GENOTOXICITY AND ANTIMICROBIAL ACTIVITY OF *MYRTUS COMMUNIS* L., *ZIZIPHUS SPINA-CHRISTI* (L.) WILLD AND *CASSIA ANGUSTIFOLIA* VAHL EXTRACTS

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Abstract

Genotoxicity and antimicrobial activity of the leaf extract of *Myrtus communis, Ziziphus spina-christi* and *Cassia angustifolia* plants were evaluated. The prophage induction assay was used to test the genotoxicity. The results showed that, no fold increase in Pfu/ml over the spontaneous release for F116 and R3 phages, so these results indicated that *Myrtus communis, Ziziphus spina-christi* and *Cassia angustifolia* leaves extract did not have genotoxic effect, so they can be used as antimutagen. On the other hand, *Myrtus communis* and *Ziziphus spina-christi* showed strong antimicrobial activity against all tested microbes. The antimicrobial effect increased by the increase of the concentration. While *Cassia angustifolia* leaves extract showed moderate antimicrobial activity against some strains. The results of antimicrobial activity suggest that these plant extracts have cytotoxic effect.

Introduction

Millions of people in the world use plants as spices or food flavors. They also use them for healing purposes as part of traditional medicine. Many local people in Al-Ahsa, Saudi Arabia are dependent on herb trading and medicinal plants as a means of livelihood and income generation. Medicinal plants are popular with people of developing countries for health care because of compatibility with human body, better cultural acceptability and fewer side effects (Amal *et al*. 2010). The traditional medicine uses some common plants for treatment some common diseases such as diabetes, diarrhea, allergies, headache, fever, arthritis, eye diseases, skin diseases, nervous disorders and stomachache. *Myrtus communis*is as very important aromatic medicinal plants because of the high essential oil contents of its leaves, flowers and fruit glands. *Myrtus communis* were used for its antioxidant, antiseptic, antimutagen, anti-inflammation and insecticide activity. In folk medicine leaves and decoction of fruits are used in treatment of cough, oral diseases and hypoglycaemic stomach pain (Touaibia 2015). In Saudi Arabian folk medicine, the leaves of *Zizyphus spina-christi* are used to treat fever, sex diseases, some skin diseases, sores, gonorrhoea, and wound healing. The fresh fruits and bark are used as a body wash and healing of newly emerging wounds while the fruits are used for the treatment of dysentery (Al-Mutairi *et al*. 2016). The *Cassia* species members are rich sources of polysaccharides, polyphenols, flavonoids and anthraquinone derivatives, which exhibit antioxidant, antimutagenic, anti-inflammatory, antiplasmodial, and anticancer activities. They are also widely used for the treatment of wounds, scabies, eczema, gastrointestinal disorders and skin diseases.

On the other hand, green plants contain substances that may be mutagenic and carcinogenic, but there is little information on the biological effects of these compounds, so it is important to assay the genotoxic and cytotoxic effects of phyto-therapeutic substances (Silva *et al*. 2012). The prophage induction is a short-term test performed with lysogenic strains allowing the assessment

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one of SOS function, so it is used to evaluate the mutagenic effects of substances. When bacterial cells are exposed to DNA damaging agents, DNA replication is repressed, this leads to prophage to enter the lytic cycle as an expression of SOS response (Silva *et al.* 2012). Various evidences indicated that, prophage induction is a broader genetic endpoint than any other bacterial assay. It is a sensitive test were addition or no addition of any activator substance will not change the results. Sometime, the mutagenic evaluation of substances may escape when tested with Ames test, the mutagenic activity using *Salmonella* was negative, whereas the prophage induction assay was positive.

The plants contain many biologically important phytochemicals and bioactive compounds such as peptides, cyclopeptides, flavonoids, sterols, tannins, alkaloids, betulinic acid and glycosides (Ads *et al.* 2017). Many plants were used to treat infectious diseases, so it is considered as important new source of antimicrobial agents (Bereksi *et al.* 2018). The excessive use of antibiotics in clinical medicine, agriculture, and veterinary is very dangerous to the environment and therefore to humans as it promotes the spread of antibiotic resistant genes between microbes, which leads to the creation of a clan of antibiotic-resistant microbes. This is due to the lack of effectiveness of antibiotics in the treatment of pathogenic microbes. So, it is necessary to search for new sources of antimicrobial agents especially among plants which contain natural products and may contain medical compounds that act as antimicrobial agents. Therefore, the aim of this study was to evaluate genotoxicity and the antimicrobial activities of plant extracts of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* that are used in traditional medicine in Al-Ahsa, Saudi Arabia.

Materials and Methods

The nutrient agar and nutrient broth media were used. Soft agar (0.7% w/v agar) was prepared in distilled water and kept at 45°C on water bath. Phosphate buffer was prepared from 1/15M KH_2PO_4 and 1/15M Na_2HPO_4. $2H_2O$. Streptomycin was added to the media after autoclaving as sterilized solutions, it was sterilized by filtration through 0.2 μm filter membrane. Whatman membrane filter, diameter 25 mm, pore size 0.2 μm was used.

The generalized transducing F116 phage and the original host bacterial strains of *Pseudomonas aeruginosa* PAO1, PU21 and lysogenic MAM2, were obtained from M. Day, University of Wales, Cardiff, UK. Other bacterial (*Bacillus thuringiensis* host strain of R3 temperate phage and *Staphylococcus aureus*), fungal (*Rhodotorula* spp.) strains were obtained from Microbial Genetics Lab., Faculty of Agriculture, Zagazig University, Egypt.

Three species of plants widely used by local people are *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* which were collected from local market. The dried leaves were ground and briefly, 100 g of plant sample was extracted with 500 ml 95% ethanol at room temperature for 48 hrs in order to extract the bioactive compounds (Nkafamiya *et al.* 2013). The ethanol crude extract products were poured into dishes and kept in dry oven at 40°C for 3 - 4 days until the extract materials were concentrated (Romha *et al.* 2018). The extracts were weighted, labeled and stored in the refrigerator at 4°C for further analysis.

The combination between methods of Siddiqui *et al.* (2011) and Silva *et al.* (2012) with some modifications was used for the prophage induction test. The lysogenic of MAM2 and Bt10 bacterial strains were grown to exponential phase (1.6×10^8), centrifuged and resuspended in10 ml of phosphate buffer solution. The test extract samples at different concentrations (5, 15 and 30 mg/ml) at 30°C for 3 hrs were treated. The treated cells were again centrifuged, washed and suspended in nutrient broth and incubated at 30°C for 3 hrs, centrifuged at 5000 rpm for 30 min and filtrated through 0.2 μm filter membrane (Whatman). The supernatant was collected, diluted

and plated with indicator cells. The number of plaques were scored using phage titration test, the plaque forming units (Pfu/ml) were calculated for each concentration. Ethylmethane sulfonate (EMS) was used as a positive control and ascorbic acid (vitamin C) as a negative control. The phage was propagated using the plate method on its bacterial host, 0.1 ml of phage diluted sample was mixed with 0.1ml of host suspension (10^8 CFU/ml) in NB broth in a sterile tube and incubated for 15 - 20 min at room temperature to facilitate adsorption of the phage to the host cells. The mixture was added to 3 - 5 ml of soft layer agar cooled to 45°C and well mixed. Then, poured over the NA Agar plate and sit for 15 min at room temperature. The plates were incubated for 18 hrs at 30°C. Clear areas of lytic plaques (Plaque forming unit, Pfu/ml) was counted. A plate showing confluent plaques was used to prepare a concentrated phage suspension by overlaying with 5 ml of NB broth. The over layer medium containing the phage was decanted and filtered through a 0.2 μm filter membrane. The filtrate was used as a phage stock solution. Several dilutions of phage solution were made (Behravan *et al*. 2011).

Antibacterial activities of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* extracts were investigated by disc diffusion method. The test suspension which was approximately 1.3×10^6 colony forming units (CFU/ml) of bacteria were spread on the agar plates. The tests were conducted with three extract concentrations 5, 15 and 30 mg/ml by dissolving in distilled water. Sterile filter paper discs (6.0 mm) were saturated with selected concentration of used extracts. The discs were placed on the surface of the inoculated medium and left for 30 min at room temperature for compound diffusion (Belmimoun *et al*. 2016). Negative controls were performed using distilled water. Positive controls were performed using 5 mg/ml streptomycin. The plates were incubated at 30ºC, the growth inhibition zones around the discs were estimated after incubation time (18 - 24 hrs). The sensitivity of the bacterial strains to the extracts was determined by measuring the size of inhibitory zones around the discs (including the diameter of disc). The inhibition zone diameters was as follows: negative = 0 mm, weak = 1 - 4 mm, moderate = 5 - 10 mm, strong = 10 - 15 mm, very strong = ≥ 16 mm (Amal *et al*. 2010). Values less than 8 mm were considered as not active but 14 mm or greater was considered as high antibacterial activity (Belmimoun *et al*. 2016). All the experiments were conducted in triplicate to minimize test error. The same procedure was followed with fungus. Bacterial host strains were prepared by directly transferring one colony from an agar plate to 5 ml NB and incubation at 30°C in shaker incubator for one hr. The 0.5 ml of phage was added to 0.5 ml of selected concentrations of plant extract. The mixture was left at room temperature for 30 min. 0.5 of previously bacterial host suspension was mixed with 5 ml soft agar (0.7 %). The mixture of phage and extract were added to soft agar and mixed, Then, the soft-agar medium was poured on nutrient agar plate and left to solidify. The plates were incubated inverted at 30ºC for 12-16 hrs. Antibacteriophage activity was then considered as the absence of plaque formation in comparison with the control, which had distilled water instead of the plant extract (Behravan *et al*. 2011).

Each experiment was repeated in triplicate and the means ± standard error from the absolute data were calculated.

Results and Discussion

Short-term experiments were conducted to identify potential genotoxicty of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* which were purchased from Saudi Arabia markets as medicinal plants. Data in Tables 1 and 2 summarize the results of prophage induction; the used concentrations (5, 15, 30 mg/ml) did not show increase in the prophage induction when compared with control. Mutagenic index indicated no fold increase in Pfu/ml over the spontaneous release of phage from the lysogenic strains. So, the tested plant extracts do not

possess genotoxic effect. According to DeMarini *et al*. (1990), the positive response of any compound is recommended to be corresponding to a three-fold increase in Pfu/ml over the spontaneous background release of the lysogenic strain.

Table 1. Prophage F116 induction from lysogenic strain by *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* extracts.

Plant extract	Concentration (mg/ml)	Phage F116		
		Pfu/ml (10^7)	Mutagenic index (fold increase)	Mutagenic response
Myrtus communis	5	0	-	-
	15	0	-	-
	30	0	-	-
Ziziyphus spina-christi	5	3.6 ± 0.12	1.71	-
	15	1.41 ± 0.04	0.67	-
	30	0	0	-
Cassia angustifolia	5	6.9 ± 0.31	3.28	-
	15	4.3 ± 0.23	2.04	-
	30	2.5 ± 0.03	1.19	-
Control (spontaneous)		2.1 ± 0.02	1.0	-
Negative control (ascorbic acid 1%)		0.53 ± 0.01	0.25	-
Positive control (EMS 1%)		9.3 ± 0.87	4.43	+

Table 2. Prophage R3 induction from lysogenic strain by *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* extracts.

Plant extract	Concentration (mg/ml)	Phage R3		
		Pfu/ml (10^7)	Mutagenic index (fold increase)	Mutagenic response
Myrtus communis	5	0	-	-
	15	0	-	-
	30	0	-	-
Ziziyphus spina-christi	5	0.02 ± 0.01	0.015	-
	15	0	-	-
	30	0	-	-
Cassia angustifolia	5	0.12 ± 0.01	0.092	-
	15	0.07 ± 0.01	0.004	-
	30	0.050.11	0.038	-
Control (spontaneous)		$1.3 \pm 0..40$	1.0	-
Negative control (ascorbic acid 1%)		0.03 ± 0.01	0.23	-
Positive control (EMS 1%)		8.6 ± 0.34	6.62	+

- < 3 = Non mutagen, + = 3 -10 = Mutagen, + + = 11 - 19 = Effective mutagen, +++ = > 19 = Very effective mutagen.

SOS induction agents able to create DNA damages and block DNA replication, one of the manifestations of SOS functions is prophage induction and entry in the lytic cycle (Silva *et al*. 2012). In bacteria, repression of DNA replication or DNA damage triggers types of functions called the SOS response. The SOS response in *E. coli* involves the RecA, LexA proteins, a repressor and an inducer. When DNA is damaged, a single-stranded DNA is produced and this possibly activates the RecA protein to a form catalyze the cleavage of LexA protein. The LexA protein represses the genes involved in repair function. Therefore, cleavage of LexA protein under SOS conditions has enhanced expression of the lexA+ and recA+ genes. The role of recA+, lexA+ genes in the SOS repair of *E. coli* K-12 is well documented (Siddiqui *et al*. 2011). The bacteriophages are also cleaved under the same conditions, leading to the prophage induction in lysogen and entering in lytic cycle. Generally, the compounds that are genotoxic to higher eukaryotes also capable of inducing the SOS response in bacteria (Siddiqui *et al*. 2011). Prophage induction and SOS response occur by a variety of mechanisms and involves a number of classes of genetic damage, including alkylation, strand scissions, oxidation and cross-links. DNA strand breaks are inducer of prophage from lysogen. Siddiqui *et al*. (2011) evaluated the genotoxicity of certain water bodies by prophage lambda induction in the lysogen defective mutants of *Escherichia coli* and the plasmid nicking assay. They recorded a significant level of phage induction in case of industrial wastewater followed by that in river and groundwater samples. Silva *et al*. (2012) used prophage induction assay to assess genotoxic effect of *Duguetia furfuracea* extract. Their results proved that there was an increase of prophage λ induction with the dose 1 mg/ml, but the high doses (2, 5, 10 mg/ml) caused a decrease of the prophage induction.

Table 3. Inhibition zone of ethanolic extracts of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* against bacteria.

Plant extract /Conc. (mg/ml)	*Bacillus thuringiensis*			Diameter (nm) ± S.E. *Staphylococcus aureus*		*Pseudomonas aeruginosa*		
	Bt3	Bt8	HD1	SM10	SM12	PAO1	PU21	MAM2
Myrtus communis								
5	9 ± 0.21	8 ± 0.13	7 ± 0.21	8 ± 0.13	10 ± 0.33	6 ± 0.40	8 ± 0.21	7 ± 0.13
15	15 ± 0.43	15 ± 0.08	9 ± 0.89	10 ± 0.23	12 ± 0.33	8 ± 0.22	13 ± 0.23	10 ± 0.43
30	20 ± 0.98	20 ± 0.23	15 ± 0.91	12 ± 0.40	20 ± 0.67	17 ± 0.40	18 ± 0.11	18 ± 0.35
Ziziyphus spina-christi								
5	7 ± 0.13	8 ± 0.13	0	8 ± 0.21	6 ± 0.40	6 ± 0.40	7 ± 0.14	8 ± 0.11
15	11 ± 0.15	12 ± 0.78	8 ± 0.22	10 ± 0.33	10 ± 0.23	10 ± 0.36	10 ± 0.27	10 ± 0.23
30	14 ± 0.24	15 ± 0.53	12 ± 0.33	15 ± 0.65	17 ± 0.28	16 ± 0.65	15 ± 0.76	13 ± 0.78
Cassia angustifolia								
5	0	0	0	0	0	0	8 ± 0.13	0
15	7 ± 0.15	0	0	7 ± 0.15	0	0	10 ± 0.23	0
30	12 ± 0.33	8 ± 0.13	0	10 ± 0.45	0	0	12 ± 0.32	0

Antibacterial activity of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* were tested against *Bacillus thuringiensis, Staphylococcus aureus* and *Pseudomonas aeruginosa* strains using disc diffusion method. Bacterial strains were subjected to 5 mg/ml streptomycin to

obtain streptomycin resistant mutants and bacterial cell mutants were tested with ethanolic plant extracts. The results obtained in this study are presented in Table 3 and Figs 1, 2. Average diameter of inhibition zone was 0 to 20 mm at used concentrations (5, 15 and 30 mg/ml). *Myrtus communis* and *Ziziyphus spina-christi* were highly efficient against all used bacterial strains at 30 mg/ml (Fig. 1). The maximum inhibition zone (20 mm) was found in 30 mg/ml of *Myrtus communis* with *Bacillus thuringiensis* and *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* (18 mm). The results of antibacterial activity of *Myrtus communis* agree with those reported by Behbahani *et al.* (2016), Jerbi *et al.* (2016), Ads *et al.* (2017) and Alyousef *et al.* (2018) but vary in the active concentration. While, Taheri *et al.* (2013) showed that *Myrtus communis* has no effect on *Pseudomonas aeruginosa.* The discs containing 5 mg/ml showed the lowest inhibitory zones (6-10 mm). *Ziziyphus spina-christi* extract was less efficient than *Myrtus communis*, the highest inhibition zone diameter of *Ziziyphus spina-christi* was 17 mm (30 mg/ml) against *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* (16 mm), while *Bacillus*
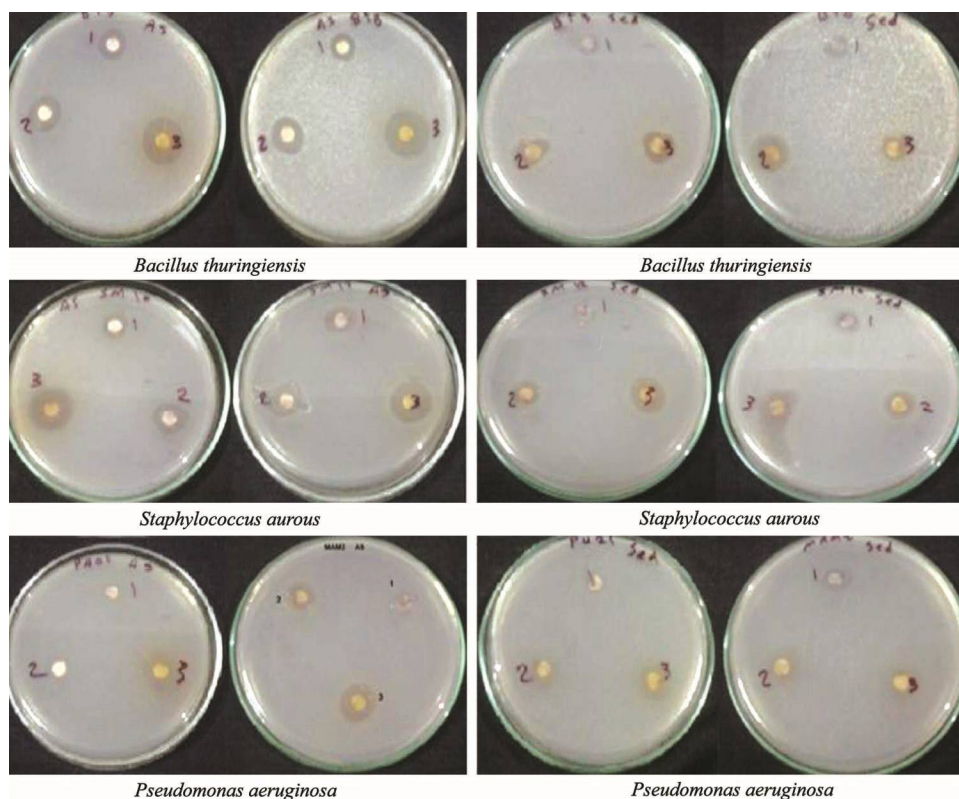

Fig. 1. Antibacterial activity of *Myrtus communis* and *Ziziyphus spina-christi.*

thuringiensis came in the last place (15 mm). Khalid *et al.* (2011) reported that the inhibitory effect of the *Ziziyphus spina-christi* leaves extract was observed up to the concentration of 50 mg/l, while Al-Mutairi *et al.* (2016) found that activity stopped at concentration of 32 mg/l. Abdallah (2017) reported that the fruit extract of *Ziziphus spina-christi* has no antibacterial activity against *Escherichia coli* and *Klebsiella pneumonia,* whereas it showed moderate antibacterial activities against *Staphylococcus epidermidis* and *Staphylococcus aureus..* According

to Belmimoun *et al*. (2016) 14 mm or greater were considered as high antibacterial activity. The present results demonstrate that ethanolic extracts of *Myrtus communis* and *Ziziyphus spina-christi* have strong antibacterial activity. *Cassia angustifolia* extract showed moderate antibacterial activity against some tested strains, the inhibition zone diameter ranged between 8-12 mm, but did not have any effect on other strains. These results are in agreement with the findings of Ahmed *et al*. (2016) and Bereksi *et al*. (2018). Singanboina *et al*. (2014) showed the remarkable inhibition of the *Cassia angustifolia* against bacterial growth. The bacterial survival assay is necessary to investigate the extracts toxic effects on the cells and demonstrate the intrinsic ability of a compound to cause cellular death because of damage to basic cellular functions.
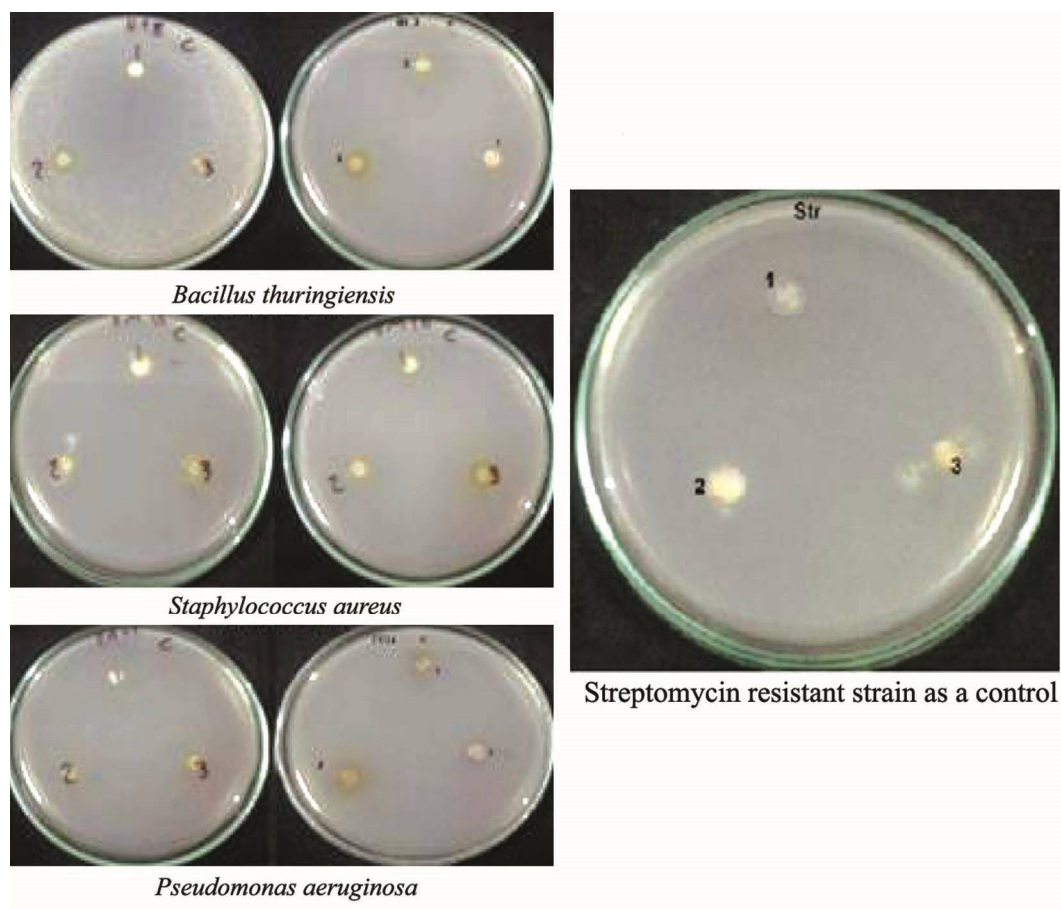

Fig. 2. Antibacterial activity of *Cassia angustifolia* and streptomycin.

With regard to the extracts of antifungal activity, the results showed a similar pattern to that of antibacterial assay. The tested fungus, *Rhodotorula* spp. when treated with discs containing 30 mg/ml of *Myrtus communis* and *Ziziyphus spina-christi* extracts showed inhibition of growth as indicated by diameter of the inhibition zones (18 and 16 mm respectively). However, *Cassia angustifolia* extract did not show any effect at all concentrations (Table 4).

Table 4. **Inhibition zone of ethanolic extracts of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* against *Rhodotorula* spp. fungus.**

Plant extract	Concentration (mg/ml)	Size of Inhibition zone (mm) ± standard error
Myrtus communis	5	0
	15	10 ± 0.23
	30	18 ± 0.40
Ziziyphus spina-christi	5	0
	15	8 ± 0.13
	30	16 ± 0.72
	5	0
Cassia angustifolia	15	0
	30	0

Davariya and Vala (2011) recorded that *Cassia occidentalis* extract was active against some fungi while was not active against others. Mehrabani *et al*. (2013) showed that methanolic extracts of *Myrtus communis* had antifungal activity against *Microsporum canis, Microsporum gypseum and Tricophyton mentagrophytes*. Ads *et al*. (2017) demonstrated that *Myrtus communis* had antifungal effects against *Aspergillus fumigate*. The antibacteriophage assay was performed using F116 against *Pseudomonas aeruginosa* and R3 against *Bacillus thuringiensis* in the direct plaque assay which indicate the antiviral activity. The obtained results revealed that ethanolic extracts of *Myrtus communis* and *Ziziyphus spina-christi* showed antiviral activity against F116 and R3 bacteriophages (Table 5). *Ziziyphus spina-christi* showed total inhibition of phage viability at all used concentrations (Pfu/ml = 0), *Myrtus communis* showed no plaque formed at high concentrations (15, 30 mg/ml), while at 5mg/ml the Pfu/ml was 4.6×10^2 with F116. These results indicate that *Ziziyphus spina-christi* and *Myrtus communis* had strong antiviral activity. *Cassia angustifolia* extract had minimal effects on tested phages, Pfu/ml range was 1.3×10^7 - 1.8×10^9. The results of the present study indicate that, *Myrtus communis* and *Ziziyphus spina-christi* extracts have strong antiviral agents against F116 and R3 bacteriophages, while *Cassia angustifolia* has moderate inactivation. Behravan *et al*. (2011) showed that *Thymus transcaspicus* essential oil had a moderate antiviral activity against phage CP51. Mohammed *et al*. (2013) demonstrated that anthraquinones isolated from *Cassia roxburghii* leaves have antiviral activity against influenza virus. The phytochemicals extracts act as antiviral by two mechanisms: one via their effect on viral particles before attachment to host cell and one after their effect on virus after entering the host cell. For example, polyphenols act by binding to the protein coat and thus repress adsorption of the virus. While flavonoids exhibit antiviral effects by inhibiting the RNA synthesis of viruses. They also inhibit replication enzymes of virus such as RT for HIV and RNA polymerase for influenza virus (Behravan *et al*. 2011).

Medicinal plants have flavonoids, tannins, alkaloids, polyphenolic components and saponins. The presence of these phytochemicals as bioactive components is responsible for its medicinal properties. Mehrabani *et al*. (2013) demonstrated that existence of flavonoids of *M. communis* leaves could be the cause of their important medicinal properties. Hussein *et al*. (2017) showed that alkaloid, phenolic and torpedoed compounds that were extracted from *Myrtus Communis* had antimicrobial activity against human gram-negative pathogenic bacteria. Al-Mutairi *et al*. (2016) showed that unsaturated fatty acids of *Ziziyphus spina-christi* were the major components (83.5%) that are responsible for the antimicrobial activity of this plant. Qualitative phytochemical screening of different extracts of *C. angustifolia* by Ahmed *et al*. (2016) revealed the presence of

alkaloids, steroids, flavonoids, saponins, terpenes, phenols, tannins, coumarins, terpenes, glycosides and anthraquinones which contribute to the antioxidant, antimicrobial and anticancer activities. Douhri *et al*. (2017) demonstrated that the antimicrobial activity may be attributed to tannin and polyphenolic compounds level in plants. This may lead to the damaging effect of these components on cell wall, proteins and DNA synthesis. Also, plant extracts and their components

Table 5. Effect of ethanolic extracts of *Myrtus communis*, *Ziziyphus spina-christi* and *Cassia angustifolia* on bateriophages viability.

Plant extract	Concentration (mg/ml)	Plaque forming unite (Pfu/ml)	
		Phage F116	Phage R3
Myrtus communis	5	$4.6 \pm 0.21 \times 10^2$	0
	15	0	0
	30	0	0
Ziziyphus spina-christi	5	0	0
	15	0	0
	30	0	0
Cassia angustifolia	5	$1.9 \pm 0.44 \times 10^7$	$1.8 \pm 0.21 \times 10^9$
	15	$1.7 \pm 0.25 \times 10^7$	$1.5 \pm 0.43 \times 10^8$
	30	$1.3 \pm 0.12 \times 10^7$	$1.2 \pm 0.23 \times 10^8$
Control (without extracts)	0	$1.4 \pm 0.33 \times 10^8$	$1.8 \pm 0.05 \times 10^9$

are hydrophobic, enabling them to fractionate the lipids of the bacterial cell membrane, disturbing the cell structures and rendering them more permeable. Excessive exit of ions and critical molecules will lead to death (Hussein *et al*. 2017). Medicinal plants are considered good resources for producing antimicrobial agents that act as natural non-harmful alternatives to antibiotics in the treatment of antibiotic-resistant microorganisms.

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(Manuscript received on 11 February, 2019; revised on 20 October, 2019)