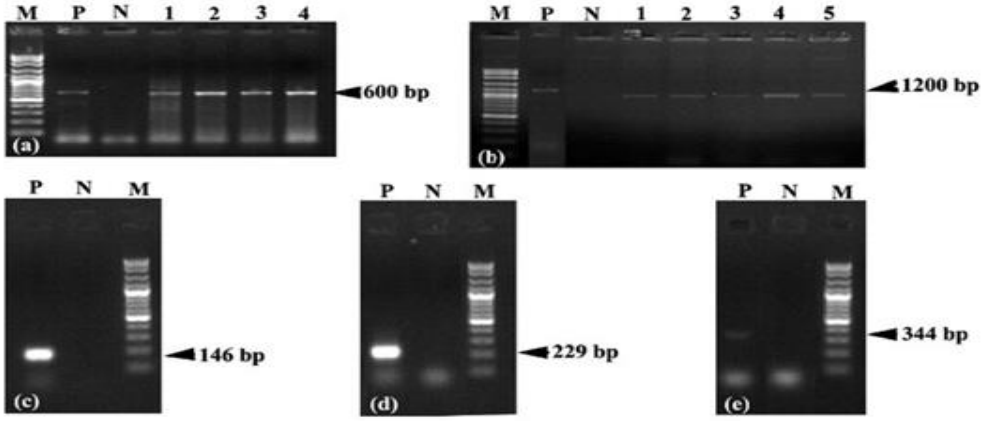

Fig 1 Molecular identification of *Rickettsia* spp. by PCR products detected in camels and *Hyalomma* species in 1.5 % agarose gels stained with ethidium bromide. In all figures, lane M: 100 bp DNA ladder and lane N: Control negative. (a) Lane P presents600 bp amplicon of *OmpA Rickettsia* positive tick sample and lanes 1 to4 present 600 bp amplicon of *OmpA Rickettsia* positive samples of camels. (b) Lane P presents1200 bp amplicon of *gltA Rickettsia* positive tick sample and lanes 1 to 5 present 1000 bp amplicon of *OmpA Rickettsia* positive samples of camels. (c) Lane P presents 146 bp amplicon of *mppA Rickettsia* positive tick sample. (d) Lane P presents 229 bp amplicon of *dksA Rickettsia* positive tick sample. (e) Lane P presents 344 bp amplicon of *rpmE Rickettsia* positive tick sample.

Sequences Analyses and Genbank Accession Numbers

The obtained Egyptian *Rickettsia* sequences of *OmpA* and *gltA* genes and intergenic spacers (*mppA*, *dksA* and *rpmE*) from *H. marginatum* tick were submitted in GenBank and registered with accession numbers KX819299, KX819298, KX819297, KX819295 and KX819296, respectively. The identities of obtained *Rickettsia* sequences were ranged from 97-100 % in comparison to *Rickettsia* strains recorded in Genbank (Table 3 and 4). The present results revealed that the Egyptian new isolates were similar to *R. africae* (KX819299, KX819298, KX819297, KX819295 and KX819296) and closely matching to the reference counterparts previously recorded in Saini-Egypt (HQ335132.1, HQ335126.1, HQ335143.1, HQ335138.1 and HQ335144.1), respectively (Table 3).Moreover, the partial sequence of *OmpA* gene of *Rickettsia* amplified from camel (no. 61) showed 48.68 % similarity with *R. africae* accession no. U83436.2 (Table 3).

Table 3 GenBank accession numbers of Egyptian Rickettsial amplified from camels and their tick vectors.

Sequence type	Animals and Ticks		Egyptian Rickettsial isolates	GenBank No.	Similarity with recorded *Rickettsia* species in GenBank		
	Species	Sex			Identity (%)	Covering (%)	Reference strains of *Rickettsia* spp.
OmpA	*H. marginatum*	♂	*Rickettsia africae*	KX819299	100	99	HQ335132.1
gltA				KX819298	100	94	HQ335126.1
mppA				KX819297	97	99	HQ335143.1
dksA				KX819295	99	99	HQ335138.1
rpmE				KX819296	99	100	HQ335144.1
OmpA	Camel (no. 61)	♂	*Rickettsia* spp.	_	48.68	_	U83436.2

HQ335132.1 = *Rickettsia africae* strain EgyRickHmm-Qalet El-Nakhl-2 outer membrane protein A (*OmpA*) gene, partial cds.
HQ335126.1 = *Rickettsia africae* strain EgyRickHd-Qalet El-Nakhl citrate synthase (*gltA*) gene, partial cds.
HQ335143.1 = *Rickettsia africae* isolate EgyRickHmm-Qalet El-Nakhl-11 *mppA-purC* intergenic spacer, partial sequence.
HQ335138.1 = *Rickettsia africae* isolate EgyRickHimp-El-Arish-4 *dksA-xerC* intergenic spacer, partial sequence.
HQ335144.1 = *Rickettsia africae* strain EgyRickHimp-El-Arish-6 RpmE (*rpmE*) gene, partial sequence; *rpmE-trnM* intergenic spacer, complete sequence; and tRNA-Met (trnM) gene, partial sequence.
U83436.2 = *Rickettsia africae*strain ESF 2500-1 cell surface antigen *rOmpA* (scaO) gene, partial cds.

Phylogenetic Relationships and Multi-genes Typing

The percent identity matrix of Egyptian Rickettsial sequences was constructed based on Clustal omega multiple alignments (Table 4), and the phylogenetic analyses of two genes and three intergenic spacers for each *Rickettsia* amplicon using two methods UPGMA (not shown) and NJ by MEGA4 using *Rickettsia felis* as outgroup (Fig.2). The NJ phylogenetic trees indicated that *R. africae* strains of *H. marginatum* were grouped together with other *R. africae* recorded in GenBank (Table 4 and Fig.2). The *dksA* and *rpmE* were fallen in a separate clade in the NJ trees (Fig.2 d, e) indicated a novel strain of *R. africae* within *H. marginatum* picked from camel from Sinai province. In addition, the similarity percent of the Egyptian *Rickettsia* camel *OmpA* amplicons was 48.68 in comparison to *R. africae* GenBank record U83436.2 (Table 4).

Table 4 Identity matrix generated with the nucleotide sequences obtained from the different *Rickettsia* spp. Isolates from *H. marginatumOmpA*(a), *gltA*(b), *mppA*(c), *dksA*(d) and *rpmE* (e) and camel 61 *OmpA* gene (f).

a) *OmpA*

1: KF791242	100.00	98.26	98.09	98.64	98.60	98.59	97.28	97.92	98.13	98.30	98.30	98.45
2: KT345980	98.26	100.00	99.83	98.99	98.98	99.13	97.64	98.92	98.32	98.48	98.48	98.65
3: DQ097082	98.09	99.83	100.00	98.82	98.81	98.96	97.47	98.66	98.15	98.32	98.32	98.48
4: U83436.2	98.64	98.99	98.82	100.00	100.00	99.48	97.79	98.96	99.05	99.21	98.58	99.33
5: GU247115	98.60	98.98	98.81	100.00	100.00	99.48	97.84	98.91	99.00	99.17	99.17	99.32
6: GQ853063	98.59	99.13	98.96	99.48	99.48	100.00	97.57	99.17	98.79	98.96	98.96	99.13
7: EU715288	97.28	97.64	97.47	97.79	97.84	97.57	100.00	98.44	97.32	97.48	96.69	97.47
8: KX819299	97.92	98.92	98.66	98.96	98.91	99.17	98.44	100.00	100.00	99.22	99.22	98.93
9: HQ335132	98.13	98.32	98.15	99.05	99.00	98.79	97.32	100.00	100.00	99.53	98.90	99.33
10: HQ335131	98.30	98.48	98.32	99.21	99.17	98.96	97.48	99.22	99.53	100.00	99.37	99.49
11: HQ335136	98.30	98.48	98.32	98.58	99.17	98.96	96.69	99.22	98.90	99.37	100.00	99.49
12: JQ691730	98.45	98.65	98.48	99.33	99.32	99.13	97.47	98.93	99.33	99.49	99.49	100.00

(b)*gltA*

1: AY743327	100.00	98.78	98.62	98.43	98.80	98.76	98.78	98.94	98.75	98.64	98.95	98.85	98.72	99.03	99.03	99.11	98.82	99.03
2: U59729	98.78	100.00	98.86	99.03	99.11	99.03	99.03	99.20	99.13	99.06	99.35	99.29	99.19	99.43	99.43	99.51	99.51	99.43
3: U59719	98.62	98.86	100.00	99.35	99.27	98.67	98.70	98.85	98.75	98.73	99.03	98.94	98.86	99.11	99.11	99.19	99.19	99.11
4: HQ335153	98.43	99.03	99a.35	100.00	99.36	98.94	98.87	99.12	99.04	98.98	99.19	99.21	98.96	99.27	99.27	99.35	99.30	99.27
5: DQ365804	98.80	99.11	99.27	99.36	100.00	99.12	98.95	99.30	99.23	99.07	99.27	99.38	99.12	99.35	99.35	99.43	99.44	99.35
6: JN043505	98.76	99.03	98.67	98.94	99.12	100.00	99.47	99.65	99.52	99.29	99.20	99.20	99.29	99.29	99.47	99.56	99.56	99.47
7: U59733	98.78	99.03	98.70	98.87	98.95	99.47	100.00	99.82	99.23	99.07	99.19	99.21	99.19	99.43	99.43	99.51	99.51	99.43
8: HM050288	98.94	99.20	98.85	99.12	99.30	99.65	99.82	100.00	99.42	99.47	99.38	99.38	99.47	99.47	99.65	99.74	99.74	99.65
9: KX819298	98.75	99.13	98.75	99.04	99.23	99.52	99.23	99.42	100.00	100.00	99.33	99.33	99.42	99.42	99.62	99.71	99.71	99.62
10: HQ335126	98.64	99.06	98.73	98.98	99.07	99.29	99.07	99.47	100.00	100.00	99.24	99.38	99.41	99.32	99.49	99.58	99.58	99.49
11: U59730	98.95	99.35	99.03	99.19	99.27	99.20	99.19	99.38	99.33	99.24	100.00	100.00	99.35	99.59	99.59	99.68	99.68	99.59
12: HM050292	98.85	99.29	98.94	99.21	99.38	99.20	99.21	99.38	99.33	99.38	100.00	100.00	99.38	99.56	99.65	99.65	99.65	99.65
13: HM050296	98.72	99.19	98.86	98.96	99.12	99.29	99.19	99.47	99.42	99.41	99.35	99.38	100.00	99.43	99.59	99.68	99.68	99.76
14: U59728	99.03	99.43	99.11	99.27	99.35	99.29	99.43	99.47	99.42	99.32	99.59	99.56	99.43	100.00	99.68	99.76	99.76	99.68
15: U59732	99.03	99.43	99.11	99.27	99.35	99.47	99.43	99.65	99.62	99.49	99.59	99.56	99.59	99.68	100.00	99.92	99.92	99.84
16: KU310587	99.11	99.51	99.19	99.35	99.43	99.56	99.51	99.74	99.71	99.58	99.68	99.65	99.68	99.76	99.92	100.00 1100.0	100.00	99.92
17: KM288711	98.82	99.51	99.19	99.30	99.44	99.56	99.51	99.74	99.71	99.58	99.68	99.65	99.68	99.76	99.92	0	100.00	99.92
18: DQ097081	99.03	99.43	99.11	99.27	99.35	99.47	99.43	99.65	99.62	99.49	99.59	99.56	99.76	99.68	99.84	99.92	99.92	100.00

(c) *mppA*

1: KX819297	100.00	48.39	46.24	48.39	48.39	47.62	48.15	48.89	48.89	49.07
2: EF140692	48.39	100.00	96.08	97.39	97.39	97.26	97.39	98.04	98.04	97.83
3: DQ008285	46.24	96.08	100.00	98.69	97.39	97.26	97.39	98.04	98.04	97.83
4: DQ008283	48.39	97.39	98.69	100.00	98.69	98.63	98.69	99.35	99.35	99.28
5: DQ008299	48.39	97.39	97.39	98.69	100.00	98.63	98.69	99.35	99.35	99.28
6: AY345087	47.62	97.26	97.26	98.63	98.63	100.00	98.75	99.38	99.38	99.34
7: HQ335142	48.15	97.39	97.39	98.69	98.69	98.75	100.00	98.98	99.49	99.35
8: KC870931	48.89	98.04	98.04	99.35	99.35	99.38	98.98	100.00	100.00	100.00
9: HQ335141	48.89	98.04	98.04	99.35	99.35	99.38	99.49	100.00	100.00	100.00
10: HQ335143	49.07	97.83	97.83	99.28	99.28	99.34	99.35	100.00	100.00	100.00

(d)*dksA*

1: KX819295	100.00	59.88	55.45	55.84	51.11	59.15	53.33	54.55	56.57	55.56	56.00
2: AY820021	59.88	100.00	87.11	92.94	88.72	93.97	92.64	84.06	87.5	88.72	94.67
3: HQ335138	55.45	87.11	100.00	100.00	93.83	92.89	96.51	97.77	93.30	93.75	98.31
4: HQ335140	55.84	92.94	100.00	100.00	96.09	98.27	96.51	97.18	97.74	98.31	98.31
5: HQ335139	51.11	88.72	93.83	96.09	100.00	93.43	95.93	94.25	90.27	90.71	97.75
6: AY428741	59.15	93.97	92.89	98.27	93.43	100.00	98.21	89.62	92.36	92.6	100.00
7: EF215902	53.33	92.64	96.51	96.51	95.93	98.21	100.00	97.09	97.67	98.26	98.26
8: AY820036	54.55	84.06	97.77	97.18	94.25	89.62	97.09	100.00	90.42	90.83	98.88
9: AY820034	56.57	87.50	93.30	97.74	90.27	92.36	97.67	90.42	100.00	97.05	99.44
10: AY820026	55.56	88.72	93.75	98.31	90.71	92.60	98.26	90.83	97.05	100.00	100.00
11: KR492916	56.00	94.67	98.31	98.31	97.75	100	98.26	98.88	99.44	100.00	100.00

(e) *rpmE*

1: KX819296	100.00	45.45	45.45	45.00	46.10	46.53	46.53	45.45	47.16	48.50	43.56	45.82
2: DQ008250	45.45	100.00	97	97.00	97	97.00	97.00	97.5	88.00	87.00	91.48	92.23
3: DQ008248	45.45	97.00	100.00	99	99.00	99	99.00	99.50	89.00	88.00	92.61	93.26
4: KF539830	45.00	97.00	99.00	100.00	98.65	98.65	98.65	99.50	91.25	90.88	92.61	93.77
5: KC870937	46.10	97	99	98.65	100	98.82	98.81	99.5	92.28	91.94	92.61	93.77
6: HQ335144	46.53	97.00	99.00	98.65	98.82	100.00	100.00	99.50	92.42	92.08	92.61	93.77
7: HQ335145	46.53	97.00	99	98.65	98.81	100.00	100.00	99.50	92.40	92.08	92.61	93.77

8: AY836521	45.45	97.5	99.50	99.50	99.50	99.5	99.50	100.00	89.5	88.5	93.18	93.78
9: HQ335165	47.16	88.00	89	91.25	92.28	92.42	92.40	89.50	100.00	99.03	88.39	95.56
10: HQ335164	48.5	87.00	88.00	90.88	91.94	92.08	92.08	88.50	99.03	100.00	87.95	95.54
11: DQ008256	43.56	91.48	92.61	92.61	92.61	92.61	92.61	93.18	88.39	87.95	100.00	90.18
12: DQ440626	45.82	92.23	93.26	93.77	93.77	93.77	93.77	93.78	95.56	95.54	90.18	100.00

(f) Camel 61

1: Camel 61	100.00	48.34	48.18	48.18	48.18	48.34	48.34	48.34	48.18	48.34	47.87	48.03	48.03	48.68	48.50	48.34	48.03	47.55	47.48	47.64	47.64	47.80
2: U83442.1	48.34	100.00	97.51	97.45	96.88	97.16	97.01	97.05	97.05	96.98	96.83	97.20	97.64	97.17	97.05	97.11	96.04	96.48	96.70	96.95	96.95	96.86
3: U83456.1	48.18	97.51	100.00	98.33	97.89	98.20	97.98	98.02	98.02	98.02	97.86	98.24	98.68	98.11	97.92	98.11	96.25	96.66	96.85	97.10	97.10	97.07
4: U83451.1	48.18	97.45	98.33	100.00	97.83	98.17	97.96	97.93	97.99	97.93	97.83	98.21	98.59	97.96	97.86	98.05	96.04	96.32	96.54	96.86	96.86	96.89
5: U83455.1	48.18	96.88	97.89	97.83	100.00	99.46	98.33	98.43	98.43	98.43	98.33	98.65	98.65	98.68	98.58	98.77	95.71	96.00	96.13	96.47	96.47	96.35
6: U83439.1	48.34	97.16	98.20	98.17	99.46	100.00	98.61	98.71	98.71	98.71	98.61	98.93	98.93	98.96	98.86	98.99	96.08	96.27	96.46	96.81	96.81	96.68
7: U83453.1	48.34	97.01	97.98	97.96	98.33	98.61	100.00	99.84	99.84	99.78	98.62	99.06	98.68	98.62	98.49	98.55	95.78	96.20	96.20	96.51	96.51	96.48
8: U83448.1	48.34	97.05	98.02	97.93	98.43	98.71	99.84	100.00	99.94	99.87	98.71	99.15	98.77	98.71	98.59	98.65	95.82	96.23	96.23	96.54	96.54	96.51
9: U83443.1	48.18	97.05	98.02	97.99	98.43	98.71	99.84	99.94	100.00	99.87	98.71	99.15	98.77	98.71	98.59	98.65	95.82	96.23	96.23	96.54	96.54	96.51
10: U83440.1	48.34	96.98	98.02	97.93	98.43	98.71	99.78	99.87	99.87	100.00	98.71	99.15	98.77	98.71	98.59	98.65	95.82	96.23	96.23	96.54	96.54	96.51
11: U83441.1	47.87	96.83	97.86	97.83	98.33	98.61	98.62	98.71	98.71	98.71	100.00	99.37	98.62	98.62	98.43	98.43	95.69	96.10	96.32	96.57	96.57	96.48
12: U83437.1	48.03	97.20	98.24	98.21	98.65	98.93	99.06	99.15	99.15	99.15	99.37	100.00	98.99	98.87	98.74	98.81	96.01	96.51	96.61	96.92	96.92	96.83
13: U83454.1	48.03	97.64	98.68	98.59	98.65	98.93	98.68	98.77	98.77	98.77	98.62	98.99	100.00	98.81	98.74	98.84	96.32	96.67	96.95	97.20	97.20	97.17
14: U83436.2	48.68	97.17	98.11	97.96	98.68	98.96	98.62	98.71	98.71	98.71	98.62	98.87	98.81	100.00	99.12	98.99	95.82	96.35	96.42	96.67	96.67	96.64
15: U83452.1	48.50	97.05	97.92	97.86	98.58	98.86	98.49	98.59	98.59	98.59	98.43	98.74	98.74	99.12	100.00	98.99	95.75	96.17	96.20	96.51	96.51	96.48
16: U83449.1	48.34	97.11	98.11	98.05	98.77	98.99	98.55	98.65	98.65	98.65	98.43	98.81	98.84	98.99	98.99	100.00	95.85	96.23	96.35	96.73	96.73	96.64
17: U83447.1	48.03	96.04	96.25	96.04	95.71	96.08	95.78	95.82	95.82	95.82	95.69	96.01	96.32	95.82	95.75	95.85	100.00	96.04	96.35	96.73	96.73	96.54
18: U83446.1	47.55	96.48	96.66	96.32	96.00	96.27	96.20	96.23	96.23	96.23	96.10	96.51	96.67	96.35	96.17	96.23	96.04	100.00	97.36	97.61	97.61	97.68
19: U83438.1	47.48	96.70	96.85	96.54	96.13	96.46	96.20	96.23	96.23	96.23	96.32	96.61	96.95	96.42	96.20	96.35	96.35	97.36	100.00	99.22	99.22	98.93
20: U83445.1	47.64	96.95	97.10	96.86	96.47	96.81	96.51	96.54	96.54	96.54	96.57	96.92	97.20	96.67	96.51	96.73	96.73	97.61	99.22	100.00	100.00	99.25
21: U83444.1	47.64	96.95	97.10	96.86	96.47	96.81	96.51	96.54	96.54	96.54	96.57	96.92	97.20	96.67	96.51	96.73	96.73	97.61	99.22	100.00	100.00	99.25
22: U83450.1	47.80	96.86	97.07	96.89	96.35	96.68	96.48	96.51	96.51	96.51	96.48	96.83	97.17	96.64	96.48	96.64	96.54	97.68	98.93	99.25	99.25	100.00

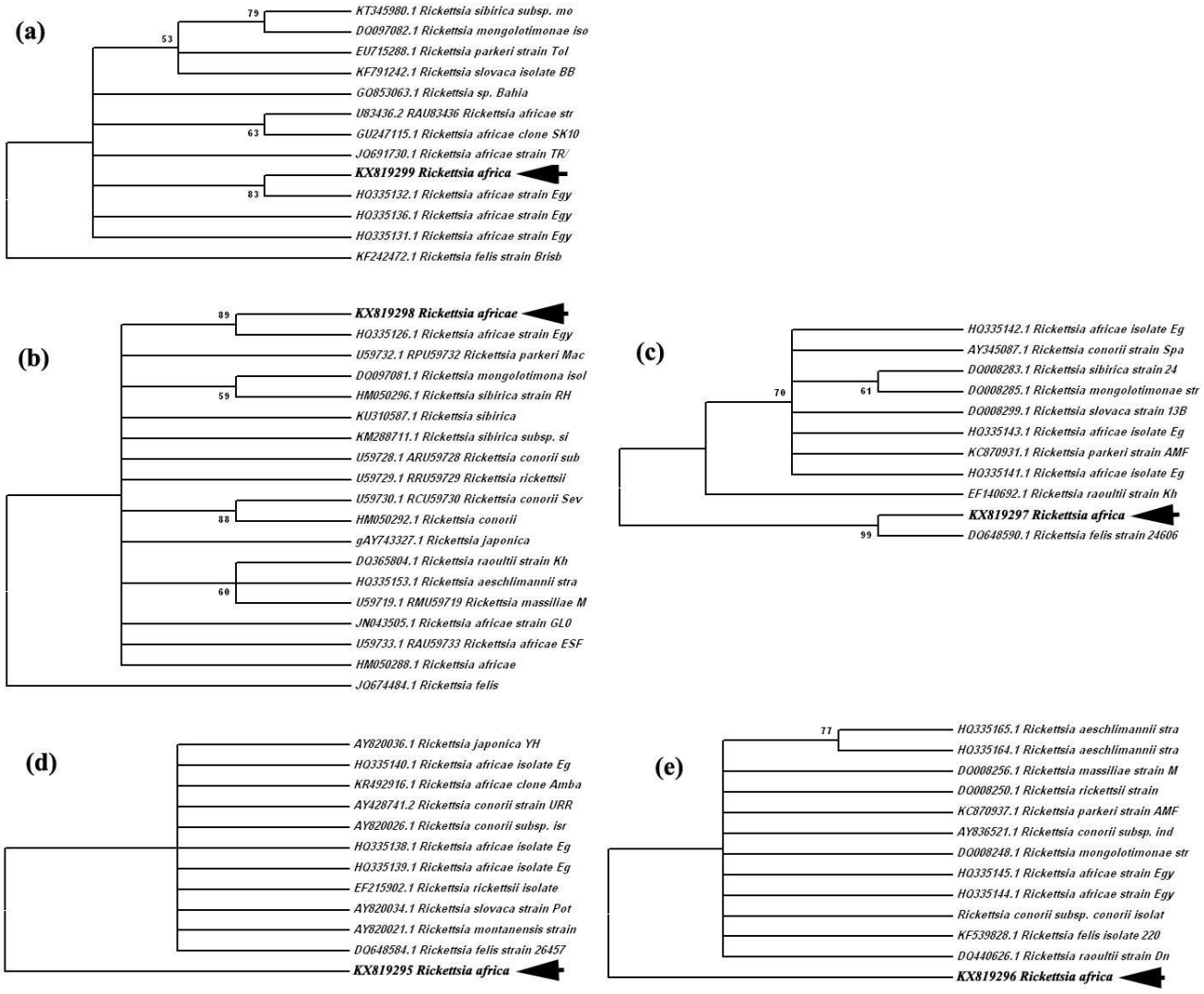

Fig 2 Phylogenetic trees of *R. africae* detected in the present study based on the sequences of two genes (*OmpA* and *gltA*) and the three intergenic spacers (*mppA*, *dksA* and *rpmE*). All sequences were aligned and Neighbor-joining trees were constructed; (a)*OmpA*, (b)*gltA*, (c)*mppA*, (d)*dksA*and(e) *rpmE*.

Epidemiological Profile on rickettsioses in the studied camels

The 61 camels investigated during the present study were divided into groups according to age, breeds and tick infestation at time of examination. The results revealed the highest prevalence of rickettsioses among aged, Abady breeds and ticks-infested camels (Tables 5 and 6).

Table 5 The prevalence of rickettsioses among camels screened by PCR with regards to age groups.

Age Groups (Years)	Total No. of Tested Camels by PCR	No. of Positive Camels by PCR	The prevalence rate (%)
< 11	16/61	4/16	25.0
11 – 13	16/61	3/16	18.7
13 – 15	6/61	2/6	33.3
15 – 17	11/61	8/11	72.7
17 – 19	5/61	4/5	80.0
> 19	7/61	4/7	57.1
Total	61	25/61	41.0

Table 6 The prevalence of rickettsioses among camels screened by PCR with regards to Breed, and Ticks Infestation.

Parameters	Breeds		Tick Infestation	
	Abady	Beshary	Ticks infested camels	Ticks free camels
No. of tested camels by PCR	37/61	24/61	14/61	47/61
No. of positive camels by PCR	21/37	4/24	6/14	19/47
The prevalence rate (%)	56.8	16.7	42.8	40.4

Hematological and biochemical changes in camels with rickettsioses

The hematological and biochemical tests were applied on 61 camels of which 25 were proved *Rickettsia* infected camels by PCR and 36 were *Rickettsia* free camels. Macrocytic anemia and leucopenia were recorded in the *Rickettsia* positive camels (Table 7), while there were no significant differences in the biochemical changes between *Rickettsia* positive and negative camels (Table 8).

Table 7 Hematological parameters of Rickettsial infected camels compared with Rickettsial free camels (Mean ± SD).

Hematological Parameters	Animal Groups	
	Rickettsial Free Camels	Rickettsial Diseased Camels
RBCs ($\times10^{6}$)	5.02±0.15	5.95±0.23**
Hb (g/dl)	13.92±0.58	19.13±1.05**
PCV (%)	40.75±1.85	55.00±3.16**
MCV (fl)	80.75±1.68	90.56±2.35*
MCH (pg)	27.72±0.54	31.62±0.75**
MCVC (g/dl)	34.46±0.40	33.78±1.31
Platelets ($\times10^{3}$)	95.16±2.06	96.66±2.59
WBCs ($\times10^{3}$)	11.48±0.31	9.15±0.51**
Neutrophils (%)	81.75±0.65	83.20±0.59
Lymphocytes (%)	11.77±0.43	11.20±0.56
Monocytes (%)	5.02±0.32	4.04±0.29
Eosinophils (%)	1.55±0.11	1.54±0.14

* = significant at P< 0.05 ** = highly significant at P< 0.01

Table 8 Biochemical parameters of Rickettsial infected camels compared with Rickettsial free camels (Mean ± Standard Deviation; SD).

Biochemical Parameters	Animal Groups	
	Rickettsial Free Camels	Rickettsial Diseased Camels
Total Protein (g/dl)	7.70±0.79	7.58±0.17
Albumin (g/dl)	2.37±0.34	2.43±0.22
Globulin (g/dl)	5.16±0.39	5.05±0.27
Albumin/Globulin Ratio	0.54±0.08	0.61±0.11
GOT (AST; IU/L)	58.40±6.59	53.30±4.95
GPT (ALT; IU/L)	47.54±5.63	35.70±2.88
ALP (IU/L)	55.97±4.73	55.61±3.98
Creatinine (mg/dl)	2.47±0.16	2.42±0.06
Urea (mg/dl)	72.37±4.80	45.57±2.86

DISCUSSION

Globalization, international trade, urbanization, climate change, travel and animals' mobility are factors that led to rapid extension of the zoogeographical range of many tick species, subsequently, tick-borne diseases (**Shaw *et al*., 2003; Harrus and Baneth, 2005**). Therefore, researches on ricketsiae are exceeded because of their public health implication, zoonotic importance and worldwide distribution (**Parola and Raoult, 2001; Dantas-Torres *et al*., 2012; Kernif *et al*., 2012b; Parola *et al*., 2013**).

In Egypt, few studies have been undertaken on the epidemiology of rickettsioses infection in camels as reservoirs of *Rickettsia* spp. The main objective of this study is to evaluate the clinical, hematological, and biochemical profiles of camel rickettsioses and their molecular diagnostic investigations to confirm the previously detected and/or novel genotypes of *Rickettsia* in Egypt.

The main clinical signs observed in the 61 studied camels were similar to those mentioned by **Wernery *et al.* (2001)** who reported some clinical characteristic of rickettsiosis as lethargy, emaciation, recumbency and enlarged edematous lymph nodes that agreed with the findings of the present study. Concerning the apparently healthy camels, the results agreed with other reportsstated thatno statistically significant differences were found between clinically healthy and sick animals (**Kelly *et al*., 1992; Solano-Gallego *et al*., 2006; Ortuno *et al*., 2009; Riveros-Pinilla *et al*., 2015**).

In the present study, it was found that *H. dromedarii* was the most abundant tick species on camels, while other *Hyalomma* spp recorded very low infestation rate in agreement with previous findings recorded by **Abdel-Shafy (2000) ; Diab *et al*. (2001); El-Kammah *et al*. (2001); Abdel-Shafy *et al*. (2012); Abdullah *et al*. (2016a).**

Gimenez staining technique of camels'blood and tick hemolymph staining revealed that the prevalence of *Rickettsia* spp. was 0 % and 10.1 %, respectively (Table 2). The negative results of Gimenez staining technique in camel blood films may return to the low numbers of rickettsiae circulating in the blood and had probably cleared from blood (**Breitschwerdt *et al.*, 1990; Parola *et al*., 2005**). While, hemolymph staining was successful as a field test for detection of *Rickettsia* in ticks which kept ticks undamaged, so that the infected ticks can be used in other purposes (**Gimenez, 1964**). However, the susceptibility of the Gimenez stain to other bacterial agents than *Rickettsia* justified the magnified prevalence percentage of infection in ticks by staining technique and needed to be confirmed by PCR; the more specific technique (**Parola *et al*., 2013; Guillemi *et al*. 2015**).

Here, PCR technique was carried out on 61 camels' blood samples and their ticks using *OmpA* and *gltA* genes; SFP specific primers (**Parola *et al*., 2013; Guillemi *et al*., 2015**). The results revealed that twenty-five camels, from Cairo, Giza and Sinai provinces and one tick (*H. marginatum*) from Sinai province, were positive for *Rickettsia* spp. infection. The samples from positive animals and ticks were additionally screened by intergenic spacers (*mppA*, *dksA* and *rpmE*) amplification and sequencing. The prevalence of *Rickettsia* spp. in camels was 41.0 % and 1.01 % in *Hyalomma* spp. (Table 2). In the previous studies, *Rickettsia* spp. were identified in camel blood film stain from Dubai (**Wernery *et al*., 2001**) and 18.8% of camel blood samples by PCR from Nigeria (**Kamani *et al*., 2015**). On the other hand, **Mentaberre *et al*. (2013)** reported that 83 % of camels were found infected with *Rickettsia* spp. serologically by ELISA. However, the detection of SFG *Rickettsia* spp. in the present study indicated the importance role of camels in the persistence of *Rickettsia* in the nature than previously thought (**Wernery and Kaaden, 2002; Kamani *et al*., 2015**). Furthermore, our results of *Rickettsia* positive ticks were similar to those were previously reported by **Niebylski *et al*. (1999)**, **Levin *et al*. (2009)** and **Socolovschi *et al*. (2009a)** that the naturally infection rate of ticks with rickettsiae almost is < 1% because of the lethal effects of *Rickettsia*. In the previous studies carried out in Egypt, SFG were detected in *Rh. sanguineus* and *Hyalomma* species at Sinai by immunostaining and PCR (**Lange *et al*., 1992**) while **Loftis *et al*. (2006a, b)** detected *R. aeschlimannii* in *Hyalomma* spp. by PCR. Moreover, **Abdel-Shafy *et al.* (2012)** were the first to report *R. africae* in *H. dromedarii*, *H. impeltatum* and *H. marginatum*, and *R. aeschlimannii* in *H. impeltatum* and *H. marginatum* collected from camels in Sinai.

Sequencing and phylogenetic analyses were performed on *OmpA* and *gltA* genes and intergenic spacers (*mppA*, *dksA* and *rpmE*) amplified from camels and *Hyalomma* spp. The present results revealed that the Egyptian obtained *R.*

africae records; KX819299, KX819298, KX819297, KX819295 and KX819296, were highly similar to the reference counterparts; HQ335132.1, HQ335126.1, HQ335143.1, HQ335138.1 and HQ335144.1, which were obtained previously from Sinai province in Egypt (**Abdel-Shafy *et al*., 2012**). Nonetheless, the topology inferred from non-coding intergenic spacers, illustrates a relationship between Egyptian isolates and other *Rickettsia* spp. The presence of obtained strains in a separate clade in the NJ trees of *dksA* and *rpmE* sequences (Fig.2d, e) were in accordance to the fact that non-coding intergenic spacers are able to identify a single *Rickettsia* spp. (**Fournier *et al*., 2004**). Therefore, the present results suggested the novelty of the obtained strain of *R. africae* in *H. marginatum* collected from camels in Sinai province. These results were in agreement with **Abdel-Shafy *et al*. (2012)** who were the first to identify *R. africae* in *H. dromedarii*, *H. impeltatum* and *H. marginatum* from Sinai province. Moreover, *R. africae* was detected in *H. dromedarii* on camels from Algeria (**Kernif *et al*., 2012a**), while in Israel it was detected in *H. dromrdarii*, *H. impeltatum*, *H. excavatum* and *H. turanicum* (**Kleinerman *et al*., 2013**). Although, *R. africae* in South Africa was associated only with *Amblyomma spp.* (**Parola and Raoult, 2001; Parola *et al*., 2005**). The present study confirmed that *Hyalomma* spp. have a potential role as a vector for *R. africae* (ATBF) in North Africa (**Abdel-Shafy *et al*., 2012; Kernif *et al*., 2012a**).

Regarding the phylogenetic analyses of camels *Rickettsia* spp., which were positive by *OmpA* amplification, the similarity percent to other reference *Rickettsia* strains published by Fournier et al. (1998) was 48.68 % with *R. africae* isolate U83436.2 (Table 4). The results revealed that the *Rickettsia* spp. detected in camel (no. 61) from Sinai province was closely matching to *R. africae*. Furthermore, the present Egyptian isolates were clustered in a separate clade with higher similarity to the reference counterparts (HQ335132.1, HQ335136.1 and HQ335131.1) which were obtained previously from Sinai province in Egypt, as well (**Abdel-Shafy *et al*., 2012**) (Fig.3). These results suggested that the presence of *R. africae* strain in camel. Accordingly, this is the first molecular detection of *Rickettsia* DNA in camels in Egypt. In addition, *Hyalomma* spp. are the main camel ticks in Egypt and North Africa (**Abdel-Shafy, 2000; Diab *et al*., 2001; El-Kammah *et al*., 2001; Abdel-Shafy *et al*., 2012**). The present study confirmed that *Hyalomma* spp. have a potential role as a vector for *R. africae* in camels. Moreover, the detection of *R. africae* in *H. marginatum* and in its camel from Sinai province indicated that *R. africae* can act as an emerging pathogen in Sinai province.

Concerning the ageand breed susceptibility, the highest infection rate of rickettsioses was recorded in age groups of 17 to 19 years and Abady camel breed, respectively as shown in tables (5 and 6). Though there were limited data on age or breed susceptibility in camels, other studies were applied on dogs and horses concluded the absence of statistical association between infection rate with *Rickettsia* spp. and age, sex and breed **Riveros-Pinilla *et al.* (2015)**. Concurrently, Cunha et al. (2014) observed that older animals were more reactive with *Rickettsia* than younger animals, which may be due to the prolonged and/or repetitive exposure of older animals to ticks infected with *Rickettsia* spp. and/or senile lower immunity. Hence, the present study revealed a significant difference among age groups.

The infection rate with rickettsioses was relatively higher in ticks-infested camels (42.8 %) than in ticks-free camels (40.4 %), as shown in table (6). The present results indicated that camels infested by ticks were at high risk to be positive for *Rickettsia* spp. because *Hyalomma* spp. were reported as the principle vector of rickettsioses in Egypt. However, some camels infested by ticks were negative for rickettsioses in the present study, this may be attributed to the fact that attached ticks were free from *Rickettsia* spp. or were infected with *Rickettsia* but they recently attached to these camels and yet to transmit the infection to their hosts. Furthermore, the ticks-free camels (at the time of examination) which were proved positive for *Rickettsia*, might be infested previously by ticks, which possibly had been removed manually and/or received various acaricide treatments.

Moreover, hematological and biochemical profiles in studied camels were recorded as shown in tables (7 and 8). The presented results revealed that macrocytic anemia and leucopenia were recorded in *Rickettsia* positive camels. The leucopenia recorded in this study may be attributed to the decrease in monocytes and lymphocytes. While, no significant differences were reported in biochemical changes between *Rickettsia* positive and negative camels. However, the available data on hematological and biochemical parameters in camels are limited, previous experimental studies were applied on dogs recorded anemia and early leukopenia during the course of disease followed by progressive leukocytosis and severe thrombocytopenia (**Gasser *et al*., 2001; Elchos and Goddard, 2003; Parola *et al*., 2005; 2013**). Similarly, **Scorpio *et al*. (2008)** reported no specific hematologic or biochemical differences between seronegative and seropositive dogs.

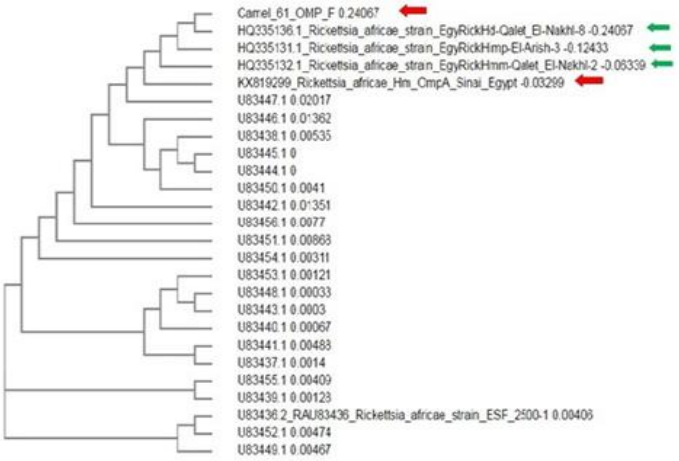

Fig 3 Cladogram of current molecular epidemiological status of the Egyptian *Rickettsia* spp. isolates that compare the ones obtained during the present study from Camels and its tick (*H. marginatum;* red arrows), with other *Rickettsia* spp. records of local (green arrows) and international isolates within Genbank database dependent on alignment of *OmpA* genes sequences constructed by the Clustal omega multiple alignments software utilizing NJ equation.

CONCLUSION

A novel strain of *R. africae* was detected in *H. marginatum* picked from camel from Sinai province that was dissimilar from previous Egyptian isolates by molecular characterization. This is the first detection of *Rickettsia* DNA in camels by PCR in Egypt with the prevalence rate 41.0 %. Moreover, the detection of *R. africa* in *H. marginatum* and its camel from Sinai indicated that *R. Africa* act as an emerging pathogen in Sinai province. Rickettsioses has tobe included during examination of imported animals as exotic diseases as well as the differential diagnosis of non-specific febrile illness of camels. Further, the detection of tick-borne *Rickettsia* in camels and their ticks not only indicates that camels' populations in Egypt are at risk, but also presents possible zoonotic implications in human populations since *Hyalomma* spp. were known to be aggressive to bite human, which likely can facilitate the transmission of *Rickettsia* to human. In conclusion, our data indicates that camels may play a role in persistence of*Rickettsia*in Egypt. Thus, further investigations are warranted to better understand the epidemiological dynamics of *Rickettsia*; survivalwithin vector populations, host species, and the horizontal transmission between vector and host species.

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SCICELL

REGULAR ARTICLE

PHYTOCHEMICALS, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF Senna alata AND Senna tora LEAF EXTRACTS AGAINST BACTERIAL STRAINS CAUSING SKIN INFECTIONS

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ABSTRACT

A study was carried out to screen for phytochemical constituents and assess the antioxidant and antimicrobial activities of *Senna alata* and *Senna tora* leaf extracts. The leaves were first dried at room temperature and 50°C in an oven prior to solvent extraction using ethanol and methanol. The *in-vitro* qualitative assays showed that both *S. alata* and *S. tora* leaf extracts contained bioactive and secondary metabolites components such as tannins, steroids, saponin, terpenoids, glycosides, flavonoids and phenols. The antioxidant activity and capacity test were carried out by conducting free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and Ferric reduction antioxidant plasma (FRAP) assays. Both assays showed *S. tora* leaf extract has higher antioxidant capacity than *S. alata* leaf extract. The efficacy of these leaf extracts were tested against skin pathogens through agar well diffusion method. *S. alata* extract showed an inhibition zone (1.15 – 1.59 mm) against *Pseudomonas aeruginosa* while *S. tora* extracts exhibited a strong antimicrobial activity against *S. epidermidis* (inhibition zone of 12 – 16.94 mm) followed by *P. aeruginosa* (inhibition zone of 1 – 1.59 mm). Nonetheless, no inhibition zone was observed for *S. aureus* by both leaf extracts. The phytochemicals and antioxidant constituents as well as inhibitory potential on skin pathogens possessed by *S. alata* and *S. tora* leave highlighted their potential utilization in the development of natural drugs or cosmetics to treat skin related diseases or infections.

Keywords: *Senna alata; Senna tora;* skin infections; antioxidant; phytochemical; antimicrobial

INTRODUCTION

Medicinal plants have been used to treat various skin diseases for many decades. Medicinal plants may seem as old and conventional in treating skin disease but the result from using the medicinal plants mostly showed a positive result. Also, medicinal plant as natural remedies in treating skin disease typically will not give any harm on human external and internal body. Medicinal plants are being used in treating skin diseases like dermatitis, atopic dermatitis, eczema, furunculosis and other inflammatory diseases (**Dawid, 2013**).

Skin microflora can be pathogenic, commensalism or mutualism depending on the human's immune system (**Cogen *et al.*, 2008**). The presence of skin flora could affect the behavior of the cell below the skin surface. For example, the ability for the skin to heal the inflammation after injury is reduced due to the presence of skin flora (**Lai *et al.*, 2004**). According to **Naik *et al.* (2015)** bacteria presences inside the mice skin have the ability to affect the immune response. Quarter to half of nowadays medicine products derived from plants are being used as antimicrobial agents. This is due to the plants attribute that is enriched with secondary metabolites components like terpenoids, tannins, alkaloid, and flavonoids (**Cowan, 1999**). For instant, flavonoid constituents that are found in the medicinal plant can exhibit inhibitory effect against various viruses (**Pengsuparp *et al.*, 1995**).

The development of plant-based product is rapidly increasing especially in many developing countries due to efficiency and ability to cure many diseases such as skin diseases (**Oluduro and Omoboye, 2010**). Besides, the effectiveness of the plants in curing diseases had been proven by our ancestor. For example, traditionally, *Senna alata* leaf is dried and grinded before it is mixed with kerosene and the mixture may then be applied to inflammation skin. In addition, in Africa, many of herbal medicines are being used as decoction in cold water, mix into food or alcohol to treat bacterial infections (**Oluduro and Omoboye, 2010, He *et al.*, 2015).**

S. alata was originated from Ghana and Brazil but today, it is spread widely into Americas and all over Africa, and Nigeria (**Adelowo and Oladeji, 2017**). In Malaysia, the plant is known as "gelenggang". Therapeutic properties such as antibacterial, antimicrobial, antifungal and analgesic are reported for different parts of the plant. For instant, the *S. alata* leaf is claimed to have an efficiency to treat skin diseases in animal and man (**Igoli *et al.*, 2005**). In addition, it is also has been reported for the treatments of constipation, haemorrhoids, intestinal parasitosis, inguinal hernia, blennorrhagia, diabetes and syphilis and proven to have the ability to cure ringworm (**Abo *et al.*, 2008**).

Senna tora is generally distributed throughout India, Sri Lanka, West China and tropics. In Malaysia the plant is known as "ketepeng cina". Different parts of *S. tora* are known to possess various ailments by rural and tribal people due to its moist quality, sweet flavor and cooling property of herb, it acts on liver and large intestine channels, clear the heat and liver fire, and nourishing the large intestine (**Kirtikar and Basu, 1998**). In addition, the leaves and seeds are also useful in treatment of leprosy, ringworm, flatulence, bronchitis, cough and cardiac disorders (**Jain and Patil, 2010**).

The present study aimed *to evaluate the phytochemical constituents,* antioxidant properties and antimicrobial activity of *S. alata* and *S. tora* leaf extracts against skin diseases causing bacteria, *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Collection and preparation of leaf extracts

S. tora and *S. alata* leaves were collected from the Taman Pertanian, Universiti Putra Malaysia. First, the leaves were washed to remove undesired substances on the surface of the leaves and were allowed to dry. The leaves were then cut into small sizes and weighed. Two types of drying methods had been used to dry the leaves prior to solvent extraction step which were oven drying at 50˚C for 8 hours and drying at room temperature for 3 days. After the drying procedures, the samples were grinded using a blender to get the desirable size. The samples in powder form were then kept in labeled bottles at 27°C.

Solvent Extraction Method

A weight of 1 g of *S. tora* and *S. alata* leaves powdered samples were mixed with 100 mL of 95% (v/v) ethanol or 80% (v/v) methanol into conical flasks (**Rabeta and Lin, 2015**). The flasks were wrapped with an aluminium foil. The mixtures were shaken for overnight at 160 rpm shaker (CERTOMAT BS-1, Boston Laboratory Equipement, MA), 27°C. The mixtures were then centrifuged (Eppendorf, Eppendorf Asia Pacific, Malaysia) at 2500 rpm for 30 minutes to obtain a clear solution. The solutions were stored at 4°C until used.

Phytochemical analysis

Total flavonoids content

Total flavonoids content was determined by using an aluminum colorimetric method and quercetin (QE) was used as a standard (**Iqbal *et al.*, 2015**). 0.5 mL of extracts and quercetin were placed in test tubes. Then, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate, 1.5 mL of 80% (v/v) methanol and 2.8 mL of distilled water were placed in the test tubes. The tubes were then incubated at room temperature for 30 minutes. The absorbance was read by using a spectrophotometer (Libra S12, Biochrom, UK) at 415 nm and the total flavonoid content was expressed as mg QE/g dw.

Total phenolic content

The total phenolic content was performed by the method as described by **Baba and Malik** (**2015**). Briefly, 3 mL of *S. alata* and *S. tora* leaf extracts were added with 0.5 mL of Follin Ciocalteu's Phenol reagent and let to sit for 3 minutes. Then, 2 mL of 20% (w/v) sodium carbonate were added and the mixture was allowed to stand in the dark for 60 min. Following that, the absorbance was read at 650 nm by using a spectrophotometer (Libra S12, Biochrom, UK). The total phenolic content was calculated from the gallic acid (GAE) calibration curve and the results were expressed as mg GAE/g dw.

Test for glycosides

Two mL of acetic acid was added to 1 mL of leaf extracts and the solutions were then cooled in an ice bath at 4°C. 1 mL of concentrated tetraoxosulphate (VI) acid (H_2SO_4) was then added drop wise to the mixture. The formation of an oil layer on top of the solution indicates the presence of glycosides.

Test for saponins

Five drops of olive oil was added to 2 mL of leaf extracts and the mixtures were shaken vigorously. The formation of a stable emulsion indicates the presence of saponins.

Test for tannins

Two drops of 5% $FeCl_3$ was added to 1 mL of leaf extracts. The appearance of a dirty-green precipitate indicates the presence of tannins.

Test for steroids

One mL of concentrated tetraoxosulphate (vi) acid (H_2SO_4) was added to 1 mL of leaf extracts. The red colouration indicates the presence of steroids.

Test for terpenoids (Salkowski test)

The leaf extracts in a final volume of 3 mL were mixed with 1 mL of chloroform and 1 mL concentration of H_2SO_4 to observe an intense red-brown coloration as an indicative of the presence of terpenoids.

Antioxidant activity assay

DPPH scavenging activity

A wavelength of 515 nm of spectrophotometer (Libra S12, Biochrom, UK) was set up to measure the radical scavenging capacity of leaves extracts. Ten-fold dilutions of both methanolic and ethanolic leaf extracts were added to 1.2 mL solution of DPPH (100 µM in methanol). A control solution was prepared by adding 0.2 mL methanol instead of the extract samples. After 30 minutes, the absorbance was measured. Determination of radical scavenging was calculated as follows (Equation 1):

$$\text{DPPH activity} = (\text{Absorbance}_{control}-\text{Absorbance}_{sample})/\text{Absorbance}_{control}\times 100 \quad \text{Equation 1}$$

Ferric reducing-antioxidant power (FRAP) assay

Modification of standard FRAP assay was performed as described by **Rabeta and NurFaraniza** (**2013**). 200 µL extracted samples were mixed with 3 mL of FRAP reagent in test tubes and the solutions were mixed thoroughly by a vortex. Blank samples were prepared with both methanol and ethanol extraction samples. Both samples were incubated in water bath for 30 minutes at 37°C. Then, determination of sample absorbances at 593 nm against blank were conducted. The FRAP value was expressed as µM of ferrous equivalent Fe (II) per gram of dried sample.

Antimicrobial tests

Determination of antimicrobial activity assay (agar well diffusion)

A modified agar well diffusion technique as reported by **Ehiowemwenguan *et al.*, (2014**) was done to determine the antibacterial activity of leaf extracts against *S. aureus*, *S. epidermidis* and *P. aeruginosa*. All the strains were generously provided by Bioprocessing and Biomanufacturing Research Centre (BBRC), Universiti Putra Malaysia. Firstly, the glycerol stock of each bacterial strain was streaked into sterile Luria-Bertani (LB) agar for *S. aureus* and nutrient broth for *S. epidermidis* and *P. aeruginosa* and incubated in an oven for 24 hours at 37˚C. Then a loop of single colony of each strain was separately transferred into 5 mL of LB broth containing *S. aureus*, and 5 mL of nutrient broth for each of *S. epidermidis* and *P. aeruginosa* colony. Then, they were incubated for overnight at 37˚C. Then, nutrient agar plate was prepared by pouring 25 mL of nutrient agar into empty plates. 60 µL of inoculum (optical density ± 0.020) of each tested microorganism (equivalent to 10^7 CFU/mL) were added into 6 mL of LB agar for *S. aureus* and 6 mL nutrient agar media for *S. epidermidis* and *P. aeruginosa*. The colony forming unit (CFU/mL) of each strains was determined based on the method as described by **Ming *et al.*, (2016**). Then, the warm LB and nutrient agar media were spread into agar plates. Four holes were made using backtips of micropipette tips onto the agars. 100 µL of methanol and ethanol made as negative control and penicillin as a positive control. Then, the plates were incubated for overnight at 37˚C. After that, the inhibition zones observed on the agars were measured by a ruler for each isolated tested microorganisms.

Determination of minimum inhibitory concentration (MIC)

MIC of the *S. alata* and *S. tora* leaf extracts were determined according to the method as described by **Bisht *et al.*, (2016**). The Muller Hinton Broth (MHB) was prepared and autoclave sterilized at 121°C for 15 minutes (Hirayama, Hirayama Manufacturing Corporation, Japan). 1.0 mL of the prepared MHB broth was dispensed into test tubes labeled from 1 to 5 using sterile syringe and needle. 1.0 mL of the 60 mg/mL leaf extract solutions were separately dispensed into tube labeled 1. After that, 1.0 mL of the solution was serially transferred into tube labeled 2 until tube labeled 5. Tube labeled 6 was filled with the sterile MHB solution to serve as a control for the sterility of the medium while tube labeled 7 contained sterile MHB solution and test organism as the control for the viability of the culture organisms. Test isolates (*S. epidermidis*, *S. aureus*, *P. aeruginosa*) were prepared in a sterile nutrient broth and were incubated in a shaker for overnight at 37˚C, 200 rpm. Following incubation, 1.0 mL of each strain inoculum was transferred into each tube (tubes 1- 7) except the tube 6 (control experiment) in which a new sterile broth was added. Leaf extracts were then added into each test tubes labeled 1 to 5 with the final concentration of each extract after dilution were 10.000, 5.000, 2.500, 1.250, and 0.625 mg/mL, respectively. All the tubes were incubated at 37°C for 24 hours. Lastly, the growths of the organisms were examined. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no or low turbidity) and lowest optical density (at 650 nm) for bacterial growth using a spectrophotometer (Libra S12, Biochrom, UK) when compared with the control tubes was regarded as MIC.

Determination of minimum bactericidal concentration (MBC)

Each test tube of MIC cultures (as described above) were then inoculated into the sterile Muller Hinton agar and incubated at 37°C for 24 hours. MBC is considered as the lowest concentration with no viable growth.

RESULTS AND DISCUSSION

Phytochemical screening of *S. alata* and *S. tora* leaves

The preliminary qualitative phytochemical components screening carried out for *S. alata* and *S. tora* leaf extracts revealed the presence of some bioactive components and secondary metabolites such as phenol, flavonoid, tannins, glycosides, steroids, saponins and terpenoids (Table 1). These secondary metabolites are reported to have many biological and therapeutic properties (**Vishnu *et al.*, 2013, Narender *et al.*, 2012**).

Table 1 Phytochemical components of *Senna alata* and *Senna tora* leaves extracted with methanol and ethanol solvents (Drying method: Room temperature and Oven dried at 50°C)

Leaves Extracts / Phytochemical constituent	*Senna alata*				*Senna tora*			
	Room Temperature		Oven Dried (at 50°C)		Room Temperature		Oven Dried (at 50°C)	
	E	M	E	M	E	M	E	M
Phenols	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+

E= ethanolic extract; M= methanolic extract

Previously **Moriyama *et al.*, (2003)** also reported that *S. alata* (synonym as *Cassia alata*) leaf contained several components of flavonoid glycosides. They found that the heat treated and sun-dried *S. alata* leaf extract contained a strong inhibitory effect on concanavalin A-induced histamine release, COX-1 and COX-2, and 5-lipoxygenase activities while K3G component that is the major flavonoid glycoside in this leaf showed a weak inhibitory effect. Besides leaf, the flower and seed coat extracts of *S. alata* are also positive for terpenoid, cardiac glycosides, steroids and terpenoids with an absence of alkaloids (**Senthilkumar *et al.*, 2013**). Nonetheless, **Uwangbaoje (2012)** reported a contradictory result of the absent of steroid compounds in the *S. alata* leaf extract.

As reported by **Supare and Mantil (2015)** beside leaf, the seeds of *S. tora* (synonym as *Cassia tora*) are also positive for the appearances of phenolic compounds, glycoside, flavonoids, saponins and tannins. They also verified the larvicidal activity of the alcoholic *S. tora* seed extracts against *Anopheles stephensi* that specifies the plant potential to fight mosquito bites related diseases. Another study that was carried out by **Sripriya (2014)** confirmed the yielding of anthraquinones and carbohydrate compounds besides glycosides, steroids, flavonoids, and saponins in the *S. tora* leaf extracts. In the meantime, the present of terpenoids in the *S. tora* leaf extract (Table 1) was in contrary of the result obtained by **Supare and Mantil (2015)**.

In general, based on this qualitative study, the drying methods (oven drying at 50°C and room temperature drying) and solvent (ethanol and methanol) extraction methods used have no influence on the phytochemical screening as a positive data was attained for all the tested samples. Nonetheless, a quantitative analysis is required to be conducted in order to confirm if there are any negative influences between these two parameters on the yields of the phytochemical constituents. Furthermore, ***Shanmugam et al.*, (2008)** stated that, different solubility capacities for different phytochemical components can be affected by different solvents used and may also distinguished the activities of different sample extracts.

Quantitative evaluations were also conducted to determine the total phenolic and total flavonoids contents in the *S. alata* and *S. tora* leaf extracts. Flavonoids are among the major constituents in phenolic components found in various plants. These compounds are known for their function in protecting plants, fruits, and vegetables from oxidative damage and hence have been used as antioxidant agents by humans (**Do *et al.*, 2014**).

As shown in Figure 1, the highest total phenolic contents in *S. alata* was the ethanolic (95 % (v/v) ethanol) extract that was dried at room temperature which is 285.53 mg GAE/g dw. Meanwhile the total phenolic content of methanolic extract (80 % (v/v) methanol) of room temperature dried leaf was reduced to approximately half of this value. This observation shows the types of solvents used during the extraction method plays important roles in influencing the yields and perhaps the stability and activity of the phytochemical components in the plant extract. Different chemical characteristics and polarities of solvents may have influences on the solubility of antioxidant compounds presence in plant matrices (**Turkmen *et al.*, 2006**). Besides types of solvents, the percentage of water content in the aqueous solvent may also plays an important role in the yield of total phenolic content. **Do *et al.*, (2014)** found that the total phenolic content form *Limnophila aromatica* plant was higher when the plant was extracted with 75% aqueous methanol extract than that of 100% methanol extract. This observation was in contrast with the ethanol extract that showed higher total phenolic content in 100% ethanol compared to 75 % aqueous ethanol. Hence, different percentages of ethanol and methanol for solvent extraction of *S. alata* and *S. tora* leaf extracts should also be studied to further understand their effects. Nonetheless, there was no apparent difference was observed for ethanolic and methanolic extracts of oven dried *S. alata* leaves for the total phenolic content. The highest total phenolic content for *S. tora* was recorded for the methanolic leaf extract that was dried at room temperature which is 385.37 mg GAE/g dw, while a lower value was obtained for the ethanolic extract (240.61 mg GAE/g dw. A palpable difference in the total phenolic content was observed for *S. tora* ethanolic and methanolic leaf extracts that were oven dried at 50°C. Nevertheless, it can be deduced that the phenolic constituents in both leaves preferred a moderate drying temperature such as room temperature compared to high temperature treatment such as 50°C or higher.

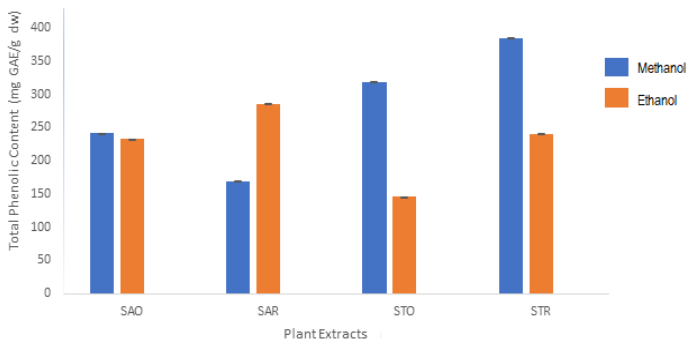

Figure 1 Total phenolic content (mg GAE/g dw) of *Senna alata* and *Senna tora* leaves extracted with methanol and ethanol solvents. SAO = *S. alata* leaves dried at 50°C (oven dried), SAR = *S. alata* leaves dried at room temperature, STO = *S. tora* leaves dried at 50°C (oven dried), STR = *S. tora* leaves dried at room temperature. The error bar represents the standard deviation about the mean (n=3).

As displays in Figure 2, the highest total flavonoid contents in *S. alata* was the ethanolic extract that was dried at room temperature which is 447.42 mg QE/g dw. In comparison, this study showed a higher total flavonoids content compared to 43 mg QE/g as previously reported by **Da *et al.*, (2014)**. A slightly lower value of 422 mg QE/g dw was measured for the ethanolic extract that was dried at 50°C. Likewise, the similar pattern was observed for the *S. tora* leaf extracts. The highest value (345.43mg QE/ dw) was recorded for the ethanolic room temperature dried extract. The drying methods seemed to have a significant effect on *S. tora* leaves but the effect was not apparent for *S. alata* leaf extracts. The flavonoid constituents in the leaf of *S. tora* were found to be heat sensitive. In the meantime, ethanol was the best solvent for extracting the flavonoid components from both leaves.

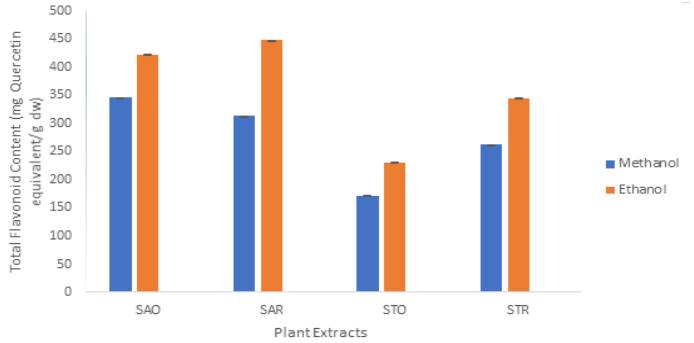

Figure 2 Total flavonoid contents (mg Quercetin equivalent/ g dw) of *Senna alata* and *Senna tora* leaves extracted with methanol and ethanol solvents. SAO = *S. alata* leaves dried at 50°C (oven dried), SAR = *S. alata* leaves dried at room temperature, STO = *S. tora* leaves dried at 50°C (oven dried), STR = *S. tora* leaves dried at room temperature. The error bar represents the standard deviation about the mean (n=3).

Antioxidant activity of S. alata and S. tora leaves

The antioxidant capability and properties of *S. alata* and *S. tora* leaf extracts were quantified by DPPH radical scavenging activity and FRAP assays. The scavenging DPPH free radical methods is well known as fast, easy and cheapest method to measure antioxidant capacity of such as in food samples (**Kirtikar and Basu, 1998**). During the reaction, the stable organic free radical DPPH becomes paired off after reacting with free radical species and hence the absorbance is decreased (**Harborne, 1984**) and a noticeable discoloration from purple to yellow can be observed (**Ghosh, 1998**).

Figure 3 (A) shows the DPPH radical scavenging activity of *S. alata* and *S. tora* leaf extracts. For *S. alata* leaves, higher percentage of free scavenging activity was obtained for the methanolic extracts compared to the ethanolic extracts of both samples dried at room temperature and 50 °C (oven dried). While comparing the drying methods, a mild condition such as room temperature was preferable than the high temperature such as 50°C for optimum scavenging activity. Room temperature kept the freshness of the plant components without exposing it to any damaging effects by heat. In the meantime, no apparent differences were observed for the DPPH scavenging activity of *S. tora* leaf extracts regardless the drying conditions and solvent extract methods used as the percentages of all tested samples were in the range of 85 – 90%.

In the meantime, the FRAP assay can be used to determine the capabilities of antioxidant agents in plants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm (**Benzie and Strain, 1996**). The absorbance value is directly proportional to the antioxidant content in the sample.

Figure 3 (B) shows the comparison between ethanolic and methanolic leaf extracts of *S. alata* and *S. tora* that were primarily dried with either in an oven at 50°C or at room temperature based on their FRAP activity. The FRAP values quantified in *S. tora* leaf extracts were much higher compared to the FRAP values of *S. alata* regardless the drying methods and solvents used for the extraction process. Despite the comparable DPPH radical scavenging percentage values were obtained for both leaf samples, a negative correlation was observed for FRAP values of *S. alata*. Among the tested samples, the highest FRAP value (5614.88 µmol/mg) was recorded by methanolic *S. tora* leaf extracts dried at room temperature. This is in accordance with the highest total phenolic content observed for the similar sample. Meanwhile the lowest FRAP value of 1016.13 µmol/mg was obtained for ethanolic, room dried *S. alata* leaf extracts. Nonetheless, the FRAP values attained in this study is slightly higher compared to 996 ± 22.16 µmol/g that was reported by **Faboro *et al.*, (2016)** for *S. alata* leaf.

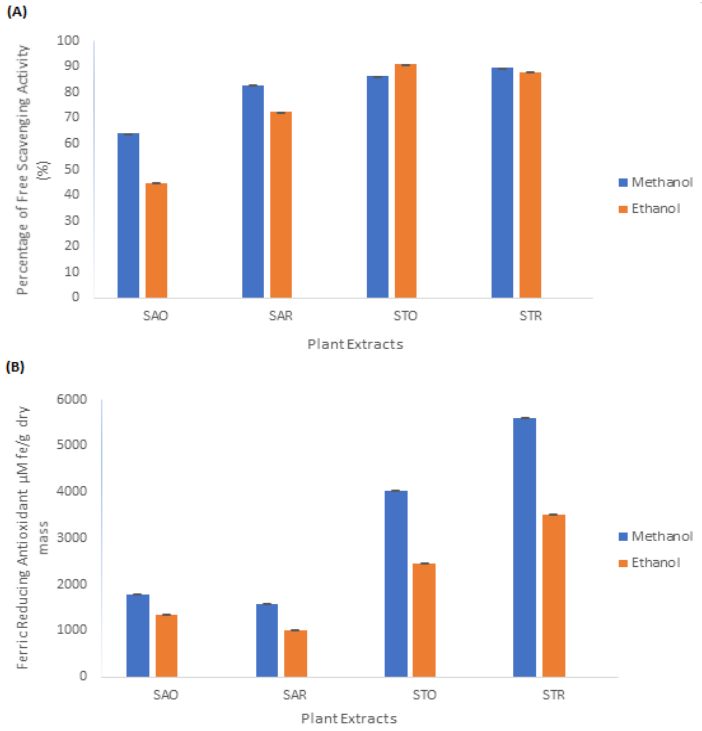

Figure 3 Percentage of free radical scavenging activity (A) and Ferric reducing antioxidant plasma (µmol fe/g (B) of *Senna alata* and *Senna tora* leaves of methanol and ethanol extracts. SAO = *S. alata* leaves dried at 50°C (oven dried), SAR = *S. alata* leaves dried at room temperature, STO = *S. tora* leaves dried at 50°C (oven dried), STR = *S. tora* leaves dried at room temperature. The error bar represents the standard deviation about the mean (n=3).

Antimicrobial activity

This study was aimed to evaluate the antimicrobial properties of the ethanolic and methnolic extracts of *S. alata* and *S. tora* leaves by examining the zone of inhibition by agar well diffusion method, minimum inhibitory concentration (MIC) as well as minimum bactericidal concentration (MBC) against skin microbial flora including *S. aureus, S. epidermidis and P. aeruginosa*.

Agar well diffusion (zones of inhibition)

Based on Table 2, the average measurement of each inhibition zones made from each alcoholic leaf extracts were taken from the ethanol and methanol of pure solvent (negative control). Both ethanolic and methanolic extracts of *S. alata* and *S. tora* leaf extracts showed no zones of inhibition in agar well diffusion assay for *S. aureus*. However, there were presence of inhibition zones for solvent extracts of *S. alata* and *S. tora* leaves for *P. aeruginosa*. While for *S. epidermidis,* zone of inhibition *was only detected for S. tora leaf*. These observations are quite contradicted with the previous work by **Sripriya (2014)** that reported on the methanolic extract of *S. tora* that was found to be active against all these three tested bacteria. In comparison, the inhibition zone measured for their *S. alata* leaf extract against *P. aeruginosa* is also much bigger than the inhibition zones exhibited by the *S. tora* leaf extracts obtained in this study.

Table 2 The antimicrobial activity of oven dried (50°C) and room temperature dried of *Senna alata* and *Senna tora* leaf extracted using ethanol and methanol solvents against *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa.*

Test strain	*Senna alata leaf*				*Senna tora leaf*			
	OD		RT		OD		RT	
	M	E	M	E	M	E	M	E
	Mean clear zonal diameter (mm)							
Staphylococcus aureus	-	-	-	-	-	-	-	-
Staphylococcus epidermidis	-	-	-	-	13.30	12.00	16.26	16.94
Pseudomonas aeruginosa	1.48	1.15	1.59	-	-	1.00	1.35	1.68

OD= oven dried (50°C) leaf extract; RT= room temperature dried leaf extract; E= ethanolic extract; M= methanolic extract

The oven dried *S. alata* leaf extracts showed zones of inhibition of 1.48 mm and 1.15 mm for methanolic and ethanolic extracts, respectively. On the other hand, the room temperature dried of *S. alata* leaf extracts showed a slightly bigger zone of inhibition which is 1.59 mm for the methanolic extracts but there was no zone of inhibition possess by the ethanolic extract. Previously, the methanolic extracts of *S. alata* leaf were reported to have inhibition zones of 3 mm and 5 mm for *P. aeruginosa* and *S. aureus*, respectively (**Ehiowemwenguan *et al.*, 2014**). No zone of inhibition was obtained for methanolic oven dried *S. tora* leaves extracts but 1.00 mm zone of inhibition was presence in the ethanolic extract. Meanwhile the ethanolic extract *S. tora* leaves dried at room temperature showed that the extract inhibited a much bigger zone than the methanolic extract (1.68 mm and 1.35 mm, respectively). Nevertheless there was a report that showed the methanolic extract of *S. tora* leaves possess 6 mm and 10 mm of inhibition zones for *P. aeruginosa* and *S. aureus*, respectively (**Sripriya, 2014**). According to **Singh and Singh** (**2000**) the yield of antimicrobial component in plant extract is commonly influenced by the non-polar or polar solvent used during the extraction process. Based on the types of solvents applied in this study, the result shows that the ethanolic extract exhibited much bigger zones than the methanolic extracts. The formation of zone of inhibitions by both *S. alata* and *S. tora* leaf extracts is shown in Figure 4.

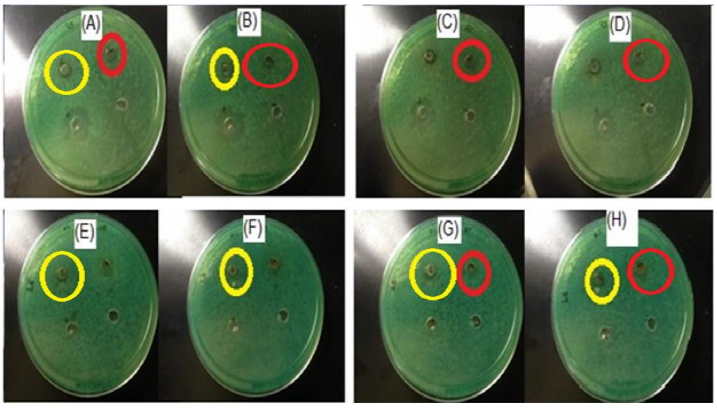

Figure 4 Inhibition zones produces by oven dried *Senna alata* leaves methanolic and ethanolic extraction of (A) methanol and (B) ethanol of pure solvent , room temperature dried *Senna alata* leaves methanolic and ethanolic extraction of (C) methanol and (D) ethanol pure solvent, Oven dried *Senna tora* leaves methanolic and ethanolic extraction of (E) methanol and (F) ethanol of pure solvent, and room temperature dried *Senna tora* leaves methanolic and ethanolic extraction of (G) methanol and (H) ethanol of pure solvent towards isolated *P. aeruginosa*. Red circles = methanolic leaves extracts and yellow circles = ethanolic leaves extracts of the inhibition zones observed.

The antimicrobial activity exerted by *S. tora* leaf extracts was found to be approximately 12 to 16 times stronger for *S. epidermidis compared to P. aeruginosa*. Higher inhibition activities were achieved for both ethanolic (inhibition zone of 16.94 mm) and methanolic (inhibition zone of 16.26 mm) extracts of *S. tora* that were dried at room temperature than the ethanolic (inhibition zone of 12.00 mm) and methanolic (inhibition zone of 13.30 mm) extracts that were oven dried at 50°C. The formation of zone of inhibition by both *S. alata* and *S. tora* leaf extracts is shown in Figure 5. The data from this study indicates that the temperature plays a role on the yielding and perhaps stability of the components responsible for the antimicrobial activity. According to **Murugan *et al.*, (2013**) the antimicrobial activity that present in the plants is associated to the presence of phytochemical constituents such as flavonoids, steroids, saponins and tannins.

In general, the antimicrobial activity of plants cannot only be relying on the agar well diffusion method to conclusively justify any findings especially the negative result (i.e, no inhibition zone) observed. This is due to some disadvantages of the agar diffusion method in determining the antimicrobial activity since it may be affected by various factors such as agar type, salt concentration, incubation temperature and molecular size of the antimicrobial component (**Eloff, 1998**). Furthermore, this method also cannot be used to distinguish between bactericidal and bacteriostatic effects. Instead, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) must be conducted to determine these effects (**Mostafa *et al.*, 2018**).

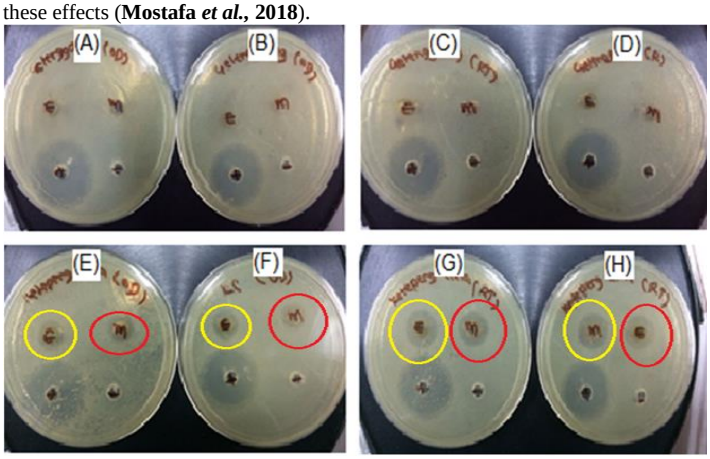

Figure 5 Inhibition zones produces by oven dried *Senna alata* leaves methanolic and ethanolic extraction of (A) methanol and (B) ethanol of pure solvent , room temperature dried *S. alata* leaves methanolic and ethanolic extraction of (C) methanol and (D) ethanol pure solvent, Oven dried *Senna tora* leaves methanolic and ethanolic extraction of (E) methanol and (F) ethanol of pure solvent, and room temperature dried *S. tora* leaves methanolic and ethanolic extraction of (G) methanol and (H) ethanol of pure solvent towards isolated *S. epidermidis*. Red circles = methanolic leaves extracts and yellow circles = ethanolic leaves extracts of the inhibition zones observed.

Minimum inhibitory concentration (MIC)

The reading of MIC for *S. alata* and *S. tora* leaf extracts has been taken by correlating it with the absorbance reading besides broth turbidity visualization. The absorbance of controls (control 1: sterile broth without test organism and control 2: test organisms grown in their respective media in absence of *S. alata* and *S. tora* leaf extracts) and each of serial dilution concentrations of *S. alata* and *S. tora* leaf extracts were taken to determine the lowest extract concentrations to inhibit the growth of tested microorganism. The tested microorganisms used were *S. aureus*, *S. epidermidis* and *P. aeruginosa*. The absorbance readings for control of broth containing tested *S. aureus, S. epidermidis* and *P. aeruginosa* (in absence of leaf extracts) were 1.230, 2.810 and 0.208, respectively. The MIC values for tested microorganisms were taken by choosing the concentration that has absorbance reading below that of the control. Hence, the absorbance reading that are exceeded the control reading showed the presence of tested microorganism growth.

Table 3 The minimum inhibitory concentration (MIC) (mg/mL) of oven-dried *Senna alata* and *Senna tora* against *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa.*

Test organism	*Senna alata*				*Senna tora*			
	OD		RT		OD		RT	
	M	E	M	E	M	E	M	E
	MIC (mg/mL)							
Staphyloccus aureus	5	5	5	10	5	2.5	5	2.5
Staphylococcus epidermidis	5	0.625	5	2.5	0.625	2.5	2.5	0.625
Pseudomonas aeruginosa	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625

OD = leaves dried using oven dried, RT= leaves dried using room temperature, M = methanol extracts of plant , E = ethanol extracts of plan

Based on Table 3, the methanolic extracts of *S. alata* leaf incubated with all tested organisms had MIC of 5 mg/mL for oven dried and room temperature dried of plant leaf, except for the *P. aeruginosa* with a much lower MIC value of 0.625 mg/mL for both types of leaf drying methods. While, the ethanolic extracts of *S. alata* leaf showed the MIC value of 0.625 mg/mL for oven dried leaf against *S. epidermidis* and *P. aeruginosa* but MIC of 5 mg/mL was observed for *S. aureus*. Meanwhile, the MIC obtained for ethanolic extracts of room temperature dried *S. alata* leaf for *S. aureus*, *S. epidermidis* and *P. aeruginosa* were of 10 mg/mL, 2.5mg/mL and 0.625 mg/mL, respectively. The presence of MIC value for *S. alata* leaf showing there is a weak inhibitory activity in the leaf extract for *S. aureus* despite none inhibition zone was observed in the agar well diffusion method.

Methanolic extracts of *S. tora* leaf had the lowest MIC values in the range of 0.625 mg/mL to 2.5 mg/mL for both type of leaf drying methods against *S. epidermidis* and *P. aeruginosa* with an exception for *S. aureus* with a MIC of 5 mg/mL. While, the ethanolic extracts of *S. tora* leaf have stronger inhibitory effect against all types of test organisms as the MIC values were in the range of 0.625 mg/mL to 2.5 mg/mL. This showed that, ethanol is a more suitable solvent in order to get the lowest MIC values. In comparison, both of leaf extracts have the strongest inhibitory effect against *P. aeruginosa* as the MIC value is only 0.625 mg/mL compared to large variation on the MIC values attained for the other two tested organisms. Previously **Saito *et al.*, (2012)** also reported that the *S. alata* leaf extract by reverse phase solid phase extraction method carried an antimicrobial activity against *P. aeruginosa*, *S. epidermidis*, *S. aureus* and *Bacillus subtilis*.

Minimum bactericidal concentration (MBC)

MBC was conducted to determine the lowest concentration of antimicrobial agent that is required to inhibit a specific microorganism. MBC is a complementary assay to MIC. In conducting the assay, a control of MBC had been prepared from the MIC assay performed earlier. The MBC values were selected by observation for the bacterial colony grown on the isolated agar plate. The plate with no growth and lowest concentration of MIC values was selected as MBC.

Table 4 shows that the MBC of *S. alata* and *S. tora* leaf extracts of different extraction solvents and different types of leaf drying methods for all three tested microorganisms (*S. aureus*, *S. epidermidis and P. aeruginosa*). The methanolic and ethanolic extracts of *S. alata* leaf against all tested microorganisms had MBCs of 10 mg/mL for both types of leaf drying methods, except for the ethanolic extracts against *P. aeruginosa* with a lower MBC of 5 mg/mL.

In the meantime, the methanol extracts of *S. tora* leaf against all the tested microorganisms for both leaves drying methods shown a similar MBC values, which is 10 mg/mL. However the ethanolic extracts of *S. tora* leaf shown that the samples possess the lowest MBC of 5 mg/mL for *P. aeruginosa*.

Table 4 The minimum bactericidal concentration (MBC) (mg/mL) of oven-dried *Senna alata* and *Senna tora* against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*.

Test organism	*Senna alata*				*Senna tora*			
	OD		RT		OD		RT	
	M	E	M	E	M	E	M	E
	MBC (mg/mL)							
Staphyloccus aureus	10	10	10	10	10	10	10	10
Staphylococcus epidermidis	10	10	10	10	10	10	10	5
Pseudomonas aeruginosa	10	10	10	5	10	5	10	5

OD = leaves dried using oven dried, RT= leaves dried using room temperature, M = methanol extracts of plant, E = ethanol extracts of plant.

CONCLUSION

S. alata and *S. tora* leaves were found to be positive for the presence of phenols, flavonoids, tannis, glycosides, steroids, saponins and terpenoids. The quantitative assays on total phenolic contents revealed the highest values were obtained for room temperature dried leaves of ethanolic extract for *S. alata* and methanolic extract for *S. tora*. While the highest total flavonoids content were recorded for extracts of room temperature-dried leaves for both *S. alata* and *S. tora* ethanolic. For *DPPH radical scavenging activity, the maximum activity for S. alata samples was observed for methanolic extract of room temperature-dried leaves (80 %) while for S. tora all the samples showed approximately the same values in the range of 85 -90%. In comparison, S. alata leaves expressed a much lower FRAP values than the S. tora leaves extracts. S. alata leaf extracts showed an inhibition against P. aeruginosa, while S. tora leaf extracts were positive against P. aeruginosa and S. epidermidis.* The MIC and MBC tests demonstrated that *S. alata* and *S. tora leaves extracts* expressed the best MIC and MBC against *P. aeruginosa* followed by *S. epidermidis* and *S. aureus.*

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REGULAR ARTICLE

ANTIBACTERIAL ACTIVITY OF *SARGASSUM CRISTAEFOLIUM* AND *DICTYOTA CERVICORNIS* AGAINST TO BACTERIA

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ABSTRACT

Nowadays the treat infection of bacterial diseases faced limitations because of the harm of industrial artificial antibiotics for the digestive system and immune system. Consequently, the scientists seek to replace conventional anti-biotics whit natural ones. The central thesis of this study is to identify potential antimicrobial properties of the *Sargassum cristaefolium* and *Dictyota cervicornis* extracts. Water-methanol, n-hexane, Ethyl acetat, methanol and water extracts of two marine macroalgae from chahbahar bay (Iran) were weighed up in terms of antimicrobial activity by Agar disk diffusion, MIC and MBC methods against (*L.monocytogens* as gram-positive bacteri, and (*E.coli* and *P. aeruginosa* as gram-negative bacteria). According to the obtained results, the hot water extract in *S. cristaefolium* and *D.cervicornis* none of the strains examined is of antibiotic effects. Ethyl acetat extract in comparison with other extracts displayed better antibacterial activity ($P<0.05$). Highest zone of inhibition (14 mm) was recorded for Ethyl acetat extract of *S. cristaefolium* contrary to *L. monocytogenes.* Maceration method extracts did not display any effect towards the studied bacteria. For extracting antimicrobial compounds, the ultrasound method was a successful method.

Keywords: *Senna alata; Senna tora;* skin infections; antioxidant; phytochemical; antimicrobial

INTRODUCTION

Human beings' morality is dependent on many causes that infection diseases among them are one of the main causes in the world particularly in developing countries **(Waldvogel, 2004)**. In recent years, life-threatening feature of illness has been increased considerably because of severe infections and resistance of the pathogenic bacteria to medicines as a result of indiscriminate use of antibiotics. Antibiotic resistor in bacteria is one of the main and important health factor in the world **(Patra *et al.*, 2008; Appelbaum, 2006)** . Algaes have been long used as food material in Asian diets because of containing carotenoids and other bioactive compounds. For inductrial manufacturing phycocolloids such as agar-agar, alginate and carrageenan, seaweeds are the main used material **(Rajasulochana *et al.*, 2009)**. For making medicine, seaweed is the most important material because of the abundance and variation of secondary metabolites. Moreover, most of the secondary metabolites existed in seaweed are in form of halogenated compounds having antimicrobial, antifungal and antiviral attributes.

MATERIAL AND METHOD

Sample collection

Sargassum cristaefolium and *Dictyota cervicornis* macroalgae (Phaeophyta) were collected in the chabahar coast of the Persian Gulf, Iran. In laboratory, algae were washed out with water and sand particles were removed. Algal samples were cleaned from epiphytes. Debries, extraneous matters, and necrotics parts were removed. The surface of algal samples was rinsed carefully with sea water and after that it was washed out with fresh water in order to remove salts from algae and to get better results. Distilled water was used was replaced several times. Algae were spread over in the shade seven days in order to be dried completely and then were grinded by an electric powder mill. After sampling, extraction procedure was performed in two following ways, namely ultrasound and maceration methods.

Maceration method

The powder was broken down in methanol, ethyl acetat, n- Hexane , water-methanol and boiling water (1:10 w/v) and kept at room temperature overnight **(Patra *et al.*, 2008).**

Ultrasound method

Ultrasound facilitated extraction experiments. Ultrasonic irradiation was run using an ultrasonic device (50 kHz, temperature of 25 °C,Type Pajohesh nasir Iran) equipped with a digital timer and a temperature controller. The ultrasonic pulse sequence was 180s on and 60 s off. In this study, solvents containing methanol, ethyl acetat, n- Hexane , water- methanol and distilled water were utilized **(Wang *et al.*, 2015).**

Microbial Test

Bacterial strains used in this study contained *Listeria monocytogenes* (PTCC 1163), *Escherichia coli* (PTCC, 1399), *Pseudomonas aeroginosa* (PTCC 1430) respectively. Antibacterial effects were used of the modified disk diffusion method on agar antibiogram test (Agar Disk Diffusion). For this purpose, suspensions of bacteria to antimicrobial susceptibility testing standards with a concentration equivalent to 0.5 McFarland was prepared from overnight cultures. The medium was used for antibiogram Hinton agar (Muller Hinton Agar), with pH 7.2 and 5 mm in diameter, respectively. After providing a uniform, culture suspensions were prepared aseptically using sterile swabs. Blank CDs antibodies Medicine Company were placed on media and were inoculated with 100µl each of the algal extracts was prepared to help Sampler. Pre-release pellets for 30 min at 4° C were then incubated at 37° C were transferred were to diameter of inhibition zone (mm) after 24 h using a digital caliper to measure and record. For Each instance replicated three times. Gentamicin and Neomycin antibiotics were used as positive controls **(Tajbakhsh *et al.*, 2011; Soltani *et al.*, 2011).**

Determination of the MICs (the minimum inhibitory concentrations) and the MBCs (the minimum bactericidal concentrations)

The MICs against all the three bacteria were determined using the Microdillution method. The determinations of the MICs were done in triplicate and the mean values were used. The 96-well plates were scanned with ELISA reader at 630 nm **(Xiaoxi , 2011).**

The MICs were taken as the lowest concentration that caused optical density reduction by more than 90% compared with control growth results. All the MIC wells, which did not show any turbidity, were pureplated on Nutrient agar plates. The lowest concentrations that did not permit any visible growth on the plates after 24 h of incubation at 37°C were recorded as the MBCs **(Mohammadpour Vashvaei *et al.*, 2015).**

Statistical Analyses

The collected data were analyzed through SPSS version 21 software. The antibacterial activities of the data are expressed by means ±SD. Statistical analyses were run by ANOVA with Tukey test. A value of P≤ 0.05 was used to show statistical significance. Finally the ANOVA statistic Test was utilized to see whether there is any relationship between extracts effect and zone of inhibition against the bacteria.

RESULT

The results of this study revealed that extracts tested by maceration method did not show a zone of inhibition towards the bacteria. The inhibitory effects of the concentration of *S. cristaefolium* and *D.cervicornis* extracts displayed the growth of two gram negative bacteria and one gram positive bacteria using disc diffusion method is shown in Table 1. The extract showed both activity against gram positive and gram negative bacteria. And inhibitory impacts enhanced with the increase of extract concentrations. The *S. cristaefolium* extracts showed different degrees of antimicrobial activities on different bacteria. The *L. monocytogenes* was more sensitive one than others among the bacteria. *E.coli* as the gram negative bacteria was found to be more resistant than *P. aeruginosa*. Overall, the gram negative bacteria were more unaffected than the gram positive bacteria. In addition, it is noteworthy to say that the *E.coli* was found to be the most resistant one among all the bacteria. Other similar studies displayed the same type of results.

Antibacterial activity

Varying amounts of antibacterial inhibition against pathogenic bacteria were obtained in the extracts of *S. cristaefolium* and *D.cervicornis*. The measured growth inhibition zone ranged from 8.66 to 14.0 mm for all the sensitive bacteria. The ethyl acetat extract indicated the highest antibacterial activity with inhibition zone of 14.0 mm against *L. monocytogenes*. As it was shown in Table 1, the methanol, ethyl acetat and n-hexan extracts demonstrated activity against all the tested pathogens. On the other hand, the degree of water extracts activity was practically zero.
Table 2 exhibited the MICs and MBCs of seaweed extracts. According to the obtained results, the MIC for *Listeria monocytogene* (gram-positive) was lower than the MIC for gram-negative bacteria.

Table 1 Antibacterial Activity Extracts of Algae Against Pathogens Bacteria (inhibition of growth expressed as mm diameter of inhibition zone).

Bacterial	Solvent	*Sargassum cristaefolium*	*Dictyota cervicornis*
E.coli	Methanol	8.66±0.5	9±0
	N-Hexan	9±1	8.66±0.5
	Ethyl Acetat	9.75±0.2	7.83±0.7
	Water-Methanol	-	-
	Water	-	-
P. aeruginosa	Methanol	11.83±0.7	9.33±0
	N-Hexan	11±0	8.83±0.7
	Ethyl Acetat	11±1	9.16±0.7
	Water-Methanol	-	-
	Water	-	-
L.monocytogenes	Methanol	10.66±1	12±1
	N-Hexan	12±1	11±1
	Ethyl Acetat	14±1	11.66±0.5
	Water-Methanol	10±1	-
	Water	-	-

Zone of inhibition (mm), including of the agar – disk (6 mm), Mean value of three replicate ± SD
(-), no activity

Table 2 MICs and MBCs extracts of marine Algae Against Bacteria Tested

Bacterial	Solvent	*Sargassum cristaefolium*		*Dictyota cervicornis*	
		MIC	MBC	MIC	MBC
E.coli	Methanol	0.1	0.3	0.2	0.4
	N-Hexan	0.05	0.2	0.2	0.4
	Ethyl Acetat	0.05	0.1	0.2	0.4
	Water-Methanol	-	-	-	-
	Water	-	-	-	-
P. aeruginosa	Methanol	0.05	0.2	0.2	0.4
	N-Hexan	0.05	0.2	0.2	0.4
	Ethyl Acetat	0.05	0.2	0.1	0.2
	Water-Methanol	-	-	-	-
	Water	-	-	-	-
L.monocytogenes	Methanol	0.05	0.1	0.05	0.1
	N-Hexan	0.05	0.1	0.05	0.1
	Ethyl Acetat	0.05	0.1	0.05	0.1
	Water-Methanol	0.05	0.2	-	-
	Water	-	-	-	-

MIC:mg/ml

DISCUSSION

The present study was an attempt to assess the antimicrobial activity of the different macro-algae in terms of their bioactive potentials **(de Quirós *et al.*, 2010; Priyadharshini *et al.*, 2012)**. have reported that seaweeds are an outstanding source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols, and carotenoids has exhibits different biological activities **(Al-Saif *et al.*, 2014; Kausalya and Narasimha Rao, 2015).**
Antimicrobial activity of the seaweed extraction depends on the utilized solvents. Many researchers reported the influence of different extraction solvents on the content of compounds in extracts. Solvents solubility effectiveness is strongly hanged on material used for extraction **(Zhou and Yu, 2004; Michiels *et al.*, 2012; Mahianeh *et al.*, 2014).** Organic solvent is of a higher capability in extracting compounds for antibacterial activities in comparison with water based methods **(TÜney *et al.*, 2006; El-Sheekh *et al.*, 2014).**
Based on the results, the n-hexane and methanol extracts of the algae *Sargassum cristaefolium* did not show antibacterial activity. Similarly, **Febles *et al.* (1995)** reported that the n-hexane and methanol extracts prepared by maceration method of *Sargassum desfonrainesii* which was gathered during winter and autumn did not show antibacterial activity against *P. aeruginosa,* **(Febles *et al.*, 1995)**. A similar study conducted by **Demirel *et al,*(2009)** reported that the methanol and n-hexan extracts *of Dictyota dichotoma var. implex* and *D. dichotoma* prepared by maceration method showed no antibacterial activity against *E. coli* (ATCC 29908) and *E. coli hemorrhagic* (O157:H7) **(Demirel *et al.*, 2009)**. *Pseudomonas*

aeruginosa was resistant to boiling water extracts of *Dictyota sp*. and *S. ramifolium*. In another study, methanol extract of *Dictyota cervicornis* could not create zone of inhibition against the studied bacteria **(El-Sheekh *et al.*, 2014)**. In the same vein, **TÜney *et al* ,(2006)** reported that the methanol extracts prepared by maceration method of *Dictyota linearis* do not create halo-zones against bacteria. The water extracts of *sargassum cristaefolium* and *dictyota cervicornis* did not manifest any antibacterial activity against all the tested pathogenic bacteria **(TÜney *et al.*, 2006)**. **Kausalya and Narasimha Rao, (2015)** study revealed that solvents in comparison with water are always better for extraction **(Kausalya and Narasimha Rao, 2015).** The result of the present study indicated the less efficiency of the extracts prepared by maceration method of *sargassum cristaefolium* and *dictyota cervicornis.* Moreover, it did not display any antibacterial activity against *E. coil,p.aeruginosa* and *L.monocytogenes. Sargassum sp*. and *Dictyota sp.* Extracts. And also, no growth of the bacteria inhibition was detected by **(Ballantine *et al.*, 1987; Chowdhury *et al.*, 2015)**. Positive and favorable antimicrobial effects were shown by extracts prepared by ultrasonic method (see Table 1 and 2).

Another important finding of the present study was that gram positive organism are more affected by the used algae extracts. Similarly,**(TÜney *et al.*, 2006; Taskin *et al.*, 2007).** suggested that gram positive bacteria were more practically dominated by the extracts of the algae used in their study than gram negative bacteria **(Salvador *et al.*, 2007; Salem *et al.*, 2011; Kavita *et al.*, 2014).**

Ultrasound-assisted extraction process employs sonic energy and solvents in order to extract the intended compounds from various plant matrices. It is generally accepted that solvents increase the extraction resulting in an acceptable level. Cavitation phenomenon occurs as a result of using diffusion of ultrasound pressure, accordingly cell membrane will be destroyed and the contents of the cell will be evacuated into the extraction medium **(Ebringerová and Hromádková, 2010).**

Interestingly, UAE was detected appropriate for the extraction of aroma compounds. UAE was also utilized for extraction of oil from soybean **(Li *et al.*, 2004)**, rapeseed **(Ibiari *et al.*, 2010)**, and Monopterus albus **(Abdullah *et al.*, 2010).** Studies on effect of different solvents and their compound, effect of solvent content, sonication power, and sonication time reported that UAE has the potential to ameliorate extraction output. The sonication-assisted extraction can be implemented at lower temperatures suitable for the thermally instable compounds **(Wu *et al.*, 2001; Gupta *et al.*, 2012).**

In a study, **Kadam *et al,* (2015)** kdam et al, reported that ultrasound technique was better in comparison with liquid-solid method in terms of observed laminarin content and molecular weight distribution in the extract **(Kadam *et al.*, 2015).**

Ultrasound may raise extraction efficiency and enhance the quality of extracts **(Altemimi *et al.*, 2016).** Ultrasound-assisted extraction was appeared to be an acceptable and appropriate method for extracting bioactive compounds from Solvia officinalis **(Salisova *et al.*, 1997).** Nowadays, UAE is widely utilized for the extraction of worthy molecules such as proteins **(Qu *et al.*, 2012)**, sugars **(Karki *et al.*, 2010)**, polysaccharides-protein complex **(Cheung *et al.*, 2012)**, oil **(Adam *et al.*, 2012)**, phenolic compounds **(Ince *et al.*, 2014; Kuo *et al.*, 2014).** oils and lipids **(Gil-Ch´avez *et al.*, 2013)**. Dent et al reported that among different extraction methods, the ultrasound-assisted extraction using an ultrasonic device with straight incitement gave rise to the increased improvement of total polyphenols combined with the used lower solvent than conventional extraction **(Dent *et al.*, 2015)**. In addition, ultrasound-assisted extraction is cheaper and easier than other new extraction techniques **(Wang and Weller, 2006)**. In their study, **Kavitha *et al*,(2015)** found that Ultrasonic-assisted extraction enlarges the efficiency of protein extraction from White button Mushroom, Agaricus bisporus **(Kavitha *et al.*, 2015)** .

CONCLUSION

Minimal inhibitory concentration (MIC) or zone diameter showed susceptible, intermediate, and resistant values as defined below**(Cockerill *et al.*, 2012).**

Table 3 Reference table for compare the results

	MIC (µg/mL)	Zone Diameter (mm)
Susceptible	≤ 4	≥ 20
Intermediate	8–16	15–19
Resistant	≥ 32	≤ 14

(Cockerill *et al.*, 2012).

Based on the above mentioned standard values, the bacteria involved in the present study were resistant against the extracts.

Recommendation: There are some cases which can be considered in future research as follows: First, Determining phytochemical compounds in algae by using GC-Mass. Second, Utilizing other extraction methods such as enzyme and soxhlet methods. Third, Decontaminating the bioactive compounds in the extract and examining the antibacterial effects of these compounds individually. Last but not the least, Assessing the antibacterial mechanism of phytochemical compounds against bacteria

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