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Chemical Study and Antioxidant Activity of Siddir (*Ziziphus spina*) Roots Extracts

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Industrial Chemistry

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استهلال

بسم الله الرحمن الرحيم

"قُلْ إِنَّ صَلَاتِي وَنُسُكِي وَمَحْيَايَ وَمَمَاتِي لِلَّهِ رَبِّ الْعَالَمِينَ"

الإنعام: الآية 162

قال رسول الله صلى الله عليه وسلم:

(إذا اردت الدنيا فعليك بالعلم, وإذا اردت الآخرة فعليك بالعلم)

صدق رسول الله

DEDICATION

To my parents,

My sisters, brothers

ACKNOWLEDGEMENT

At First, my respectfully thanks should be to Allah, almighty who gave me the strength, Patience to complete this work. I would like to express my deep gratitude to **Dr.Inas Osman Khojali Mohammed** my thanks also extend to all the staff in the department of chemistry .thanks to all those who helped me.

ABSTRACT

The roots of *Ziziphusspina* was collected from Omdurman. It was dried in shade at room temperature then powdered. *Z.s* was extracted by two methods (maceration and soxhlet). Phytochemical screening indicated the presence of some active constituent in *Z.s* roots (terpenes, steroids, tannins, flavonoids and saponins). All extracts were examined by thin layer chromatography to detect the right solvent system for separation. The ethyl acetate crude gave a good separation using chloroform: methanol: petroleum ether (5:1:3). Ethyl acetate extract was purified using preparative thin layer chromatography. The isolated band was subjected to IR analysis. The antioxidant activity of the crude extracts was tested using 2,2-Di(4-tert-octylphenyl)-1-picryl-hydrazyl. Ethanol extract (maceration) showed high antioxidant activity. Methanol and ethyl acetate extracts (soxhlet) exhibited moderate antioxidant activity. The gas chromatography- mass spectroscopy study was carried on the ethyl acetate extract. The result revealed the presence of twenty six different compounds.

المستخلص

تمت عملية تجميع جذور نبات السدر من منطقه امدرمان. جففت الجذور في الظل في درجه حرارة الغرفة وطحنت في شكل بدوره. تمت عملية الاستخلاص بطريقتين النقع وايضا تم استخلاص الجذور باستمرار باستخدام مذيبات مختلفه في الدرجه القطبيه وهي بتروليوم ابثر . كلوروفورم . خلات الايثيل والميثانول باستخدام جهاز السوكسلت. المسح الكيمياءى على جذور نبات السدر اوضح وجود بعض المواد الفعاله مثل التيربينات, الاستيرويدات, التانينات, الفلافونيدات والصابونينات.تم اختبار كل المستخلصات باستخدام مذيبات مختلفه لاختيار افضل مذيب للفصل. تمت تنقيه مستخلص خلات الايثيل باستخدام كروماتوغرافيا الطبقة الرقيقه التحضيريه(كلوروفورم:ميثانول:بتروليوم ابثر 5:1:3). تم فصل الحزمه الرئيسيه واخضعت لتحليل الاشعه تحت الحمراء.تم اختبار المستخلصات كمضادات اكسده وجد أن مستخلص الايثانول(نقع) عالي الفعاليه اما مستخلص الميثانول وولات الايثيل (سوكسلت) احدثت نشاط معتدل.اجريت دراسه كروماتوغرافيا الغاز - طيف الكتله علي مستخلص خلات الايثيل. اشارت النتائج لوجود ستة وعشرين مركب مختلف.

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LIST OF ABBREVIATION

AlCl₃	AluminumChloride
CCl₄	Carbon tetrachloride, chloroform
CH₃COO⁻	Acetate Ion
CNS	Thiocyanate
HCl	Hydrochloride Acid
H₂SO₄	Sulfuric Acid
KBr	Potassium Bromide
K₃[FeCN]₃	Potassium Ferricyacide
KOH	Potassium Hydroxide
NH₄OH	Aluminum Hydroxid
CAT	CATalase
DMSO	Di Methyl SulfOxide
DPPH	Di(4-tert-octylPhenyl)-1-Picryl-Hydrazyl
EC₅₀	Effective ConcentrationHalf maximal
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	<i>Gas Chromatography</i> –Mass Spectrometry
GsH-PX	GlutatHionePeroXides
IC₅₀	Inhibitory Concentration Half maximal

IR	Infra Red Spectroscopy
MDA	MalonDialdehyde
MIC	Minimum Inhibitory Concentration
NIST	National Institute of Standards and Technology
PTLC	Preparative Thin Layer Chromatography
SOD	Super Oxide Dismutase
TLC	Thin Layer Chromatography
UV	Ultra Violet

LIST OF SYMBOLS

g	Gram
m	Meter
mm	Millimeter
ml	Milliliter
min	Minute
μm	Micrometer
%	Percent
$^{\circ}\text{C}$	Degree Celsius
μg	Milligram

CHAPTER ONE

INTRODUCTION

1.1 Introduction

By definition, the word natural is an adjective referring to something that is present in or produced by nature and not artificial or man-made. When the word natural is used in verbiage or written, many times it is assumed that the definition is something good or pure. However, many effective poisons are natural products (Weil, 1965). However, in most cases the term natural products today is quite commonly understood to refer to herbs, herbal concoctions, dietary supplements.

Natural products have long been of interest to the fields of chemistry, biology and medicine because of their immense molecular diversity, biological activities and medicinal properties.

Natural product chemistry is a branch of chemistry that deals with the isolation, identification, structure elucidation, and study of the chemical characteristics of chemical substances produced by living organisms. Strategies for research in the area

of natural products have evolved quite significantly over the last few decades. These can be broadly divided into two categories, older strategies which focus a straightforward isolation and identification of compounds from natural sources followed by biological activity testing (mainly in vivo). The second is modern strategies, which focus on bioassay-guided (mainly in vitro) isolation and identification of active compounds from natural sources (Sarker and Nahar, 2006) .

Plant constituents comprise a wide variety of organic substances that are formed and accumulated by plants. They include: Primary metabolites which are produced by primary metabolism. The primary metabolites such as carbohydrates, proteins, fats, and nucleic acids, are essential for life and are commonly present in all organisms in large amounts. Secondary metabolites, also known as natural products, are those products of metabolism that are not essential for normal growth, development or reproduction of an organism. In this sense they are "secondary". Each plant family, genus, and species produces a characteristic mix of these secondary metabolites, and they can sometimes be used as taxonomic characters in classifying plants. Humans use many of these compounds as medicines due to their variable biological activities. However, some groups of natural products could be assigned to both divisions" e.g. fatty acids and sugars. Most of these compounds are described as primary metabolites, whilst some representatives are of rare occurrence and characteristic to certain plant species and are thus closer to secondary metabolites. Secondary compounds are grouped into

classes based on similar structures, biosynthetic pathways, or the kinds of plants that make them. The largest such classes are the alkaloids, terpenoids, and phenolics (Samuelson, 1999)

.Secondary compounds often occur in combination with one or more sugars. These combination molecules are known as glycosides. Usually the sugar is a glucose, galactose or rhamnose. But some plants have unique sugars.

Examples of plant phenolics are the flavonoids and their glycosides, the phenyl propanoids, anthocyanins, xanthenes, coumarins, tannins and quinones. Many of the phenolic compounds are reported to have antioxidant and anticancer activities (Prior, and Cao, 2000). Terpenoids are characterized by being lipophilic in nature. They are sub-classified according to the number of their isopentene (or isoprene, C = 5) building units into: monoterpene, sesquiterpene, diterpene, triterpene and carotenoids. mono- and sesqui-terpenoids are the main constituents of volatile oils, carotenoids are example of tetraterpenoids. Terpenes are reported for their antimicrobial, anticancer activities and some also affect the CNS (Breitmaier, 2008)

Organic acids, lipids and related compounds: They have common acetate precursor (CH_3COO^-). Examples of simple organic acids accumulating in plants are citric, malic, fumaric, oxalic, tartaric, malonic, shikimic, quinic and ascorbic acids. Fatty acids occur mainly in esterified forms with glycerol or higher aliphatic alcohols in the form of fixed oils, fats or

waxes. Nitrogen-containing compounds are distinguished from other classes by being usually basic. They give a positive response to either ninhydrin or Dragendorff's reagents. Examples are amines, alkaloids, cyanogenic glycosides, and chlorophylls.

1.2 Objectives of the study

1. To investigate the presence of different photochemical in crude extracts with different solvent of *Ziziphus spina* roots.
2. To screening the crude extractives for their antioxidant potential.
3. To separate some compound extract gives good separation using PTLC technique.
4. To analyze the constituents of the above extract using GC-MS technique

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

LITERATURE REVIEW

2.1 Literature Review

2.1.1 The Genus *Ziziphus*

The genus *Zizyphus* belongs to the buckthorn family (Rhamnaceae). It is a genus of about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world. Some species, like *Z. mauritiana* and *Z. jujuba*, occur on nearly every continent, whereas other species, like *Z. nummularia*, *Z. spina-Christi* and *Z. mucronata*, are restricted in their distribution to distinct areas. *Ziziphus* species can grow either as trees and shrubs (*Z. mauritiana*, *Z. rotundifolia*, *Z. jujuba*, *Z. mucronata*) or exclusively as small shrubs or bushes (*Z. nummularia*, *Z. lotus*, *Z. spina-christi*, *Z. obtusifolia*) (Lev and, Amar, 2002)

2.2 Botanical Description and Chemical Composition

It is a shrub or small tree up to 9 m height. The bark is pale grey. Thorns in pairs, one upright and one recurved. Branchlets pale or nearly white, glabrous. Leaves are

ovate-lanceolate, sometimes thickened on the edges, strongly nerved from the base, lateral nerves obscure, glabrous below. The flowers are greenish yellow in sub sessile sometimes dense cymes. Fruits are fleshy, subglobose. Leafing, flowering and fruiting: evergreen in wet sites but drops some or all of the leaves during dry season on sites where less water is available. Flowers: August- December. Fruits: October- April.

The plant has been extensively studied and its chemical composition is well known (Aynehchi and Mahoodian,1973) (Mahran et al.,1996) The main constituents of the essential oil were alpha-terpineol (16.4%) and linalool (11.5%). The main neutral hydrocarbons were n-pentacosane forms (81%). Methyl esters isolated from leaves included methyl palmitate, methyl stearate and methyl myristate. Beta-Sitosterol, oleanolic acid and maslinic acid were the main aglycones of the glycosides present in leaves. Sugars present in leaves included lactose, glucose, galactose, arabinose, xylose and rhamnose. The plant also contains four saponin glycosides (Mahran et al.,1996) .The highest flavonoid content was found in the leaves (0.66%). No significant influence of growing site or year of harvesting on the flavonoid content was observed. As quercetin 3-Orhamnoglucoside 7-O-rhamnoside and rutin are the main flavonoid compounds present in all plant parts investigated (Brantner and Males,1999) .

The composition of the plant has always proved complex in its chemistry, in addition to the known alkaloids, *zizyphine-F*, *jubanine-A* and *amphibine H*, a new peptide alkaloid spinanine-

A has been isolated from the stem bark of *Zizyphus spina-christi*. *Spinanine-A* is a 14-membered cyclopeptide alkaloid of the amphibine-B type (Abdel-GalilF and El-Jissry, 1991).

2.2.1 Taxonomy

Table 2.1 shown Botanical classification of *Zizyphus spina*, and

Figure 2.1 shown description of *Zizyphus spina*.

Table 2.1: Botanical classification of *Zizyphus spina*

Kingdom	Plantae
class	Mangoliophyta
Order	Rosales
Family	Rhamnaceae
Genus	Zizyphus
Species	Z. Spina- Christi



Figure 2.1: Description of *Ziziphus spina*

2.3 Different Uses of *Ziziphus spina*

Fruits of *Z. spina-christi* are used as food especially for people in western and central Sudan and other Saharan regions. Fruits are collected by women and children and sold on local markets. This provides an additional source of income for local people, who may use the revenue to buy important non-food items. Similarly, in Oman fruits are collected from wild and cultivated plants and sold on local markets (Miller and Morris,1988). Fruits are consumed either fresh or dried and the sweet pulp of fruit is dried to produce fine flour. Typically, this flour is placed in small metal cups and cooked under steam. This process solidifies the flour to the shape of the container. The dried pulp flour and water are also mixed with sesame and formed into small balls. The fruit pulp prepared in these two ways can be consumed either immediately or stored for future use. In Oman fruits including kernels are ground to produce an edible mealy substance which is either eaten raw or cooked in water, milk or buttermilk (Miller and Morris,1988).

The leaves provide valuable animal forage and fodder under open grazing conditions (Verinumbe,1993). This is especially important during the dry season when grazing is limited. Small branches are often lopped as dry season fodder for camels and goats and later used to make thorn fences (Bunderson et al.,1999), (Miehe,1986).

The wood is used as a source of fuel and it produces an excellent charcoal. The timber is used for tool handles, fence posts,

bedstead legs, walking sticks, furniture, bent wood chairs, roofing beams, doors, windows and turned items. It is hard and heavy and is known to resist termites (Sudhersan and Hussain ,2003).

In some parts of West Africa *Ziziphus* trees are planted to stabilize sand dunes and erosion-prone areas because of their deep taproot and spreading lateral roots. The tree is planted around towns and villages for shade as it makes useful windbreaks and shelterbelts. The tree is also intercropped with millet as it is known to improve soil fertility by increasing available phosphorous (Bunderson et al.,1999) .

The honey collected from the flowers of *Z. spinachristi* is of excellent flavour (Robinson,2006)and is normally sold at a price higher than that derived from the flowers of other trees.

For Muslims seeds are used as rosaries, while for Christians the thorns are reported to have made up the crown of thorns of Jesus. In natural religions the tree's roots are used in superstitious practices (Robinson,2006).

The vast majority of rural people in Sudan has no access to modern medicine and relies heavily on traditional cures, mostly prepared from plants(Saied,2007) .

The genus *Ziziphus* is known for its medicinal properties as a hypoglycemic, hypotensive, antiinflammatory, antimicrobial, antioxidant, antitumour, and liver protective agent and as an immune system stimulant (El-Kamali and El-Khalifa,1999). In central Sudan, *Z. spina-christi* fruits are eaten to treat diarrhoea and malaria and as an antispasmodic. The powder of the twigs is used externally to treat rheumatism and scorpion sting (El-Kamaliand El-

Khalifa,1999). In northern Kordofan the poultice of the powdered leaves is used to heal swellings and macerated roots are used as an antipurgative (El Ghazali et al.,1997).

In the White Nile states the decoctions of the bark are used to treat intestinal spasms (El Ghazali et al.,1994).. In the Sahel region the roots are reported as a treatment against headaches, while spines and ashes are applied to heal snake bites. Boiled leaves are applied to various surface wounds and against diarrhea. In Egypt a beverage made from fruits is considered to be a sedative. In Palestine leaves young branches are used as an anti-inflammatory for eye wash, treat toothache and stomachache and as an ant rheumatic (Ali-Shtayeh et al.,1998).

In the United Arab Emirates leaves are boiled in water and used as a shampoo or mixed with lemon and applied to the face and hair to soften or to soothe it. Ash of wood mixed with vinegar is applied to heal snake bites and a tea made of fruit is used to treat measles. Fruits and crashed kernels are eaten to treat chest pains, respiratory problems and as a tonic (Jongbloed,2003). Moreover, in Oman fruits are regarded as having purifying properties such as cleansing the stomach, removing impurities from blood as well as being a restorative for the whole system. The water in which crushed leaves had been boiled is given to women in prolonged labour or with a retained placenta for its *oxytoxic* properties (Verinumbe,1993). In India, a preparation from bark is used to clean wounds and sores, while the gum that exudes from the tree in hot weather is used in eye remedies. The chewed leaves are said to numb the taste buds, which provides a method of suppressing the unpleasant flavor of

some oral medicines. Conservation and genetic improvement in Sudan stands of *Z. spina-christi* are endangered as a consequence of intensive browsing, excessive lopping and cutting (Anonymous,2000). Thus, there is a need for germplasm preservation of existing local types as well as for improvement through selection and breeding programs. This could be carried out in central and western Sudan where the species is found to grow abundantly. In India, some high quality commercial cultivars of *Ziziphus spp.* have been developed through selection of productive natural variants propagated by grafting on wild rootstocks and used for orchard plantations(Singh et al., 2006). Improved Indian cultivars of *Z. mauritiana* such as „Gola“ and „Seb“ have been imported to Israel and West Africa where they have been successfully grafted onto native rootstocks of *Z. spina-christi* and *Z. abyssinicaHochst.*, respectively . No specific cultivars are known for Sudan, however, a large variety of fruits size, color and taste are found which indicates the existence of local types. This distinct could be used to establish a breeding program for better quality and higher yielding cultivars. Subsequently, species with high quality fruits could be planted or grafted onto local *Z. spinachristi* types known for their resistance against harsh climatic conditions and multiple stresses on marginal.

2.4 Chemical Composition

A survey of the literature revealed that a number of cyclopeptide and isoquinoline alkaloids, flavonoids, terpenoids and their glycosides have been found to occur in various amounts in most *Ziziphus species*. The leaves of these plants contain

betulinic and ceanothic acids, various flavonoids, saponins, erols, tannins and triterpenes (Ali,2006) . The extract of *Z. spina-christi* was shown to contain butic acid and ceanothic acid (a ring-A homologue of betulinic acid), cyclopeptides, as well as saponin glycoside and flavonoids, lipids, protein, free sugar and mucilage (Adzu, et al.,2003). Cardiac glycosides and polyphenols (such as tannins) are also reported from the leaves.

Geranyl acetate (14.0%), methyl hexadecanoate (10.0%), methyl octadecanoate (9.9%), farnesyl acetone C (9.9%), hexadecanol (9.7%) and ethyl octadecanoate (8.0%) were characterized as the main components of *Z. spina-christi* leaves essential oil (Ghannadi et al., 2002). *Zizyphine-F*, *jubanine-A* and *amphibine-H* and a new peptide alkaloid spinanine-A have been isolated from the stem bark of *Z. spina-christi*. *Spinanine-A* is a 14-membered cyclopeptide alkaloid of the *amphibine-B* type(Fathy et al.,1990). is the major saponin of the leaves. Dodecaacetylprodelphinidin B3 has been also isolated from the leaves (Weinges and Schick,1995). New flavonoid, quercetin 3-xylosyl(1→2)rhamnoside-4'-rhamnoside(Pawlowska et al.,2008) . accompanying with rutin, hyperin, quercetin, apigenin-7-O-glucoside, isovitexin and quercetin-3-O lucoside-7-O-rhamnoside were characterized from *Z. spina-christi* fruits. A flavonoid, C-glycoside, 3',5'-di-C-β-d-glucosylphloretin, was also identified in *Z. spina-christi* leaves. In addition, 4-hydroxymethyl-1-methyl pyrrolidine-2-carboxylic acid and 4-hydroxy-4-hydroxymethyl-1-methyl pyrrolidine-2-carboxylic acid were characterized as two

new cyclic amino acids from *Z. spina-christi* seeds (Said et al., 2010).

2.5 Pharmacological activities

2.5.1 Antibacterial and antifungal properties

The aqueous extract of *Z. spina-christi* stem bark has shown significant antibacterial activity against *S. aureus*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella paratyphi B* and *Klebsiella pneumoniae* (El-Kamali and Mahjoub, 2009). *spina-christi* stem bark aqueous extract has shown highly significant antibacterial effect activity on some Gram negative bacterial growth including *Brucella abortus*, *Brucella melitensis*, *Proteus spp.*, *Klebsiella spp.*, *P. aeruginosa*, *E. coli* and *Enterobacter spp.* in comparison with eight antibiotics (Korji, 2012). Alcoholic extract of the leaves has also shown good antibacterial activity against *S. aureus* isolated from eye infections (conjunctivitis). An inhibition zone of 20 mm was recorded for 1 mg/ml of the extract (Alsaimary, 2012). Additionally, the leaves were active against *Salmonella typhi*, *Proteus mirabilis*, *Shigella dysenteriae*, *E. coli*, *K. pneumoniae*, *B. melitensis*, *Bordetella bronchiseptica* and *P. aeruginosa*. The highest activity (20 mm) was against *B. bronchiseptica* by concentration of 100 mg/ml (Motamedi et al., 2009). The pulp aqueous extract of *Z. spina-christi* also showed inhibitory activity on *E. coli*, *P. aeruginosa* and *Candida albicans* in vivo. The extract showed MIC of 6.25 mg/ml against *E. coli* and *C. albicans*. The minimum bactericidal concentration of the pulp aqueous extract was 12.5 mg/ml for

Streptococcus pyogenes(Adamu et al.,2006) . Methanol extract of *Z. spina-christi* roots showed antifungal activity against dermatophytes, including *Trichophytonrubrum*, *T. mentagaphytes*, *Microsporumcanis* and *Aspergillus fumigates* (Tom et al, 2009). The fruits were also active against *C. albicans*(Ghasemi et al, 2009).

2.5.2 Antidaibetic properties

Oral administration of *Z. spina-christi* leaf extract, plain and formulated for 28 days, reduced blood glucose level with significant increase in serum insulin and C-peptide levels. Marked elevation in total antioxidant capacity with normalization of percentage of glycated hemoglobin (HbA1C %) was reported. Moreover, they succeeded in reducing the elevated blood lactate level and to elevate the reduced blood pyruvate content of diabetic rats. In line with amelioration of the diabetic state, the extract, plain and formulated, restored liver and muscle glycogen content together with significant decrease of hepatic glucose-6-phosphatase and increase in glucose-6-phosphate dehydrogenase activities. In vitro experiments showed a dose-dependent inhibitory activity of the extract against α -amylase enzyme with IC50 at 0.3 mg/ml. Such finding has been supported by the in vivo suppression of starch digestion and absorption by the extract in normal rats. The results revealed that *Z. spina-christi* leaf extract improved glucose utilization in diabetic rats by increasing insulin secretion, which may be due to both saponin and polyphenols content, and controlling hyperglycemia through

attenuation of meal-derived glucose absorption that might be attributed to the total polyphenols(Michel et al., 2011).

2.5.3 Antioxidant activity

An antioxidant is defined as „any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Rhee et al.,2009). Antioxidants are of interest to biologists and clinicians because they help to protect the human body against damages induced by reactive free radicals generated in atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and even in aging process (Hemati et al.,2010). There are many evidences that natural products and their derivatives have efficient anti-oxidative characteristics, consequently linked to anti-cancer, hypolipidemic, anti-aging and anti-inflammatory activities (Cho et al., 2006; Rhee et al., 2009).

The anti-oxidative capacities of ethanol and petroleum ether extracts of *Z. spina-christi* leaves were evaluated by hydroxyl radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, lipid peroxidation and superoxide radical standardization methods (Abalaka et al., 2011).The EC50 values for hydroxyl radical with ethanol and hexane extract of *Z. spina-christi* were found to be 198.34 and 234.11 µg, while that of ascorbic acid was found to be 219.31 µg. The EC50 values for the two plant extracts were ethanol 101.02 µg and hexane 124.21 µg. These results compare favorably with that of standard ascorbic acid which had the EC50 value of 78.12. Moreover, the EC50 values for lipid peroxidation with ethanol extract and hexane extract of *Z. spina-christi* were 298.65

and 376.35 μg , while that of ascorbic acid was 191.42 μg . The EC50 value for superoxide radical scavenging with ethanol and hexane extract of *Z. spina-christi* were 156.45 and 265.22 μg , while that of ascorbic acid was 138.26 μg (Abalaka et al.,2011). These activities indicate that the extracts from *Z. spina-christi* are good antioxidants.

It was also indicated that the fruits contained high level of total phenolic compounds (7.55mg /g as gallic acid) (Yossef HE, *et-al*,2011). The fruit administration inhibited lipid peroxidation at higher level after CCL₄ treatment. Interestingly, the methanolic extract of these fruits with dose of 200 mg/kg was able to increase the activities of endogenous antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)) and levels of GSH in hepatic tissue. The fruits extract pretreatment demonstrated to inhibit malondialdehyde (MDA) of the reactive oxygen radical production (Xiangchun et al., 2009).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

3 Materials

3.1 Plant material

The roots of *Ziziphus spina* was collected in November -2017 from Omdurman city. Botanical identification and authentication was performed in the herbarium of National Research Center, Khartoum.

3.2 Chemicals

1. All chemicals are Analar ,and water is deionized
2. Petroleum ether
3. Chloroform
4. Ethyl acetate
5. Ethanol
6. Methanol
7. Concentrated Sulfuric acid
8. Concentrated Hydrochloric acid
9. Aluminum chloride (AlCl_3)
10. Mercuric Chloride
11. Ferric Chloride

12. Acetic anhydride
13. Glacial acetic acid
14. Potassium hydroxide
15. Iodine
16. Olive oil
17. $K_3 [Fe(CN)_6]$
18. Bismuth Nitrite
19. Potassium iodide

3.3 Apparatuses and Equipments

1. Soxhlet Apparatus.
2. Heating mantle.
3. Water bath
4. Petri dishes.
5. Test tubes.
6. TLC Apparatus :
7. Plates (10x10 cm)
8. Slides
9. Measuring cylinder.
10. Filter papers.
11. Beakers.

3.4 Methods

3.5 Extraction Methods

3.5.1 Maceration

In this method the plant material is soaked usually in a volatile organic solvent, over a period of time with constant or occasional stirring or agitation. The supernatant liquid (extracted) is then either decanted or filtered through plug of cotton or glass wool. The process continues to complete extraction (Sarker et al., 2006).

Here 25 g of the ground roots was soaked in 250 ml of ethanol for 3 days then it was filtered. The filtrate was dried at room temperature and the crude extract was collected into small bottles.

3.5.2 Continuous Soxhlet Extraction

In this continuous process of extraction with a hot organic solvent soxhlet extractors are particularly used for this method of extraction.

The powdered plant material is taken in a thimble which is placed in the soxhlet extractor. The extractor, which has a siphoning system, is fitted on top of a round-bottom flask. A condenser is fitted at the top of the extractor. Enough quantity of the extracting solvent is poured into the flask placed on a heating mantle, on heating the solvent vapor rises to the condenser where it condenses and drains back to the extractor.

which is holding the thimble with the plant material. When the extractor becomes full with the hot solvent, the solvent siphons down the flask along with the extracted constituent the recycling of the evaporated solvent is allowed to continue until the extraction is complete (Harborne,1984).

Here 30 g of the roots was powdered. It was placed in the thimble of soxhlet . It was extracted successively using solvent of increasing polarity (petroleum ether, Chloroform ,ethylacetate and ethanol) . The extracts were allowed to dry at room temperature, after that photochemicalscreening for all extractsby Maceration and by Soxhlet were done.

3.6 phytochemical screening

3.6.1 Test for flavonoids

The extracts were evaporated to dryness on water bath; the cooled residue was defatted with petroleum ether. The defatted residue was dissolved in 95% ethanol and filtered. The filtrate was used for the following tests.

(a) To apportion of filtrate, few drops of methanolic aluminum chloride (0.1g aluminum chloride in 100 ml methanol) were added, formation of a yellowish color indicate the presence of flavones and chalcones.

(b) To apportion of filtrate, few drops of aqueous potassium hydroxide solution (KOH) (0.1g potassium hydroxide in 100ml

water) were added. A dark yellow color is taken as positive test for flavonoid compounds.

(c) To apportion of filtrate 0.5ml of conc. HCl and a small amount of magnesium turning were added. Color changes to pink or red were taken as presumptive evidence of the presence of flavonid compounds.

(d) To apportion of filtrate, few drops of ferric chloride solution (5.0g of ferric chloride in (100ml) 95% ethanol). Were added. Development of a blue coloration was taken as appositve test for flavonoids.

3.6.2 Test for Alkaloids

50 mg of the extracts was stirred separately with ten ml of dilute 2% hydrochloric acid or 10% NH_4OH in a water bath, it was cooled and filtered. The filtrate was tested carefully with various alkaloid reagents as follows.

A. Mayer's Test

One ml of the above acidic or basic filtrate was added to 0.5ml of Mayer's reagent (was prepared by mixing 1.3g of mercuric chloride with 5.0g potassium iodide and completed with distilled water up to 100ml)by the side of the test tube. Formation of Cream –white precipitate was indicated as the presence of alkaloids.

B. Dragendoff's Test

One ml of the above acidic or basic filtrate was added to 0.5 ml of Dragendoff's reagent (Stock solution A: 0.8g of bismuth nitrate was dissolved in ten ml acetic acid. 40 ml of water was added. Stock solution B: 8g of Potassium iodide was dissolved in 20ml of water). The formation of orange brown precipitate / reddish brown precipitate was indicated the presence of alkaloids.

C. Hager's Test

One ml of the above acidic or basic filtrate was added to 0.5 ml of Hager's reagent (Saturated solution of picric acid). Formation of yellow precipitate was indicated the presence of alkaloids.

3.6.3 Test of Glycosides

50 mg of the extracts was taken into a dry test tube and hydrolyzed with hydrochloric acid for two hrs on a water bath. It was filtered. This solution was subjected to the following test to detect the presence of different glycosides.

A. H₂SO₄ Test

One ml of concentrated H₂SO₄ was added to 1ml of the above solution. It was allowed to stand for two min .The formation of reddish color indicates the presence of glycosides.

3.6.4 Anthraquinone Glycosides (Borntrager,s Test)

1ml of the above solution was treated with 5 ml of chloroform. It was shaken for five min and then the chloroform layer was separated. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. Formation of pink /violet /red color in the ammoniacal layer (lower layer) was indicated the presence of anthraquinone.

3.6.5 Cardiac Glycosides (Keller Killiani's Test)

100ml of the extract was dissolved in 1ml of glacial acetic acid containing one drop of ferric chloride solution. One ml of concentrated sulphuric acid was added. A brown ring was obtained at the interface was indicated the presence of deoxy sugar characteristic of cardenolides.

3.6.6 Test of Saponins by Foam Test

Two g of crude powdered plant material was dissolved in 5ml of distilled water. The suspension was shaken for 15 min if two cm layer of foam produced persists for 10 min it indicates the presence of saponins.

3.6.7 Test for Tannins

0.5 g of the extract powder samples was boiled in 20 ml of water in a test tube. It was filtered. Few drops of 0.1 % Ferric chloride were added. Formation of brownish green or blue coloration indicates positive result.

3.6.8 Test for Steroids

The extract was evaporated to dryness in a water bath then it was cooled. The residue was stirred with pet. Ether many times to remove most of the coloring materials. The residue was extracted with (20ml) chloroform. The chloroform was dehydrated over anhydrous Sodium Sulphate. Apportion of the solution was mixed with (0.5 ml) acetic anhydride. Two drops of concentrated sulphuric acid were added to the filtrate. Formation of green colored indicating presence of steroids.

3.7 Chromatographic Methods

3.7.1 Thin Layer Chromatography

Analytical thin layer chromatography (TLC) was carried out using small glass slides of dimensions 5*8 cm pre-coated with silica gel and distilled water slurry (30:60). The aim was to detect a right solvent system for separation. A fine glass capillary tube was used to spot the sample onto the TLC slides and then developed in the saturated vapor chromatographic tanks by using suitable solvent systems at room temperature. Visualization was achieved by spraying with suitable spray reagent or by UV lamp.

Preparative thin layer chromatography (PTLC) plates were prepared by mixing 100g of F2324 type 60 silica gel with 200ml distilled water. This mixture was spread as thick slurry on a glass plate 20*20cm (0.5mm thickness). The resultant plate

was dried and activated by heating in an oven for thirty minutes at 110C⁰. The mixture was not spotted on the TLC plates as dots, but rather was applied to the plate as a thin even layer horizontally to and just above the solvent level. When developed with solvent, the compounds separated in a horizontal bands rather than horizontally separated spots. Each band (or a desired band) was scraped of the packing material. The packing material was then extracted with a suitable solvent and filtered to give the isolated material upon removal of the solvent.

Here all extracts were tested to detect the right solvent system for separation. The ethyl acetate crude gave a good separation using chloroform: methanol: petroleum ether (5:1:3) as a solvent system. It was dissolved in a mini amount of 95% ethanol and applied on (20*20 cm) silica gel plates as a narrow strip. The plates were developed with the solvent system; chloroform: methanol: petroleum ether (5:1:3) and the chromatograms were located under UV light. The sole major band was scratched. The packing material was then extracted with 95% ethanol and filtered to give the isolated material upon removal of the solvent. The isolated fraction was studied using IR analysis.

3.7 .2 Infra Red Spectroscopy (IR)

Dried powdered ethyl acetate extract was used for IR analysis. 10mg of the dried extract powder was encapsulated in 100mg of KBr pellet, in order to prepare translucent sample

disc. The powdered sample of the plant specimen was loaded in the FTIR spectroscope (Shimadzu, IR Affinity 1, Japan), with a scan range from 400-4000 cm^{-1} with a resolution of 4 cm^{-1} .

3.7.3 Gas Chromatography –Mass Spectrometry Analysis (GC-MS)

The qualitative and quantitative analysis of the sample was carried out by using GM/MS technique model (GC/MS- QP2010-Ultra) from japons "Simadzu Company, with serial number 020525101565SA and capillary column (Rtx-5ms- 30m \times 0.25 mm \times 0.25 μm).The sample was injected by using split mode, helium as the carrier gas passed with flow rate 1.61 ml/min, the temperature program was started from 60c with rate 10c/min to 300c as final temperature degree with 3 minutes hold time , the injection port temperature was 300c, the ion source temperature was 200c and the interface temperature was 250c.The sample was analyzed by using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 26 minutes .Identification of components for the sample was achieved by comparing their retention times and mass fragmentation patents with those available in the library ,the National Institute of Standards and Technology (NIST). , results were recorded.

3.8 Antioxidant activity of methanol extract

3.8.1 2,2-Di(4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging was determined according to the method (Shimada et al.,1992). with some modifications. In 96-wells plate, the test samples were allowed to react with 2,2- Di(4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical for half an hour at 37°C. The concentration of DPPH was kept as (300 μM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiple reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate

CHAPTER FOUR

RESULTS AND DISCUSSION

CHAPTER FOUR

RESULTS AND DISCUSSION

4. Results

4.1 Percentage Yield

The Percentage Yield of extracts by maceration and using hot extraction were are shown in Table 4.1 and 4.2

$$\text{Percentage Yield \%} = \frac{\quad \times 100}{\quad}$$

Table (4.1) percentage Yield % of ethanol extracts using maceration method

Extract	Percentage Yield %
Ethanol	4%

Table: 4.2 percentage Yield % of extracts using Hot Extraction method

Extract	Percentage Yield %
1-Peterolum ether	7.04 %

2-Chloroform	6.65 %
3-Ethylacetate	7.65 %
4-Methanol	6.74 %

4.2 Results of photochemical Screening

The following results were obtained when the roots extracts was screened against different reagent as were are shown in Table 4.3 and Table 4.4.

Table :4. 3 photochemical screening of ethanol extract by Maceration

Flovanoids	Alkaloids	Tannins	Terpenoids	Steroids	Sponin	Cardiac Glycosides
+	+	+	+	+	+	+

Table: 4. 4 photochemical screening results of crude extracts by Soxhlet

Extract	Flovanoids	Alkaloids	Tannins	Terpenoids	Steroids	Sponin	Cardiac Glycosides
1-Peterolum ether	-	+	+	+	+	+	+
2-Chloroform	-	+	-	+	+	-	+
3-Ethylacetate	+	+	+	+	+	+	+
4-Methanol	+	+	+	+	+	+	+

(+) = The Present of Active Constituents.

(-)= The Absent of Active Constituents.

The photochemical screening summarized in Table 4.3 and 4.4 showed the presence of major classes of secondary metabolites such as tannins, steroids, flavonoids, alkaloids, etc. It is known that secondary metabolites have good antioxidant properties.

These result to camper with study (Essam et al., 2018) our work is to photochemical investigate the plant's bark in our laboratory with Chromatographic approach. The crude ethanol extract of dried bark of *Z. spina-christi* (L.) was fractionated by diethyl ether, chloroform, ethyl acetate and finally with n-butanol.

4.3 The antioxidant activity

These tests worked in Medical Biochemistry, Research Department. Medicinal and Aromatic Plants for research , the antioxidant activity of the ethanol crude extract by Maceration and crude of ethyl acetate and methanol were are shown in Table 4.5 and 4.6 .

Table: 4.5 the antioxidant activity of the ethanol crude extract by Maceration

No.	Extract	% RSA \pm SD(DPPH)
1	Ethanol	90 \pm 0.00
Standard	Propyl Gallate	95 \pm 0.00

Table :4.6 the antioxidant activity of ethyl acetate and methanol crude extracts by Soxhlet.

No.	extract	% RSA \pm SD(DPPH)
1	Methanol	77 \pm 0.05
2	Ethyl acetate	79 \pm 0.03
Standard	Propyl Gallate	93 \pm 0.01

In table 4.5 and 4.6 the antioxidant activity of the crude extracts was tested using 2,2-Di(4-tert-octylphenyl)-1-picryl- hydrazyl radical scavenging antioxidant assay. The results showed high activity of the ethanol extract by maceration and moderate activities of both extracts by soxhlet. Previous studies on the ethanol and petroleum ether extracts of *Z. spina-christi* leaves showed high activities indicate that the extracts from *Z. spina-christi* are good antioxidants (Abalaka et al ., 2011) . It was also indicated that the fruits contained high level of total phenolic compounds (7.55mg /g as gallic acid) (Shimada et al., 1992).All these suggest that the antioxidant activity of *Ziziphusspina* in vitro could be related to the high concentration of flavonoids and phenolics. These agree with the photochemical results of this study table 4.3 and 4.4.

These results to camper with study (Khaleel et al ., 2016) showed all extracts presented a good scavenging activity. Lower value of IC50 indicates higher antioxidant activity. In addition, the reductive abilityof the extracts reflected the reducing power of the *Z. spina-christias* a potential source of antioxidants. The reducing ability of a compound generally depends on the presence of reductantswhich have been exhibited antioxidative potential.

4.4 The IR results of the fraction isolated from ethyl acetate crude

The ethyl acetate crude showed antioxidant activity and gave a good separation by TLC (chloroform: methanol: petroleum ether 5:1:3). The isolated fraction was studied IR analysis were are shown in figure 4.1

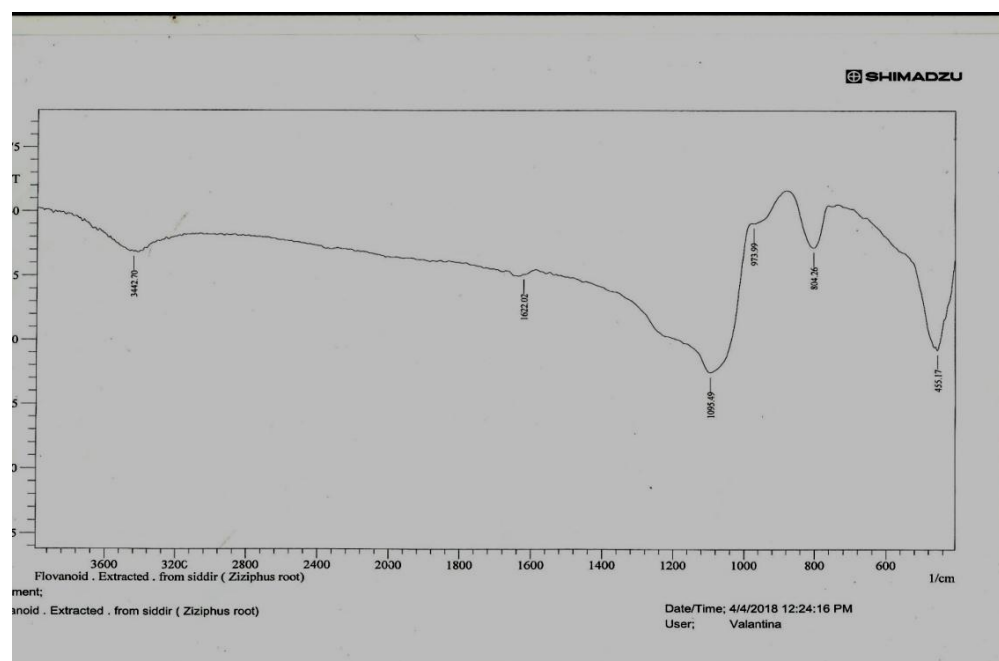


Figure 4.1: IR Result for PTLC ethyl acetate fraction using solvent system (chloroform: methanol: petroleum ether) (5:1:3)

The IR spectrometer is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting infrared absorption spectrum, the chemical bonds in a molecule can be determined.

The spectrum figure (4.1) showed ν (cm^{-1}) 804 (C-H, Ar bending), 1095(C-O), 1622 (C=C) and 3442(OH) .

4.5 The GC-MS results of the ethyl acetate extract

The GC-MS analysis of the ethyl acetate extract was conducted using the database of University of Medical Sciences and Technology (UMST). The spectrum of the unknown components was compared with the spectrum of the known components stored in the (UMST) library. The peak area%, name, molecular formula, molecular weight and chemical structures of some components were shown in Table 4.7 and Figure 4.2 .

Table :4.7 some components detected in the *Ziziphus spina* ethyl acetate extract

Area%	IUPAC name	Molecular formula	Molecular weight
15.37	7-Methyl-6-oxo-1,2,3,4-tetrahydro-6h-pyr	C ₈ H ₁₁ N ₃ O	165
13.00	Ethyl format	C ₃ H ₆ O ₂	74
12.59	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256
10.62	Gamma –Sitosterol	C ₂₉ H ₅₀ O	414
10.34	Stigmasterol	C ₂₉ H ₄₈ O	412
7.88	D-Limonene	C ₁₀ H ₁₆	136
6.47	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	284
4.24	Hexadecanoic acid ethyl ester	C ₁₈ H ₃₆ O ₂	284
3.62	3-Isopropyl -4-methyl-1-pentyn -3-ol	C ₉ H ₁₉ O	140
3.57	Octadecanoic acid, ethyl ester	C ₂₀ H ₄₀ O ₂	312
1.45	Heptane 2,4-dimethyl	C ₉ H ₂₀	128
1.40	Octane ,2,3,6,7-tetramethyl	C ₁₂ H ₂₆	170
1.14	Nonane ,5-(2-methylpropyl)	C ₁₃ H ₂₈	184
1.07	1,3,6-Heptatriene ,2,5,5-trimethyl	C ₁₀ H ₁₆	136
1.03	11-Methyldodecanol	C ₁₃ H ₂₈ O	200

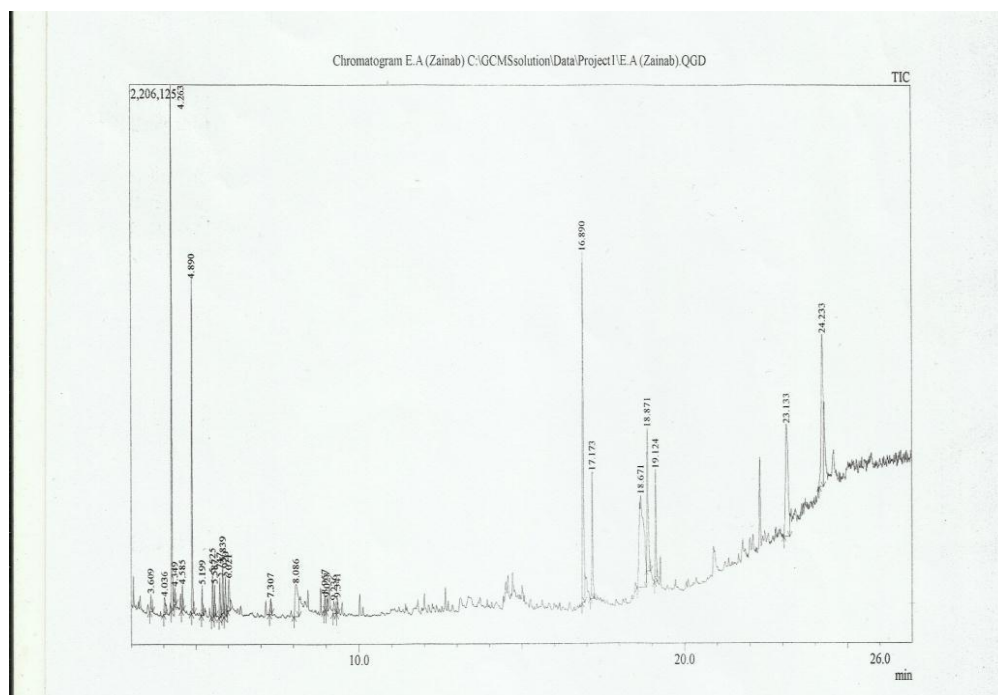


Figure 4.2: GC-MS chromatogram of the ethyl acetate extract

The ethyl acetate extract was found to contain 26 different compounds, representing 100% of the total extract. The identified compounds are listed in Table 4.7 and figure 4.2 according to their elution order on GC column. The major compounds detected were 15.37% 7-Methyl-6-oxo-1,2,3,4-tetrahydro-6hpyr ,13.00% Ethyl formate ,12.59 % n-Hexadecanoic acid , 10.62% Gamma –Sitosterol ,7.88% D- liomonene, 4.24% Hexadecanoic acid ethyl ester ,3.57% Octadecanoic acid, ethyl ester. Comparing these results with one of the reported photochemical evaluation and GC-MS analysis conducted on the ethyl acetate extract from stem bark of *Ziziphus spina* growing in Saudi Arabia showed that: the photochemical results of both the stem bark and the roots gave positive results for most secondary metabolites. The ethyl

acetate stem bark extract was found to contain 11 different compounds, representing 100% of the total extract. The major compounds detected were α -sitosterol (43.89%), stigmasterol (15.98%), and betulin (15.45%). The minor components of the ethyl acetate extract are found to be 8,11-octadecadienoic acid, methyl ester (1.80%), hexadecanoic acid, methyl ester (Palmetic methyl ester) (2.15%) and phytol (2.66%) (Essam et al., 2018). These differences might be attributed to geographical and environmental factors as well as time of harvest and age of the plant which were known as key factors affecting the chemical composition.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The interest of consumers towards natural bioactive compounds as functional ingredients in the food products has arisen due to their various health beneficial effects. Besides their nutritional and sensorial properties, functional foods have been recognized as acting as protective agents. The health promoting benefits of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species.

In this study, the photochemical constituents like flavonoids, phenolic compounds, terpenes and other secondary metabolites were present in the root extracts of *Ziziphus spina*. May be due to the presence of these photochemical constituents the roots of *Ziziphus spina* exhibited antioxidant activity.

The crude extracts of *Ziziphus spina* were tested for antioxidant activity by radical scavenging method. The results showed high activity of the ethanol extract by maceration and moderate activities of both ethyl acetate and methanol extracts by soxhlet. To our knowledge, this is the first study dealing with the **free radical** scavenging capacity of *Ziziphus spina* roots grown in Sudan. This study proved the potential

antioxidant activity of *Ziziphus spina* roots extracts grown in Sudan which can be used as easy accessible source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

5.2 Recommendations

Plants of Siddir (*Ziziphus spina*) are of high therapeutic important. Most of the previous studies were conducted on the leaves, fruits and seeds. This study showed the presence of bioactive components in Sudanese *Ziziphus spina* roots extracts which confer the antioxidant.

Further research needs to be carried out on the roots of *Ziziphus spina* extracts to identify the active compound(s) responsible for their antioxidant.

Further research needs to validate the therapeutic potential of this roots by assessing the activity and protective action of *Ziziphus spina* antioxidants against cell damage, first **in vitro** and then in vivo.

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