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ANTI-BIOFILM ACTIVITY OF CRUDE ALKALOIDS EXTRACT FROM ROSELLE CALYCES AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA).

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Abstract

The aim of current study was evaluation of antibiofilm and antibacterial activity of crude alkaloids extract against methicillin resistant *Staphylococcus aureus* (MRSA). Crude alkaloid extract was prepared from the dried crushed calyces of Roselle (*Hibiscus sabdariffa*), then characterized by Gas Chromatography- Mass Spectrometry (GC-MASS). The antibacterial activity and MIC were determined for this extract by using agar well diffusion method and the modified broth macro-dilution method respectively against fifteen isolates of MRSA. Detection of biofilm production was assayed by tissue culture plate method. The results revealed that the antibacterial activity of crude alkaloids extract against MRSA bacteria was 18mm as inhibition zone at concentration 20mg/ml while the MIC of extract were 15 mg/ml. Biofilm assay results showed that ten isolates were strong biofilm production, all of these isolates changed to weak and moderate biofilm production isolates after treating with MIC of crude alkaloid extract of testing plant. From this study, the anti-biofilm activity of crude alkaloid was highly effective on biofilm degree of *Staphylococcus aureus* forming biofilm.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is adangerous pathogen because it have the ability to cause diseases by employing a varied number of virulent factors and resistance mechanisms (Butterly *et al*., 2010). Microorganisms adherence to their host is mediated by a number of general factors such as bacterial cell surface hydrophobicity (CSH). This factors was given initial bacterial adhesion to different surfaces such as teeth, medical devices, glass surface and contact lenses. The leading causes of device related infections are *Staphylococcus aureus* and Coagulase negative Staphylococci biofilms (Go¨tz, 2004). Many studies have proposed that the effect ofanti-infective agents on CSH of bacterial cells would be essential for anti-biofilm formation and anti-adhesion of the treated bacterial cells (Nithyanand *et al.*, 2010). The increasing number of multiple antibiotic-resistant microorganisms and failure to treat (infectious diseases) are main the problem in the medical area (Jafari *et al*., 2015). Therefore, many scientists has researched to produce new efficient agents that exceed the resistance of these microorganisms and are also cost-effective (Abo-Neima and El-Kholy, 2016).

Finding new anti-staphylococcal agents that could stop the adherence become an essential challenge to the scientific community. In the recent years, the use of plant extracts as alternative treatments has become widespread. The plant products which is considered as secondary metabolites produced by plants, these substances act as the defense mechanisms for plant against predation by microorganisms, insects and herbivores. *Hibiscus sabdariffa* (*H.sabdariffa*), commonly known as Roselle nearly 2.4m tall herbaceous subshrub. The calyx of this plant is thick, red and cup-shaped (Medina *et al*., 2009). This part of plant was rich in secondary metabolites that have medicinal properties since it contain flavonoids like alkaloids, gossypetin, hibiscetin and saponins (Hirunpanich *et al.*, 2005). Also Roselle calyx extract contains hibiscus acid, anthocyanins, flavonols and other polyphenolic compounds. Roselle extract has been used in folk medicine. The extract has anti-hypertensive, hepatoprotective, anti-oxidant, anti-inflammatory, anti-cancer and anti-microbial properties (Tsai *et al.*, 2014). Although there are many studies found the effects of Roselle as an herbal medicine but there are only a few trainings have studied the anti-biofilm activity of Roselle

calyx compounds. Thus, the purpose of this study was to examine the anti-biofilm activity of Roselle calyx compounds like alkaloids on pathogenic bacteria methicillin resistant Staphylococcus aureus (MRSA).

Material and Methods

Collection and drying of *H. sabdariffa* calyces

The calyces of Roselle was collected from the local market in Babylon Province (Iraq). Then it was washed three times by D.W. and then dried by using oven at 40°C for 5 days and pulverized to a fine powder using a sterilized mixer grinder. The powdered samples were stored in a clean container until the time of the extraction.

Extraction of Crude alkaloids

Extraction of crude Alkaloids from *H. sabdariffa* were carried out by using 100 g of plant powder was homogenized with 350 ml of 4:1 ethanol: D.W., in electrical blender for 5 minutes, then filtered with muslin cloth and Buchner funnel under reduced pressure by using Whatman No. 1 filter papers. The supernatant was evaporated at 45°C in a rotary evaporator, drops of 2% sulphuric acid were added until the pH became (1-2), then extracted with chloroform three times in separating funnel. The solution was separated into two layers, the lower layer was chloroform, was neglected. The upper layer was the aqueous layer to be used. Addition of drops of concentrated ammonium hydroxide was added to this layer until pH became (9-10), then extracted was again with chloroform: methanol mixture in ratio of 3:1 twice and one time with chloroform alone. Two layers appeared, the lower layer was evaporated at 40°C for (1-2) hours. The upper layer, the aqueous layer, was evaporated at 40°C for (1-2) hours and kept in refrigerator (Harborne, 1984).

Characterization using Gas Chromatography- Mass Spectrometry

Characterization of alkaloids was done by Gas Chromatography- Mass Spectrometry. GC-MS were recorded in a GCMS-2010 Shimadzu instrument operating in EI mode at 70ev. A Restek-5MS column (30m × 0.25mm × 0.25µm) was used. The oven temperature program was 1000 to 2500°C at 50°C min^{-1} and held for 5 min at 2500°C and from 2500°C to 2800°C at 100°C min^{-1} and held for 10 min at 2800°C. The injector temperature was 2500°C with normal injection mode. The flow rate of carrier gas helium was 1.21ml min^{-1}. The identification of alkaloids was confirmed by comparing the mass spectral data with those of authentic compounds and with data obtained from the literature.

Bacterial isolates

• Collection of samples:

A total of 73 clinical samples (wound, urine and stool) were collected from patients admitted at Al-Hashemia hospital during a period of four months (from January to May 2019). These samples were inoculated on nutrient, Mannitol and Blood agar plates, then incubated at 37°C for 24 hours under aerobic conditions. *S. aureus* isolates were identified according tomorphological, cultural and biochemical characters. Vitek2 system was used to confirm the characterization of the isolates.

• Screening for MRSA by Disc Diffusion method:

Screening for MRSA was done by Kirby-Bauer disc diffusion method by cefoxitin discs (30µg). Bacterial suspension was made by emulsify three to five colonies into 3 ml of sterile normal saline, then compared with the 0.5 McFarland turbidity standard. This suspension was inoculated on Muller Hinton agar plates and incubated at 37°C for 24 hours. An inhibition zone diameter of 21 mm around the cefoxitin disc was considered resistance; and 22 mm is considered sensitive (CLSI, 2016).

Determination of Antibacterial Activity of Crude alkaloid Extract (Agar Well Diffusion Method)

Muller Hinton agar plates were prepared and inoculated with the tested isolates by spreading the inoculums on the surface of media by sterile swab. By sterile cork borer (6 mm in diameter), wells were made in the agar and these wells were filled with alkaloid plant extract with concentrations (2.5, 5, 10, 20, 40, 80 mg/ml). The plates were incubated at 37°C for 18 hours. Then the diameters of the inhibition zones were measured to assess the antibacterial activity of these extract (Dodson *et al.*, 2000).

Determination of Minimum Inhibitory Concentration (MIC) of Crude alkaloid Extract

To assess the MIC of the crude alkaloid extract of tested plant, modified broth macro-dilution method was used. A twofold serial dilution of the extract was set by dissolving the dried extract in sterile distal water in four tube to gain a decreasing concentration range from (60mg/ml to 7.5mg /ml.) Then 500 µL volume from each dilution is transferred into test tubes contain about 4ml of sterilized Muller Hinton broth media. Standardized inoculums of 500 µL *S. aureus* strain that compared with McFarland standard tube No 0.5 (1.5×10^8 cell/ml) were inoculated for the tube that contain Muller Hinton broth media and plant extract and incubated at 37°C for 24 h. Then the turbidity of the medium was observed, the tube that have no turbidity is considered as the MIC (CLSI, 2012).

Biofilm Production by Tissue Culture Plate Method (TCP)

This method was considered the gold-standard method that detect for biofilm production, it was described by (Christensen *et al.*, 1985). The organisms were isolated from fresh agar plates and inoculated in 10 mL of BHI broth with 1% glucose. After the period of incubation, the cultures were diluted 1:100 with fresh medium. The wells of sterile 96 well-flat bottom polystyrene tissue culture were filled with 200µL of the diluted cultures. The control organisms were also added to tissue culture plate, while the negative control wells contained only sterile broth. Then the tissue culture plate was incubated at 37°C for 24 h. After incubation, the contents of all wells were detached by gentle tapping. Then 0.2 mL of phosphate buffer saline (pH 7.2) was used to wash all the wells four times to remove free floating bacteria. Biofilm produced by bacteria that adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was detached by using deionized water and plates were kept for drying. Optical density (OD) at 570nm of stained adherent biofilm was obtained by using Spectrophotometer.

Results and Discussion

Out of 73 clinical samples, 20(27.3%) were found to be *S. aureus*. The distribution of *S.aureus* isolates according to the site of infection was presented in the table 1. Others researcher (Kadhum, 2017) found the rate of *S. aureus* isolations were (16.66%) with distribution rate as (20.6%) from urine and (15.7%) from wound.

S. aureus is the main causes of community and1 hospital acquired infections which can cause various consequences (Hamza *et al.*, 2015). It's consider the cause for serious infections such as skin infections, burn and wounds infections, osteomyelitis, urinary tract infections, meningitis, septicemia and toxic shock syndrome (McCaig *et al.*, 2006). *S. aureus* able of long survival on environmental surfaces, it can be found in distilled water and in any part of hospital and it is resistant to numerous conventional antibiotics and chemical disinfectants (Azize, 2015).

Table 1: The numbers and percentage of *S. aureus* isolated from clinical specimens.

Sources of isolates	No. of samples	No. (%) of *S.aureus* isolates
Urine	35	10(28.5%)
Wound	25	5 (20.0%)
Stool	13	5(38.4%)
Total	73	20(27.3%)

Table 2: Means of antibacterial activity of crude alkaloid extract of *H.sabdariffa* calyx on *S. aureus* isolated from urine samples.

Concentration of extracts (mg/ml)	Inhibition zone in (mm) Mean ±SD
1.25	0
2.5	0
5	8±0.7
10	14±0.6
20	18±0.8

Detection of MRSA By Disk Diffusion Method

To detect MRSA, Disk Diffusion Method was done to tested bacterial isolates based on resistance ability to cefoxitin and Oxacillinantibiotics disc according to the (CLSI, 2016).

Current study showed that the rate of MRSA isolates were 75.0%. This result was agree with (Salman and Taj Aldeen, 2018) which found that 86.9% of *S. aureus* isolated from urinary tract infection is MRSA. Additional studies by (Sina *et al.*, 2011) exhibited that 53.6% of *S. aureus* isolated from urine samples are MRSA. Oxacillin considers stable at storage conditions (Weigelt, 2007). Rendering to that, the isolates that has been resistance tocefoxitin and oxacillin were interpreted as MRSA. Both of cefoxitin and oxacillin discs were used in this test for screening about MRSA isolates, other than methicillin. Methicillin is not used in our study because it is more sensitive to experimental conditions in contrast with oxacillin, also some evidence of another studies refer to failure the detection of resistance by using this antibiotic (Derek *et al.*, 2005).

Antibacterial Activity of crude alkaloid *H. sabdariffa* calyces extract

Antibacterial activity of crude alkaloid *H. sabdariffa* calyces extract has been determined via the agar well diffusion method. The findings of this study show that this extract showed inhibition zone diameter (18 mm) at concentration 20 mg\ ml against *S. aureus* as shown in table 2.

Characterization using Gas Chromatography- Mass Spectrometry

The crude alkaloid was extracted from the dry crushed powder of *H. sabdariffa* calyx. Identification of alkaloids was made by GC-MS (Fig. 1). Total of fifteen compounds were identified of which fifteen compounds namelyas shown in table 3.

The compounds that found in the extract are given this activity against testing bacteria. These compounds

Table 3: The compounds that identified in crude alkaloids extract from Roselle calyces by GC-MASS.

	The compounds
1	Trichloromethane
2	Methane,oxybis
3	Trichloromethane
4	Phenol, 2,4-bis(1,1-dimethylethyl)
5	Methane,bromodichloro-
6	Fumaric acid, 2-methylpent-3-yl tridecyl ester
7	Acetyl chlorid
8	Ethane,2,2-dichloro-1,1,1-trifluoro
9	Ethane,1,2,2-trichloro-1,1-difluoro
10	Dichloroacetic acid,heptadecyl ester
11	Pentaflouropropionic acid,pentadecyl ester
12	Acetic acid,chloro-,octadecyl ester
13	Erythro-9,10-Dibromopentaacosane
14	Fumaric acid,cyclohexylheptadecyl ester
15	Phenol,2,2,methylenebis (6-(1,1-dimethylethyl)-4-methyl-

are doing by numerous mechanisms and employ antimicrobial activity. The anti-microbial activities also may be due to numerous free hydroxyl ions that able to associate with the carbohydrates and protein of the bacterial cell wall. They may linked with the sites of cell enzymes and make them inactive (Harborne and Baxter, 1995).

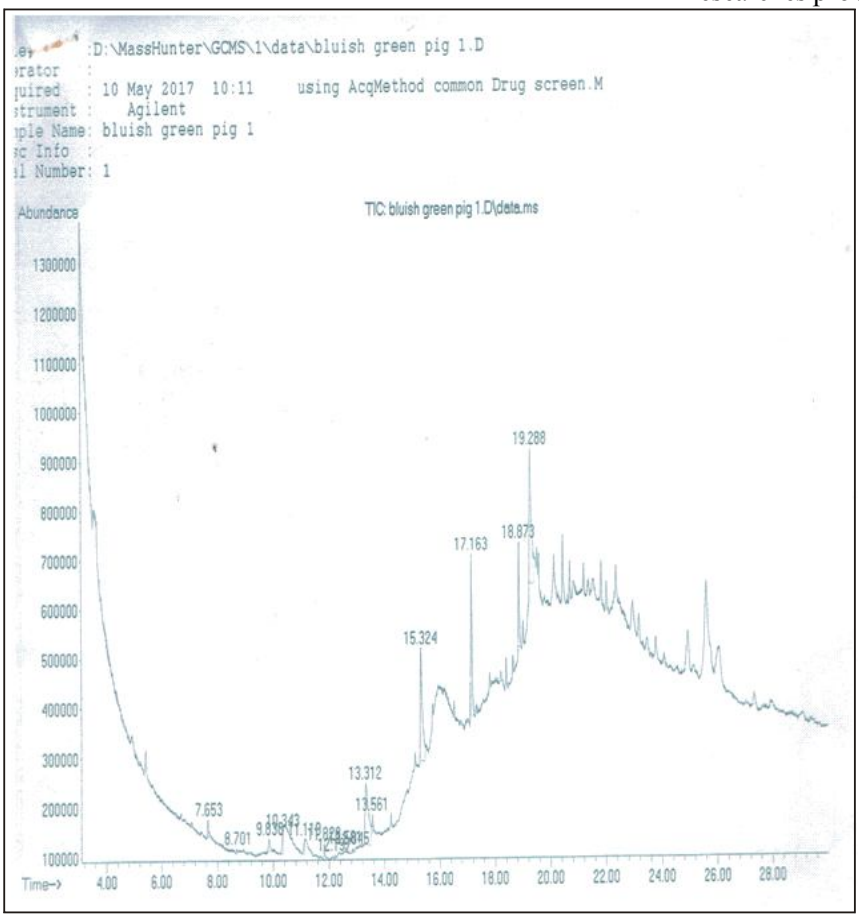

Fig. 1: GC-MASS characterization of alkaloid extract from *H. sabdariffa.*

Table 4: Biofilm production of MRSA according to with and without MIC of crude alkaloid *H. sabdariffa* calyces extract.

Bacterial isolates	Weak No. (%)	Moderate No. (%)	Strong No. (%)
S.aureus without plant extract	2(13.33%)	3(20.00%)	10(66.66%)
S.aureus with crude alkaloid *H. sabdariffa* calyces extract	13(86.66%)	2(13.33%)	None

The micro dilution assay gave MIC value 15 mg/ml. An outer peptidoglycan layer of gram positive bacteria is not effective barrier that make this type of bacteria is susceptible. Phytochemicals act as antimicrobial agent by different mechanisms, tannins for example do their role by iron removal, hydrogen bounding or non-specificinteractions with important proteins like enzymes (Scalbert, 1991), the mechanism of indoloquinoline alkaloids remains unclear (Sawer *et al.*, 2005). Many researches proved that the main indoloquinoline alkaloid, cryptolepine, makes cell lysis and morphological alterations of *S. aureus*, but the antimicrobial effects of the alkaloid may be through another mechanism, since the compound is known to be a DNA intercalateor and an inhibitor of DNA synthesis through topoisomerase inhibition (Lisgarten *et al.*, 2002).

Biofilm Assay by Tissue Culture Plate Method (TCM)

Biofilm is bacterial derived sessile groups described by the bacterial cells that are attached to each other or to a substratum continuously. Then this cell begin to make a material of extracellular polymeric substances (EPS) to fix themselves and exhibit a new phenotype with respect to growth rate and the transcription of the gene. Biofilm safe bacteria from phagocytosis, environmental pressure and antibiotics (Donlan and Costerton, 2002). In present study, biofilm production was identified by using tissue culture plate method (TCM).

TCM is appropriate method to

quantify biofilm production (Harvey *et al*., 2007). Crystal violet dye is linked to molecules that found on the surface of the cell such as polysaccharides, nucleic acid and negatively charged, so it clarifies the whole amount of the biofilm production. This method was applied to the bacterial isolates without extract and with MIC of crude alkaloid *H. sabdariffa* calyces extract.

Our results found that the isolates are ranged as weak, moderate and strong biofilm, as shown in table 4.

The result of biofilm production showed that 65.2% of bacterial isolates give strong biofilm,this result was agree with a local study in Babylon province, that found 61% of *S. aureus* isolates able to produce strong biofilm, most of these isolates from ulcer tissues and urine (Al-Hassnawi, 2012). Biofilm production is the dangerous feature of *S. aureus* infection, which be consisting of numerous layers of bacteria sheathed within an exopoly saccharideglycocalyx. This material protected the coated bacteria from host defenses and antibiotics (Stewart, 2002). While the result after treated with crude alkaloid *H. sabdariffa* calyces extract showed that no one of these isolates was strong biofilm production. The compounds which inhibited the biofilms might interfere with the adherence of bacteria to the surface, which is the initial step in the formation of biofilm. Biofilm provides microorganisms shelter against antibiotic and host defense, hence these compounds might be used as an alternative to antibiotics or used as an adjuvant with different antimicrobials to treat different bacterial infections (Ahmed *et al.*, 2014).

Conclusion

The crude alkaloid *H. sabdariffa* calyces extract revealed highly antibiofilm activity against methicillin resistant *Staphylococcus aureus* isolated from different clinical specimens.

References

Abo-Neima, S.E. and S.M. El-Kholy (2016). Antibacterial Characterization Studies of Silver Nanoparticles Against *Staphylococcus Aureus* and *Escherichia Coli*. *Internal J. Basic Appl. Scien*., **16(06):** 1-11.

Ahmed, A., A. Azim, M. Gurjar and A.K. Baronia (2014). Current concepts in combination antibiotic therapy for critically ill patients. *Indian J. Crit. Care Med.*, **18:** 310-314. 10.4103/0972-5229.132495.

Al-Hassnawi, H.H.M. (2012). Molecular Characterization of Antibiotic Resistance and Virulence Factor of Methicillin Resistance *Staphylococcus aureus* (MRSA) Isolated from Clinical Cases in Babylon Province. Ph.D thesis. Babylon university. Collage of medicine

Azize, H.W. (2015). Antibacterial Activity of Titanium Dioxide (Tio2) Doped with H_2O_2 against staphylococcus aureus Human pathogen in aqueos solution. *J. Baby Univ./Pure Appl. Scie.*, **23(2):** 617-625.

Butterly, A., U. Schmidt and J. Wiener-Kronish (2010). Methicillin resistant *Staphylococcus aureus* colonization, its relationship to nosocomial infection and efficacy of control methods. *Anesthesiology,* **113:** 1453-1459.

CLSI (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

Clinical and Laboratory Standards Institute (CLSI) (2016). Performance standards for antimicrobial susceptibility testing; Twenty-sixth informational supplement. CLSI document M100-S26. Wayne, PA: Clinical and Laboratory Standards Institute.

Christensen, G.D., W.A. Simpson, J.J. Yonger, L.M. Baddor, F.F. Barrett, D.M. Melton and E.H. Beachey (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol*., **22:** 996-1006.

Derek, F.J., Brown, David, I. Edwards, Peter, M. Hawkey, D. Morrison, Geoffrey, L. Ridgway, Kevin, J. Towner and W.D. Michael (2005). Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA). Oxford University Press. *J. Antimicrob. Chemother*., **56:** 1003.

Dodson, C.D., L.A. Dyer, J. Searcy, Z. Wright and D.K. Letourneau (2000). Cenocladamide, adiydropyridone alkaloid from piper. Cenocladum. *Phytochemistry*, **53:** 51-54.

Donlan, R.M. and W. Costerton (2002). Biofilms: Survival mechanisms of clinically relevant Microorganisms. *Clin. Microbiol. Rev*., **15(2):** 93-167.

Go¨tz, F. (2004). Staphylococci in colonization and disease: prospective targets for drugs and vaccine. *Curr. Opin. Microbiol*., **7:** 477-487.

Hamza, L.F., A.H. Al-Marzoqi, G.M. Aziz and Z.M. Al-Taee (2015). Molecular Study of Virulence Genes of Staphylococcus aureus Isolates from Various Clinical Origins by PCR. *Med. J. Babyl*., **12(3):** 677-688.

Harborne (1984). Phytochemical methods, London. Chapman and Hall, Ltd. 100-101. CLSI (2016) Clinical and Laboratory Standards Institute (CLSI) (2016): Performance standards for antimicrobial susceptibility testing; Twenty-sixth informational supplement. CLSI document M100-S26. Wayne, PA: Clinical and Laboratory Standards Institute.

Harborne, J. and M. Baxter (1995). Phytochemical Dictionary, A handbook of bioactive compounds from plants. Tylor and Francis; London.

Harvey, J., K.P. Keenan and A. Gilmour (2007). Assessing biofilm formation by Listeria monocytogenes strains. *Food*

Microbiol., **24:** 380-392.

Hirunpanich, V., A. Utaipat, N.P. Morales, N. Bunyapraphatsara, H. Sato and A. Herunsalee *et al*. (2005). Antioxidant effects of aqueous extract from dried calyx of *Hibiscus sabdariffa* Linn. (Roselle) *in vitro* using rat low-density lipoprotein (LDL). *Biol. Pharm. Bull.*, **28(3):** 481-484.

Jafari, A., L. Pourakbar, K. Farhadi, L.M. Golizad and Y. Goosta (2015). Biological synthesis of silver nanoparticles and evaluation of antibacterial and antifungal properties of silver and copper nanoparticles. *Turk. J. Biol*., **39:** 556-56.

Kadhum, S.A. (2017). The Effect of two Types of Nano-Particles (ZnO and SiO_2) on Different Types of Bacterial Growth. *Biomed. Pharmacol*., **10(4)**.

Lisgarten, J.N., M. Coll, J. Portugal, C.W. Wright and J. Aymami (2002). The antimalarial and cytotoxic drug cryptolepine intercalates into DNA at cytosine-cytosine sites. *Nature Structural Biol*., **9:** 57-60.

McCaig, L.F., L.C. McDonald, S. Mandal and D.B. Jernigan (2006). *Staphylococcus aureus*–associated Skin and Soft Tissue Infections in Ambulatory Care. *Emerg. Infect. Dis.*, **12(11):** 1715-1723.

Medina, I.C.R., R.B. Debon, V.M. Molina, C.A. Villaverde, J. Joven and J.A. Menendez *et al*. (2009). Direct characterization of aqueous extract of *Hibiscus sabdariffa* using HPLC with diode array detection coupled to ESI and ion trap MS. *J. Sep. Sci*., **32(20):** 3441-3448.

Nithyanand, P., R. Thenmozhi, J. Rathna and S.K. Pandian (2010). Inhibition of *Streptococcus pyogenes* biofilm formation by coral-associated actinomycetes. *Curr. Microbiol*., **60:** 454-460.

Salman, S.A. and W. Taj Aldeen (2018, Feb.). Antibacterial, Anti-virulence factors of *Hibiscus sabdariffa* extracts in *Staphylococcus aureus* isolated from patients with urinary tract infection. *Research J. Pharm. and Tech*., **11(2)**.

Sawer, I.K., M.I. Berry and J.L. Ford (2005). The killing effect on *Staphylococcus aureus*. *Lett. Appl. Microbiol*., **40:** 24-29.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, **30:** 3875-3883.

Sina, H., F. Baba-Moussa, T.A. Ahoyo, W. Mousse, S. Anagonou, J.D. Gbenou, G. Prévost, S.O. Kotchoni and L. Baba-Moussa (2011). Antibiotic susceptibility and toxins production of *Staphylococcus aureus* isolated from clinical samples from Benin. *African Journal of Microbiology Research,* **5(18):** 2797-2803.

Stewart, P.S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology*, **292(2):** 107-113.

Tsai, T.C., H.P. Huang, Y.C. Chang and C.J. Wang (2014). An anthocyanin-rich extract from *Hibiscus sabdariffa* Linnaeus inhibits N-Nitrosomethylurea-induced leukemia in rats. *J. Agric. Food Chem*., **62(7):** 1572-1580.

Weigelt, J.A. (2007). MRSA Informa Healthcare. USA, Inc.