Sudan University of Science & Technology
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Antimicrobial and Antioxidant Activity of *Hibiscus sabdariffa* (Roselle) against Selected Microorganisms

نشاط نبات الكركدى كمضاد للأكسدة والميكروبات ضد بعض الكائنات الحية الدقيقة

A Dissertation Submitted in Partial Fulfillment of the Requirements of M.Sc Degree in Medical Laboratory Sciences (Microbiology)

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الآية

قال تعالى :

(وَتَرَى الْجِبَالَ تَحْسَبُهَا جَامِدَةً وَهِيَ تَمُرُّ مَرَّ السَّحَابِ صُنْعَ اللَّهِ الَّذِي أَتْقَنَ كُلَّ شَيْءٍ إِنَّهُ خَبِيرٌ بِمَا تَفْعَلُونَ)

صدق الله العظيم

(النمل : 88)

Dedication

To my parents the source of tenderness

I am honored to have you as my parents

To my brothers and sisters

To all my family and my friends

Acknowledgement

First and all thanks to the ALMIGHTY ALLAH for giving me the strength to run this research. My gratitudes and thanks to my supervisor Dr. Ahmed I. Hashim for his great efforts and valuable advices. My gratitudes and appreciation to Suliman Ismael and Ahmed Saeed and all members of Department of Microbiology and Parasitology in Medicinal Aromatic Plants and Traditional Medicine Research Institute(MAPTMRI), National Center for Research, for their assistance and cooperation. I would also like to thank the staff of Department of Biochemistry in (MAPTMRI). A special thanks goes to Amina Mahmood for her assistance. Last but not least I would like to express my gratitudes to everyone encouraged me, supported me, or assisted me throughout this study. Without you all this work would have been impossible.

Abstract

Introduction : Prevalence of resistant microbial strains is increasing with time; due to the extensive and indiscriminate use of the broad spectrum antimicrobial drugs that render the current used antimicrobial agents insufficient to control some microbial infections.

Objective: The present study was aimed to investigate the antimicrobial and antioxidant potential of methanolic extract of the medicinal plant *Hibiscus sabdariffa* L.

Method: The agar well diffusion technique was used to determine the antimicrobial activity of *Hibiscus sabdariffa* L and the antioxidant activity was determined through the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) method. In this study the methanolic extract of *Hibiscus sabdariffa* L was examined against six reference bacterial strains, one fungal reference strain, and one parasitic species including *E. coli* (ATCC 25922) *Ps. aeruginosa* (ATCC27853), *K. pneumoniae* (ATCC 15380), *S. typhi (ATCC), B. subtilis* (NCTC 8236), *S. aureus* (ATCC 25923), *C. albicans* (ATCC 7596) and *G. lamblia.*

Result: The results of the preliminary screening reveals that the extract showed activity against all microorganisms tested and the inhibition zones were between 20 and 25 mm. The minimum inhibitory concentrations were determined for the extract against the selected microorganisms. The methanolic extract of the *Hibiscus sabdariffa* L screened for antigiardial activity against (*G. lamblia*) trophozoites *in vitro* showed antigiardial activity with an inhibition concentration (IC50) more than 180µg/ml. The radical scavenging potential of methanolic extract of *Hibiscus sabdariffa* L showed 53% .

Conclusion: In conclusion, methanolic extract of *Hibiscus sabdariffa* L used in this study had antimicrobial and antioxidant potential that justify it is use in folkloric and traditional medicine. Further studies are required to confirm these results, identify active compounds and toxicity.

المستخلص

ان ازدياد انتشار السلالات الميكروبية المقاومة للمضادات الحيوية غالبا مايزداد بمرور الزمن نتيجة للاستخدام المكثف وغير الرشيد للمضادات الميكروبية مما يجعل هذه المضادات غير فعالة في السيطرة علي بعض الامراض الميكروبية. . هدفت هذه الدراسة لتقييم الأنشطة المضادة للميكروبات والاكسدة لنبات الكركدي و تم اختبار المستخلص الميثانولي للكوؤس ضد مجموعة من الجراثيم و الفطريات والطفيليات المعيارية. اتبعت طريقة الانتشار الطبقي في الاجار للتقصي عن وجود مضادات ميكروبية في نبات الكركدى وطريقة DPPH لمعرفة نشاط النبات كمضاد للاكسدة.

تم اختبار فعالية المستخلص الميثانولي لنبات الكركدى ضد ستة انواع من البكتريا و فطر وحيد واخر طفيلى (الاشريكية القولونية ،،الزائفة الزنجبارية، الكلبسيلا الرئوية، السلمونيلة التايفية ، العصوية الرقيقة، العنقودية الذهبية ، البيضاء المبيضة والجياردية اللمبلية).

اظهرت النتائج الأولية أن المستخلص له فعالية ضد الاحياء الدقيقة القياسية تراوحت مناطق التثبيط من 20-25 ملم.

باستخدام طريقة الانتشار في الآجار.ايضا نم تحديد اقل تركيز مثبط لنمو الكائنات الدقيقة.

كما اظهرت النتيجة بأن النبات فعال ايضا ضد طفيل (الجياردية اللمبلية) فى التركيز (500 -250-125 مايكرو جرام)، وايضا تم تحديد الفعالية المضادة للاكسدة والتى اظهرت فعالية 53 %.

تمخضت هذه الدراسة عن ان المستخلص الميثانولي لكؤوس نبات الكركدي المستخدم في هذه الدراسة ذو فاعلية عالية كمضاد للميكروبات ومضادات الأكسدة التي تبرر استخدامها في الفلكلور والطب الشعبى، مزيد من الدراسات لتأكيد هذه النتائج وتحديد المركبات النشطة وسمية النبات.

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List of Abbreviations

1	ATCC	American Type Culture Collection
2	C.F.U	Colony Formed Unit
3	CONS	coagulase negative staphylococci
4	DNA	deoxyribonucleic acid
5	DPPH	2, 2-Diphenyl-1-picrylhydrazyl
6	IC50	IC50 The concentration of sample required for 50% inhibition
7	MAPTMRI	Medicinal Aromatic Plants and Traditional Medicine Research Institute
8	MDIZ	Mean diameter inhibition zone.
9	MIC	Minimum inhibitory concentration
10	NCTC	National Collection of Type Culture
11	RPMI	Roswell Park Memorial Institute
12	SD	Standard Deviation

CHAPTER ONE

INRODUCTION

Infectious diseases pose a constant threat to human. Every individual on the earth can be affected by a disease. The emergence and re-emergence of infectious diseases have become worldwide problem. An infectious disease is caused by various microbes or pathogen. Most of them are usually microorganisms. Few of them are visible by naked eyes. The most common pathogens are different types of viruses and bacteria. Fungi and Protozoa are also known as pathogens and are responsible for various diseases. One of the effective ways to control a disease is to reduce contacts. Vaccines and drugs are the two widely used prevention tools that can potentially reduce transmissions and control diseases. Antibiotics are one of the most important weapons in fighting infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. Resistance development is an even bigger problem since the bacterial resistance is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class. Bacterial resistance and its rapid increase is a major concern of global public health and are emerging as one of the most significant challenges to human health. The global emergence of antibiotics resistance is fueled by the wide spread use of broad- spectrum antimicrobial agents, creating continuous selective pressure, and by lapses in infection control, which facilitate transmission of resistant pathogenic microorganisms. The dynamics of antibiotic resistance within hospital settings are determined by introduction of resistance, cross-transmission and induction of

resistant strains during antibiotic therapy (Bonten *et al*., 2001). Nature has been a huge source of antimicrobial and other medicinal product since pre -historic times, the importance of using herbal products in the treating of various human diseases are not limited. It is obvious that the plant kingdom harbours inexhaustible sources of active ingredients that valuable in the management of many serious and complicated diseases. Therefore, medicinal plants are significant for the study of their conventional uses through the confirmation of their pharmacological effects (Rabia and Asghari, 2012).Treating bacterial infections by antibiotics is beneficial but their indiscriminate use has led to an alarming resistance among microorganisms as well as led to re-emergence of old infectious diseases. One approach to treat infectious diseases is the use of plant extracts individually and /or as an alternative approach is the use of combination of antibiotics with plant extracts.

1.2. Rationale

Currently, the main therapy for bacterial infections is synthetic antibiotics. However, the misuse and overuse of antibiotics has become key factor in the emergence of drug resistant strains of several groups of microorganisms. Drug resistance is now a global public health threat that involves all major microbial pathogens and antimicrobial drugs (Stuart and Bonnie, 2004). Therefore, researchers are now turning their attention to herbal products, investigating for new hints to develop better drugs against resistant strains (Braga *et al.,* 2005). Plant-based therapeutics are known to be easily biodegradable, with minimal adverse side effects and being easily accessible at low prices (Fullerton *et al*., 2011). Therefore the need to find new herbal antimicrobial agents in this era of rapid global spread of resistant isolates to commonly used antibiotics.

1.3. Objectives

1.3.1. General objectives

To study the the Antioxidant and Antimicrobial Activity of *Hibiscus sabdariffa* (Roselle) against some microorganisms

1.3.2. Specific objectives

1- To determine the antimicrobial activity of reference bacterial strains against selected antibiotics

2- To determine the antimicrobial activity of methanolic extract of *Hibiscus sabdariffa* L against reference bacteria, fungi and *G.lamblia* trophozoite clinical isolate

3- To detect the radical scavenging potential of *Hibiscus sabdariffa* L through the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) method

CHAPTER TWO
LITERATURE REVIEW

2.1. Antimicrobial activity of medicinal plants

Historically, plants have provided a good source of antimicrobial agents. Plants such as *Hibiscus sabdariffa* L have been used effectively in folk medicines for treatment of inflammatory diseases (Dafallah and Al-Mustafa, 1996), The significant biological action of medicinal plants is their antimicrobial activity against infectious diseases, which are the foremost deadly treat worldwide (Balandrin *et al.,* 1993). Plant based antimicrobial compounds became popular, and it is known that, now, almost half of the clinically used drugs are originated from natural products with one quarter coming from higher plants (Bandow *et al.,* 2003). The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of certain medicinal plants for their potential antibacterial activity (Pakekh and Chanda, 2007). Thus, scientists tended to look for more effective plant based antimicrobial sources as an alternative to synthetic ones and now it is estimated that more 50% of the Western drugs are plant derived, which have been once used in crude form in traditional or folk healing practices (Özge, 2011). The use of plant extracts and phytochemicals, with known antibacterial properties, may be of immense importance in therapeutic treatments. In the past few years, a number of studies have been conducted in different countries to prove such efficiency (Bhattacharjee *et al.*, 2006).

2.2. Botanical, phytochemical and ethno-pharmacological profiles of selected plant

Medicinal and aromatic plants and their derivatives represent an integral part of life in Sudan (Khalid *et al.,* 2012).The selected plant are recognized as components of the traditional medicine in Sudan used for treatment.

2.2.1. *Hibiscus sabdariffa* L

Family: Malvaceae

Genus: *Hibiscus*

Species: *H. sabdariffa*

Vernacular name: karkade.

2.2.1.1. Description

Hibiscus sabdariffa L is a species of *Hibiscus* native to the old world tropics. It is an annual or perennial herb or woody-based subshrub, (2–2.5 m) tall. The leaves are deeply three- to five-lobed .The flowers are 8–10 cm in diameter, white to pale yellow with a dark red spot at the base of each petal. Have a stout fleshy calyx at the base, fleshy and bright red as the fruit matures. It takes about six months to mature (Copley, 1975). The size of the calyx varies with each variety, but ranges from ½ to 1 ½ inches in diameter (Stephens, 1994). Roselle may have been domesticated in western Sudan before 4000 BC; (Wilson and Menzel, 1964). Sudan is currently the major producer of Roselle; however, farmers regard it as a famine food. When drought is expected, farmers prefer to cultivate Roselle rather than cereals because of its hardiness under adverse conditions (Mohamad *et al.,* 2002). Roselle or Karkade (Arabic name) is grown in various parts of the Sudan, particularly Kurdofan and Darfur. It is one of the cash crops cultivated by traditional farmers in Kurdofan and Darfur States under rain-fed conditions, where large quantities are produced both for local consumption and for export. There are two main types white, red Kerkrade. The sensory evaluation of cold and hot drinks

made from both white and red Kerkrade revealed that there was no significant different as regard to the overall preference (Suliman *et al*., 2011).

2.2.1.2. Uses of *Hibiscus sabdariffa* L

Many parts of *Hibiscus sabdariffa* L including seeds, leaves, fruits and roots are used in various foods. *H. sabdariffa* L is a multi-use plant, whose outer leaves (calyx), also known as natal sorrel; (Ageless, 1999) is frequently used in the production of jelly, jam, juice, wine, syrup, gelatin, pudding, cake, ice cream and flavoring. Its brilliant red color and unique flavor make it a valuable food product (Tsai and Ou, 1996). Roselle is an annual crop used in food, animal feed, nutraceuticals, cosmetics and pharmaceuticals. The calyces, stems and leaves are acidic in flavor. The juice from the calyces is claimed to be a health-enhancing drink due to its high content of vitamin C, anthocyanins and other antioxidants (Mohamed *et al*., 2002).

In Sudan, the dry calyx is used to produce a flavorsome and healthy and dried calyces are used for tea, jelly, marmalade, ices, ice cream, sorbets, butter, pies, sauces, tarts, and other desserts (Duke and Ayensu, 1985). The seeds have also been used as an aphrodisiac coffee substitute.

2.2.1.3. Medicinal uses

H. sabdariffa L is used in many folk medicines. It is claimed as a Thai traditional medicine for kidney stones and urinary bladder stones (Hirunpanich *et al*., 2006). *H. sabdariffa* L also is said to have diuretic effects, used effectively in folk medicines for treatment of inflammatory diseases (Dafallah and Al-Mustafa, 1996), and cancer (Chewonarin *et al*., 1999). The positive effect of *H. sabdariffa* L extract consumption to decrease blood pressure has been proved in study on both man and rats (Faraji *et al.,* 1999; Onyenekwe *et al.,* 1999). More recently, the antihypertensive action of *H. sabdariffa* L has been confirmed with experimental hypertension (Odigie *et al.,* 2003). However, there is no reliable evidence to support recommending hibiscus tea in the treatment of primary hypertension

(Wahabi, 2010). Hibiscus has one of the highest levels of antioxidants of any widely available food; antioxidants have been shown in several studies to enhance nitric oxide production in the body, reducing blood pressure and oxidized lipids. Antioxidants have also reduced cancer promotion in several studies, and plants containing large amounts of antioxidants are regularly studied for their known anti-cancer effects, and promotion of good health by enabling oxidative balance (Burton, 2010).

2.2.1.4. Phytochemicals

The plants are rich in anthocyanins, as well as protocatechuic acid. The dried calyces contain the flavonoids gossypetin, hibiscetine and sabdaretine. The major pigment, formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), Chrysanthenin (cyanidin 3-monoglucoside), and delphinidin are also present. *H. sabdariffa* seeds are a good source of lipid-soluble antioxidants, particularly gamma-tocopherol (Mohamed *et al*., 2007).

2.3. Microscopic examination of test microorganisms

The identity of the reference bacterial strains was confirmed using conventional methods described in medical microbiology (Brooks *et al* ., 2012)

2.3.1. *Candida albicans* Identification

On routine media, cream colour pasty colonies usually appear after 24-48 hours, the colonies have a distinctive yeast smell.

Budding yeast in stained or non-stained preparation, from corn meal agar pseudo hyphae and budding yeast. Germ tube test (the ability of *C. albicans* to produce pseudo germ tube in serum) positive.

Germ tube test (GTT)

This is rapid test for presumptive identification of *C.albicans*. Three drops of serum were put into small tube by using a Pasteur pipette, a colony of yeast was

touched by sterile wire loop and emulsified it in the serum. After incubation at 37oC for 2-4 hours then a drop of the serum was transferred to a slide and examine microscopically. Presence of short lateral filament (germ tube) indicate positive test for *C.albicans*.

2.3.2. *Giardia lamblia* Identification

The protozoan *G. lamblia* is the most frequently isolated intestinal protozoan parasite around the world and it is the causal agent of the disease known as giardiasis (Campanati and Monteiro-Leal, 2002). *G. lamblia* is a unicellular, flagellated intestinal protozoan parasite isolated worldwide and is ranked among the top 10 human parasites (Harris *et al.,* 2000; Harris *et al*., 2001). The morphology of *Giardia* is encountered in two forms: trophozoites and cysts. The trophozoite stage is approximately 12-15 microns by 6-8 microns (Morgan *et al.,* 1993). The cyst of *Giardia lamblia* is elliptically shaped, ranged in size from 6 to 10 microns and contains two to four nuclei (Petri, 2003).

2.4 2, 2 diphenyl-2-picryl hydrazyl (DPPH) radical scavenging

The 2, 2 diphenyl-2-picryl hydrazyl (DPPH) radical widely used in screening the antioxidant potential of natural compounds such as phenolic or crude extracts of plants. The assay is based on the measurement of the scavenging potential of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent (Mensor *et al*., 2001). DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The colour changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 518 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant by its hydrogen donating ability.

CHAPTER THREE

MATERIALS & METHODS

3.1. Study design

This was an experimental laboratory based study.

3.2. Study area

This research was conducted in Medicinal Aromatic plants and Traditional Medicine Research Institute (MAPTMRI), National Center for Research. Khartoum, Sudan.

3.3. Study population

Reference strains of S. *aureus* (ATCC 25923), *E. coli* (ATCC 25922), *Ps. aeruginosa* (ATCC 27853), *B. subtilis* (NCTC 8236), *C. albicans* (ATCC 7596), *K. pneumoniae* (ATCC 53657) *S. typhi* (ATCC 14028) *G. lamblia* clinical isolate.

3.4. Data analysis

Data was analyzed statistically through Microsoft Excel

3.5. Methods

3.5.1. Collection of the Plant materials

Calyx of *H. sabdariffa* L was bought from Omdurman supermarket, Khartoum, Sudan in May 2016. They were authenticated by the Herbarium Department, at (MAPTMRI).

3.5.2. Preparation of Crude Extracts

Extraction was carried out according to the method described by (Khalid *et al*., 2012) 100 gram was grounded using mortar and pestle and successively extracted by soaking in 80 % methanol for seventy two hours with daily filtration and evaporation. A rotary evaporator was used for evaporation of the solvent under reduced pressure then the extract were exposed to dryness using rotary to air until

complete dryness. Each residue was weighed and the yield percentage was determined.

3.5.3. Preparation of the test organisms

3.5.3.1. Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used.

3.5.3.2. Preparation of fungal suspension

The fungal cultures were maintained on Sabouraud dextrose agar (SDA), incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspension in 100 ml of sterile normal saline, and the suspension were stored in the refrigerator until used.

3.5.3.3. *In vitro* testing of extracts for antimicrobial activity by agar well diffusion method

The antimicrobial activity of the plant extract was determined using the agar well diffusion method. The extract (*Hibiscus sabdariffa* L) were dissolved in methanol to prepare 100 (w/v) % of each. The individual test organisms were standardised by adjusting the absorbance of the inoculum to (0.08–0.13) at OD 625 nm (Wiegand *et al*., 2008). Hundred µl of the standardised inoculums were spread on the surface of Nutrient agar using disposable sterile glass spreader, and the surface was allowed to dry. Wells (10 mm in diameter) were cut from the inoculated medium using a flame-sterilized cork borer, and then filled with 100 µl of plant extract concentration 0.1g/ml. The plates were incubated at 37°C for 24 hours. And then the zones of inhibition were measured around each well.

3.5.3.4. *In vitro* testing of extract for antigiardial activity

In vitro susceptibility assays were performed following the sub-culture method of (Cedillo-Rivera *et al*., 2002) which is a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *E. histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis* (Arguello-Garcia *et al*., 2004). Five mg from plant extract and compound was dissolved in 50 µl of dimethylsulfoxide (DMSO) in an Eppendorf tube containing 950 µl in order to reach the concentration of 5 mg/ml (5000 ppm). The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution. The concentrates were stored at -20°C for further analysis. Sterile 96 multi-well plate (8 columns (C) × 12 rows (R)) was used, positive control and negative control with three columns used for extract. 40 µl of the plant extract solution (5 mg/ml) were added to the first column wells C-1, On the other hand, 20 µl of complete RPMI medium were added to the other wells of the second column and third column (C-2 and C-3). Serial dilutions of the extract were obtained by taking 20 µl of extract from C1 to the second column wells and mixing then, 20 µl were taken out from the solution in C-2 wells to C-3 wells and discarding 20 µl from the solution of C-3. 80 µl of culture medium was Completed with parasite (1 $X10^3$ cell/ml) and added to all wells. The final volume in the wells was 100 µl. Metronidazole (a trichomonocide) pure compound ((1-(2-hydroxyethl)-2-methyl-5 Nitroimidazole), was used as positive control at a concentration of 312.5 µg/ml, whereas untreated cells were used as a negative controls (culture medium plus trophozoites). For counting, the samples were mixed with Trypan blue in equal volume. The final number of parasites was determined with haemocytometer three times after 24, 48, 72 and 96 hrs.

The mortality % of parasite for each extract was calculated according to the following formula:

Mortality of cells % =

$$\frac{(\text{ n° of cells in negative Control -n° of cells in tested sample with extract})\times 100}{\text{n° of cells negative Control}}$$

100% inhibition of the parasite was considered, when there was no motile parasite observed.

3.5.3.5. Antioxidant activity assay

DPPH radical scavenging assay

The radical-scavenging activity of the plant extracts was evaluated with the DPPH assay (Zaouali *et al*., 2010). One millilitre of plant extract was added to 3 ml of the methanolic DPPH solution. The mixture was then shaken and allowed to stand at room temperature in the dark for 30 minutes then the decrease in absorbance at 517 nm was measured against a blank (methanol solution) using a Jenway spectrophotometer. A mixture consisting of 1 ml of Propyl Gallate and 3 ml of DPPH solution was used as the control. The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH, was calculated according to the formula % inhibition = [(AB _ AA)/AB]*100, where AB and AA are the absorbance values of the control and of the test sample, respectively.

CHAPTER FOUR

RESULTS

The antimicrobial potential of the methanolic extract of the medicinal plant *Hibiscus sabdariffa* L was evaluated against six standard bacterial strains, one fungal, and one parasitic species .Ciprofloxacin and Gentamicin were used against six strains, *E. coli* was resistant for all Ciprofloxacin concentration, concentration (40-5) whereas it susceptible to Gentamicin. The higher concentration the higher effect on the bacterium. Both of the antibiotics were effective in treatment of the other pathogenic bacteria in all concentration (table1). *H. sabdariffa* L gave slightly similar inhibition effect on all the tested organisms (table2). The plant showed similar inhibition effect to antibiotics for all the tested strains (Fig1)

Table (1): Antibacterial activity of selected antibiotics against reference strains

Antibiotic	Conc. (µg/ml)	Reference strains /MDIZ mm					
		E.coli	*Ps.aeruginosa*	*K. pneumoniae*	*S. typhi*	*B. subtilis*	*S.aureus*
Ciprofloxacin	40	0	38	26	33	34	30
	20	0	33	24	30	29	29
	10	0	31	23	29	28	28
	5	0	23	20	28	26	20
Gentamicin	40	19	23	18	19	18	21
	20	16	20	16	17	17	20
	10	15	17	15	16	16	19
	5	14	15	14	14	15	15

Table (2): Antimicrobial activity of methanolic extracts of *Hibiscus sabdariffa* L against selected reference strians

Plant name	Reference strains /MDIZ mm						
	E. coli	*Ps. aeruginosa*	*K. pnemoniae*	*S.typhi*	*B. subtilis*	*S. aureus*	*C.albicans*
H. sabdariffa L	20	23	20	22	20	25	21

MDIZ: Mean diameter inhibition zone.

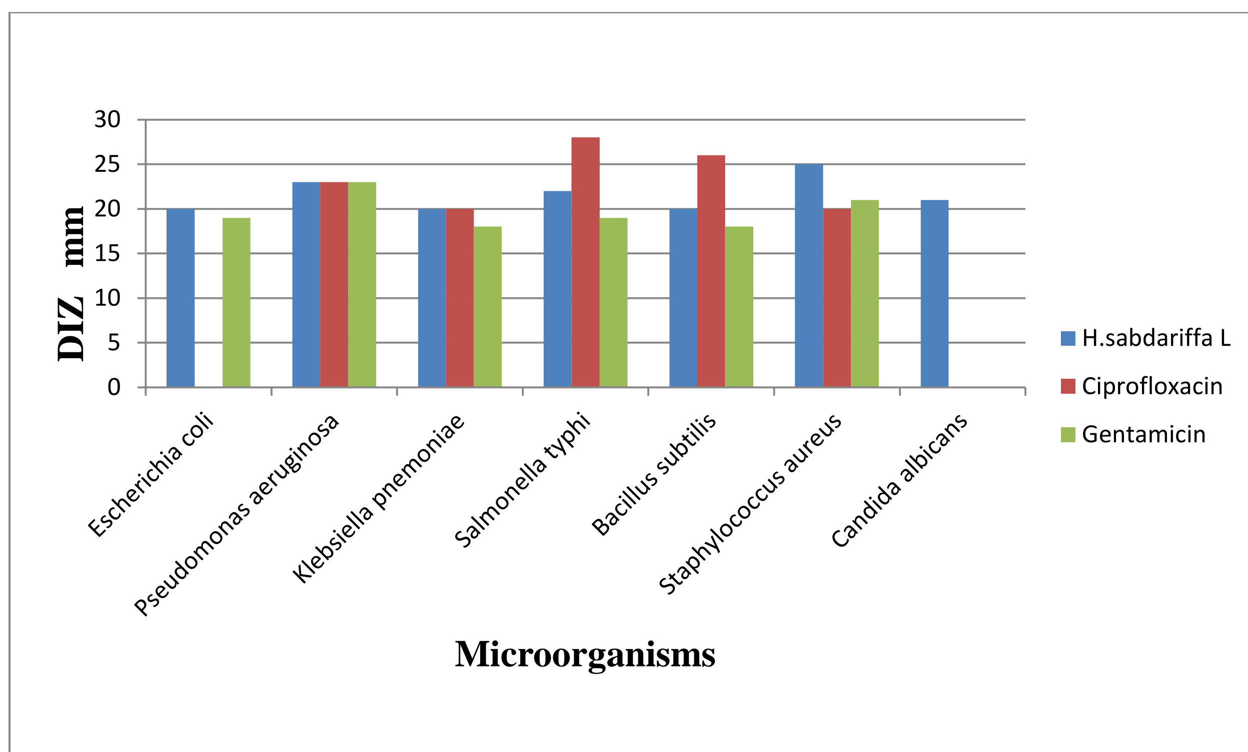

Fig (1): Antimicrobial Activity of Roselle extract & selected drugs against reference strains

Minimum Inhibitory Concentration of *H. sabdariffa* L was 12.5 to four of the strains and 6.25 to *B. subtilis* and *C.albicans*

Table (3): Minimum Inhibitory Concentration(MIC) of the methanolic extract of *Hibiscus sabdariffa* L

Plant name	MIC of reference bacterial strains mg/ml						
	E. coli	*Ps.aeruginosa*	*K. pneumoniae*	*S. typhi*	*B. subtilis*	*S. aureus*	*C.albicans*
H. sabdariffa L	12.5	12.5	12.5	12.5	6.25	6.25	12.5

H. sabdariffa L also showed antigiardial activity and the mortality rate was related with concentration (higher at high concentration) (table4)

Table (4): Anti-giardial activity of the methanolic extract of *Hibiscus sabdariffa* L

Plant name	Mortality (µg/ml)			IC_{50} (µg/ml)
	Mortality (%) ± SD			
	500	250	125	
H. sabdariffa L	72.57 ± 0.4	53.09 ± 0.9	44.02 ± 0.03	184.28
Control	95.03 ± 0.03			

H. sabdariffa L pose antioxidant potential (table 5)

Table (5): Radical-scavenging potential of the methanolic extract of *Hibiscus sabdariffa* L

No.	Sample	%RSA ±SD (DPPH)
1	*H. sabdariffa* L	53± 0.09
2	Propyl Gallate	93± 0.01

CHAPTER FIVE

DISCUSSION

The therapeutic use of plants especially as antimicrobials has been reported by many scientists (Sharaf *et al*., 1966; Cowan, 1999; González-Lamothe *et al.,* 2009). Reports of antimicrobial activity of *H. sabdariffa* L showed various levels of microbial growth inhibition against Gram positive and Gram negative bacteria (Fullerton *et al*., 2011). This indicates the broad spectrum nature of the *H. sabdariffa* L. In This study the antimicrobial potential of the methanolic extract of *H. sabdariffa* L was evaluated against six reference bacterial strains, one fungal and one parasitic clinical isolate. The mean diameter of inhibition zone produced by *H. sabdariffa* L extract against the tested microorganisms is presented in (Table 4.2). On the other hand, (Table 4.1) showed antimicrobial activity of the selected drugs. Based on the results of (table 4.2), Plant extract resulting in 15 mm or more MDIZ were considered active and those resulting in less than 15 mm were regarded as inactive (Cruickshank *et al*., 1975). The minimum inhibitory concentration (MIC) of the methanolic extract of *H. sabdariffa* L calyces against reference microorganisms (Table 4.3) revealed that *H. sabdariffa* L calyces was inhibiting at different concentration (12.5 mg/ml and 6.25). The extract was screened for antigiardial activity against (*Giardia lamblia*) trophozoites *in vitro* in (Table 4.4). The radical scavenging potential of methanolic extract of *H. sabdariffa* L calyces at concentration (5 mg) scavenged 53% of DPPH radical .

The calyces methanolic extract of *H. sabdariffa* L revealed high activity against *S. aureus* (25mm), *C. albicans* (21mm), *Ps. aeruginosa* (23mm), *K. pneumoniae* (20mm), *B. subtilis* (20mm), *E. coli* (20 mm) and *S. typhi* (22mm). *S. aureus* was found the most sensitive organism being inhibited by the extract. Therefore, these results showed that *H. sabdariffa* L methanolic extract inhibited the growth of all reference bacterial strains in a range between 20 and 25 mm. The results of the

present study were similar to that reported by Abd-Ulgadir *et al*., (2015) and Abdallah (2016). The findings are in accordance with those of (Samuel *et al*., 2014; Olaleye, 2007) with respect to the inhibition of *P. aeruginosa* and *S.aureus*. The calyx methanol extract of *H. sabdariffa* L exhibited higher activity against Gram positive strains compared to Gram negative strains and *C. albicans*. This could be due to the fact that the cell wall of Gram positive bacteria is less complex and lack the natural sieve effect against large molecules due to the small pores in their cell envelope (Walsh *et al*., 2003). The comparison of observation, which provided in (table 4.1 and 4.2) illustrates that the calyces methanolic extract of *Hibiscus sabdariffa* L showed high activity against *S. aureus* (25mm) which is almost more than 40 µg/ml Gentamicin and similar to 5 µg /ml Ciprofloxacin. MDIZ of *K. pneumoniae* was (20mm) which is more than the activity produced by 40µg/ml Gentamicin. MDIZ (25mm, 23mm, 20mm, 20mm, 20mm , 22mm and 21mm) of *S. aureus, P. aerginosa, K. pneumoniae, B. subitilis , E. coli, S. typhi*, and *C. albicans* respectively, which are higher than 40µg/ml Gentamicin.

The antigiardial potential of the methanolic extract of the *H. sabdariffa* L , with different concentrations (500, 250 and 125 ppm) and Metronidazole (the control) with concentration (312.5 µg/ml) was investigated against *G. lamblia* trophozoites *in vitro*. The methanolic extract of the *H. sabdariffa* L showed 72% inhibition at a concentration of 500µg/ml after 72hrs; which was compared with Metronidazole giving 95% inhibition at concentration 312.5 µg/ml at the same time against *G. lamblia* (table 4.4). The calyces extract screened for antigiardial activity against (*G. lamblia*) trophozoites *in vitro* showed antigiardial activity with an inhibition concentrations (IC50) more than 180µg/ml .

The antioxidant properties of the methanol extract of *H. sabdariffa* L calyx were tested, and then compared with the activity of the well known antioxidants Propyl Gallate. The radical scavenging potential of methanolic extract of *H. sabdariffa* L calyces at concentration (5 mg) scavenged 53% of DPPH radical. The

effectiveness of *H. sabdariffa* L calyx in scavenging free radicals was reported by so many research workers, (Chinedu *et al.,* 2011) found methanolic extract scavenged 78% this more high than our results due to different in concentration that used, The current study vary with results found by (Alaa, 2012) (14% and 19%) because different in solvent (aqueous, alcoholic extract) and concentration (20mg), and similar to found by (Liuqing *et al*., 2012) 60% alcoholic extract at concentration (8 mg), The present study indicated that methanol extract from the calyx *H. sabdariffa* L have significant antioxidant activity.

5.2. Conclusion

Hibiscus sabdariffa L plant extract has antimicrobial properties which might justify the use of this herb in traditional medicine. In conclusion the screening of *H. sabdariffa* L calyces against reference strains as well as the antigiardial activity and radical scavenging potential showed that the methanolic extract has broad antimicrobial and antioxidant activity that explain the use of *Hibiscus sabdariffa* L in traditional therapies.

5.3. Recommendations

1. Further studies using solvents other than methanol for extracting the important compounds from the *H. sabdariffa* L plant .
2. Investigations of the plant extract against other reference strains and clinical isolates.
3. Screening others parts of *H. sabdariffa* L plant (like leave , seeds…) for antimicrobial and antioxidant activity.
4. Identify the safe level of the extract and the active compounds responsible for biological activity.
5. The compounds which are found in the plant must be determined using other techniques like (GC)

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Appendix 1

Chemicals and Reagents:

Analytical grades of the following chemicals were used:

Crystal violet The British Drug House, England.

Glucose phosphate peptone water The British Drug House, England.

Hydrogen peroxide British Drug House, England.

Immersion oil British Drug House, England.

Iodine Hopkins and William England

Lactose Hopkins and William England

Lead acetate paper British Drug House, England.

Methanol Loba Chemie PVT Ltd.

Methyl red British Drug House, England.

Para-dimethyl-aminobenzaldehyde Oxoid Ltd

Phenol red British Drug House, England.

Safranin red British Drug House, England.

Sodium chloride British Drug House, England.

Sucrose Oxoid limited, England Sulphuric acid British Drug House, England.

Tetra methyl-p phenylene diamine- Dihydrochloride. British Drug House, England.

Urea powder Abott Ltd.

Chemotherapeutic agents:

Antibacterial drugs:

Ciprofloxacin powder Amipharma laboroteries Lt Sudan

gentamycin	Shanghi pharmaceutical Co. Ltd
china	

Culture media:

Blood agar base	Oxoid limited, England
Koser citrate agar	Oxoid limited, England
Lactose	Oxoid limited, England
MacConkey's agar	Oxoid limited, England
Mannitol salt agar	Oxoid limited, England
Nutrient agar	Oxoid limited, England
Nutrient broth	Oxoid limited, England
Nutrient gelatin	Oxoid limited, England
Peptone	Oxoid limited, England
Urea agar	Oxoid limited, England
RPMI 1640 with L-Glutamine	Gibco-Brl, Life Technology

Equipment and Instruments:

Autoclave	Griffin and George Ltd, England
Balance type H 6T	Mettler, England
Colony counters	Gallenkamp, England
Glass ware	Griffin And George Ltd.
Hettichi centrifuge	Tuv Bayern.Germany
Hot air oven	Gallenkamp, England
Human count plus	Human GMBH Germany
Incubators	Baird and TatlockL td, England
Microscope	Will Wetzlar, Germany
Water bath	Grant Instruments Ltd.

Test microorganisms:

Bacillus subtilis	NCTC 8236 (Gram + ve bacteria)

Escherichia coli	ATCC 25922(Gram -ve bacteria)
Klebsiella pnemoneae	ATCC 53657(Gram -ve bacteria)
Pseudomonas aeruginosa	ATCC 27853 (Gram -ve bacteria)
Staphylococcus aureus	ATCC 25923(Gram +ve bacteria)
Salmonella typhi	ATCC 14028
Candida albicans	ATCC 7596 (Fungus)
Giardia lamblia	clinical isolate (parasite)

National Collection of Type Culture (NCTC), Colindale, England.

American Type Culture Collection (ATCC) Rockville, Maryland, USA.

Appendix 2

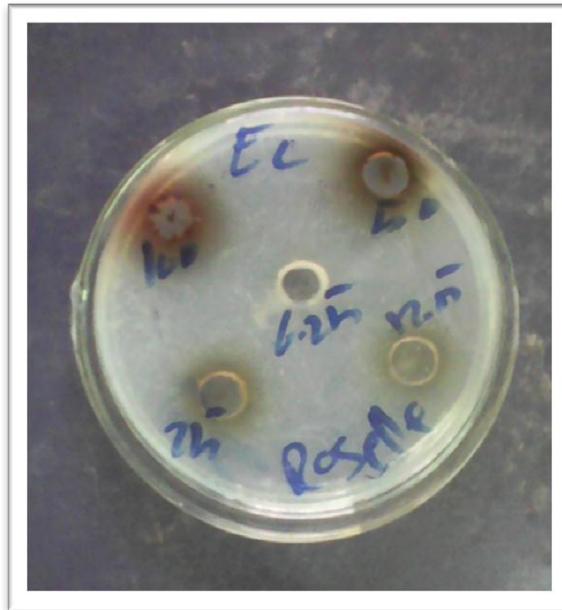

Figure No (2): Antimicrobial activity of *Hibiscus sabdariffa* against *Escherichia coli*

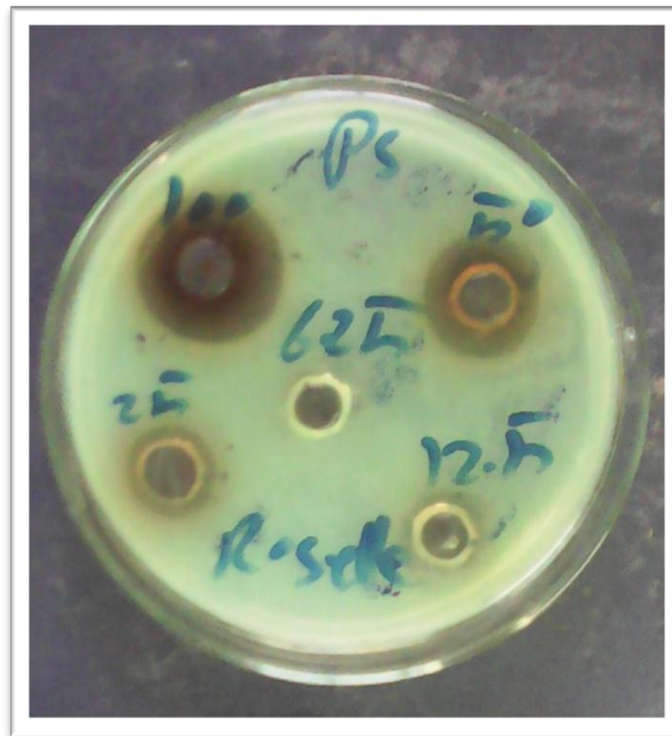

Figure No (3): Antimicrobial activity of *Hibiscus sabdariffa* against *Pseudomonas aeruginosa*

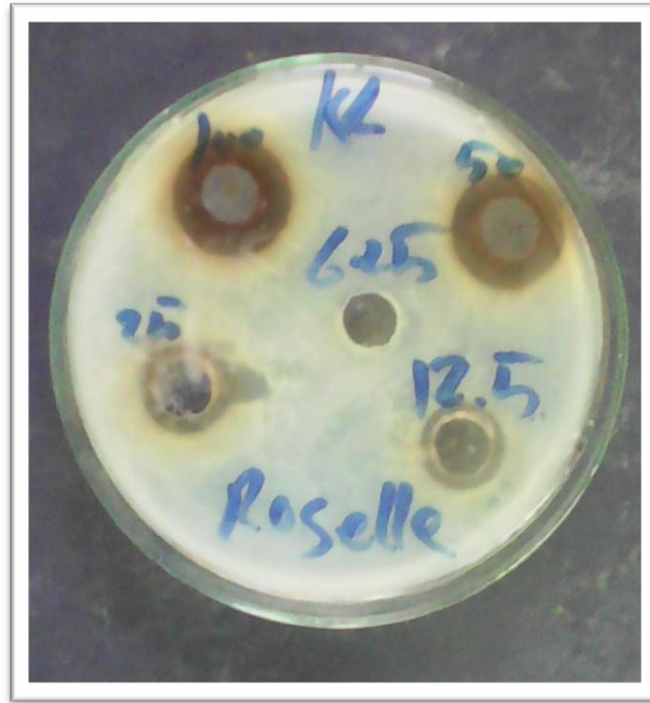

Figure No (4): Antimicrobial activity of *Hibiscus sabdariffa* against *Klebsiella pnemoneae*

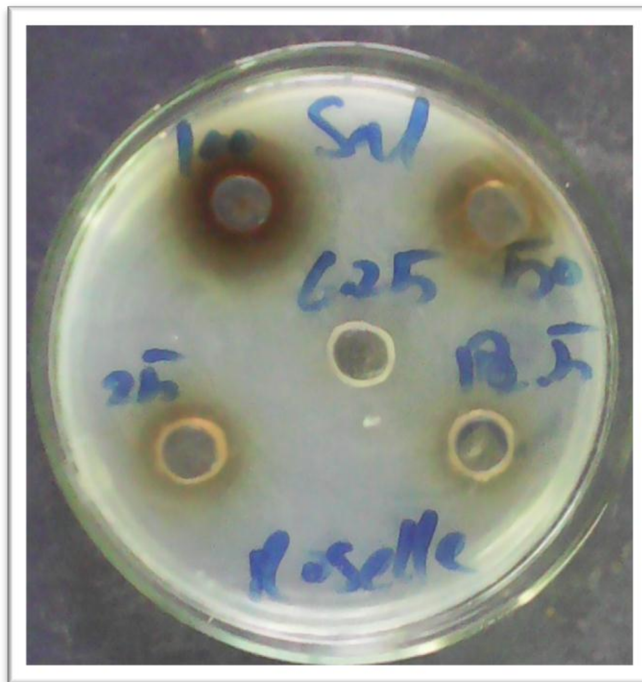

Figure No (5): Antimicrobial activity of *Hibiscus sabdariffa* against *Salmonella typhi*

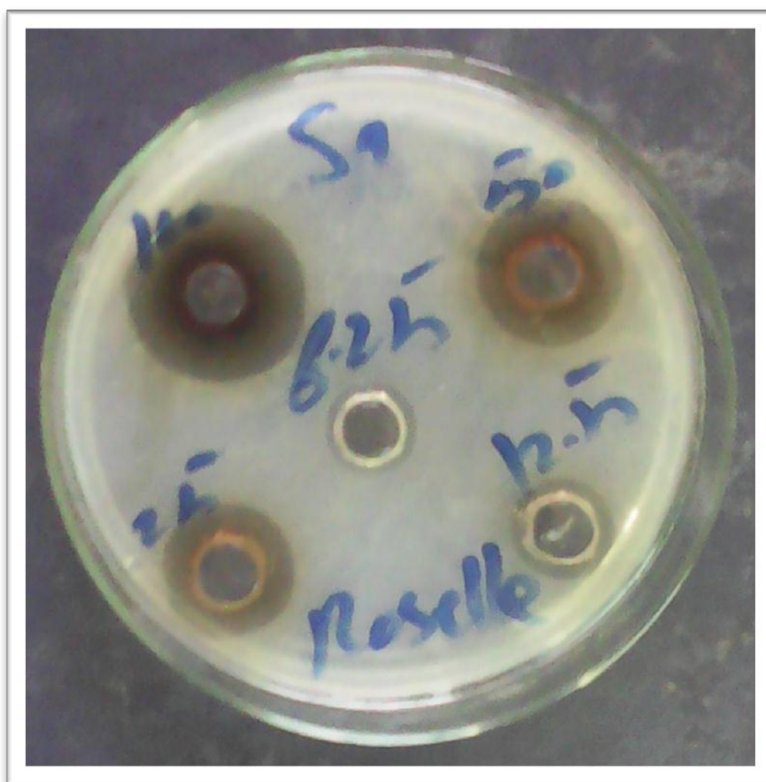

Figure No (6): Antimicrobial activity of *Hibiscus sabdariffa* against *Staphylococcus aureus*

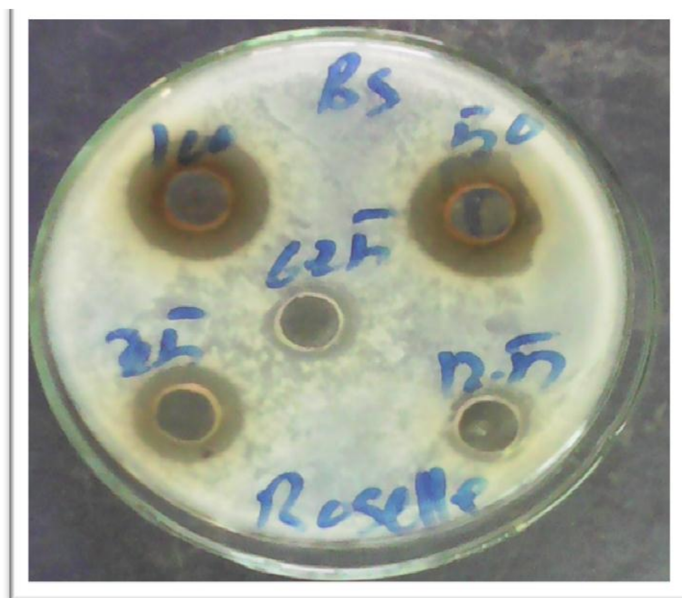

Figure No (7): Antimicrobial activity of *Hibiscus sabdariffa* against *Bacillus subtilis*

Figure No (8): Antimicrobial activity of *Hibiscus sabdariffa* against *Candida albicans*