Master's thesis :

Microscopic Examination, Phytochemical Screening, Nutritional Value, Antifungal and Antibacterial Activities of Cynomorium coccineum L. Grown Wild in Libya

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منشورات البرنامج الوطني لتطوير المجلات العلمية

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Microscopic Examination, Phytochemical Screening, Nutritional Value, Antifungal and Antibacterial Activities of *Cynomorium coccineum* L. Grown Wild in Libya

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DEDICATION

To my late father

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Praise to Allah for providing me this opportunity and granting me the capability to proceed.

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ABSTRACT

Cynomorium coccineum L. locally known as "tarthuth" belongs to the family Cynomoriaceae and is distributed mainly in the Mediterranean region in dry rock or sandy soil. It is found in Libya in Tobruk, Nalut, Ghade, Wadi Al-athal, Ghadames and Zwara. Traditionally, it is used to treat dysentery and haemorrhoids, for nasal and uterine bleeding, as a tonic, laxative and astringent.

This study included a microscopic examination, phytochemical screening, and evaluation of the nutritional value, antifungal and antibacterial activities of *C. coccineum*. The plant was collected from the Zwara region of Libya. The aerial parts of the plant were dried in the shade and ground to a powder. The microscopic character of fresh and dried stem samples were evaluated. Powdered *C. coccineum* was extracted (hot extraction) with methanol as a solvent, using the Soxhlet apparatus. Preliminary phytochemical screening of the methanolic extract was carried out using standard testing procedures for secondary metabolites. Powdered *C. coccineun* was also used for proximate analysis (crude protein, crude fat, crude fibre, moisture, ash, carbohydrate content, and energy value) using standard analytical methods. Analysis of calcium, potassium and sodium content was performed using Flame Photometer, while content manganese, iron, copper, zinc, and cobalt were determined using Atomic Absorption Spectrophotometer. Different concentrations of the methanolic extract of *C. coccineum* stem were tested against fungi (*Candida albicans, Cryptococcus neoformens, Aspergillus niger* and *Aspergillus flavus*) and bacteria (*Escherichia coli*, *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus*) by the agar well diffusion method and minimum inhibitory concentration (MIC).

The transverse section of stem showed the presence of large parenchyma cells and vascular bundles, but no definite endodermis; whereas, the central part of the pith consisted of light coloured parenchyma cells. Microscopy of the powder revealed that the stem had many starch grains, xylem vessels, cork cells, pollen grains, and the crystals were absent. The phytochemicals identified of the stem were flavonoids, terpenoids, saponins, tannins, cardiac glycosides and phenols. Proximate analysis showed the crude protein, crude fat, crude fibre, ash, moisture and carbohydrate content to be 9.633 ± 0.205 %, 1.656 ± 0.004 %, 3.073 ± 0.12 %, 5.276 ± 0.016 %, 10.513 ± 0.026 % and 69.849 ± 0.074 % respectively. The computed energy value was 332.834 ± 1.152 Kcal/g. Analysis of mineral elements showed that the plant stem contained high levels of calcium, sodium and potassium 19940 ± 53 μg/g, 2273± 20 μg/g and 13840± 53μg/g respectively and low levels of manganese, iron, copper, zinc and cobalt 156 ± 4.8μg/g, 124 ± 7.7μg/g, 112 ± 2.8 μg/g, 54 ± 3.7μg/g and 20 ±2.4 μg/g respectively. The methanolic extract showed inhibitory activity against fungi *C. albicans,* and *C. neoformens.* The zones of inhibition for these two species were between (10.6±1.2 mm and 17±0.8 mm) for all concentrations of the extract, with lowest MICs of 125 mg/ml and 250 mg/ml respectively. The methanolic extract showed inhibitory of growth against bacteria *S. aureus* and *P. aeruginosa.* The zones of inhibition ranged from (12 ± 0.8 mm to 25.3 ± 0.4 mm) for all concentrations of the extract, with lowest MICs of 125 mg/ml and 62.5 mg/ml respectively. The fungi *A. niger, A. flavus* and the bacteria *E. coli, K. pneumonia* were resistant to all concentrations. The result of this study showed that the plant's stem contained an appreciable amount of nutrients, mineral elements, and phytochemical constituents, and further supports its potential use as an antimicrobial agent and in nutraceutical formulations.

الملخص

نبات الطرتوت المعروف علميا بأسم *Cynomorium coccineum* L. يتبع العائلة الكلبية (Cynomoriaceae) ينتشر في منطقة حوض البحرالأبيض المتوسط ، أما في ليبيا ينتشر النبات في مناطق متفرقة منها طبرق و غات وغدامس ووادي الأثل وزوارة و الحمادة. يستخدم نبات الطرتوت في الطب الشعبي لعلاج الزحار والبواسير و لوقف نزيف الانف والرحم وأيضا مقوي وملين وكمادة قابضة.

يستهدف هدا البحث دراسة نبات الطرتوت تشريحا والكشف علي المركبات الكيميائية الفعالة و تقديرالقيمة الغذائية وأيضا تأثيره المضاد علي بعض أنواع من الفطريات والبكتريا.

تَم تجميع النبات من منطقة زوارة (ليبيا) تمُ جففت الأجزاء الهوائية وطحنت الي مسحوق خشن. تَم أجراء الفحص المجهري لنبات لتعرف علي عناصر النبات المميزة. من ناحية أخري فلقد تم أستخدام مسحوق النبات لأستخلاص المركبات الفعالة بأستخدام المديب العضوي الميثانول وايضا تقدير القيمة الغذائية بأستخدام الطرائق القياسية وتَم الكشف علي العناصر المعدنية الكالسيوم والصوديوم والبوتاسيوم بأستخدام جهاز مطياف اللهب بينما أُستخدم جهاز طيف الأمتصاص الذري للكشف علي المنجنيزوالحديد والنحاس والزنك والكوبلت . وتم اختبار فاعلية المستخلص الميثانولي ضد أنواع من الفطريات والبكتريا وهي *Candida albicans, Cryptococcus neoformens*, *Aspergillus niger*, *Aspergillu flavus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* علي التوالي، بأستخدام طريقة الأنتشار بالحفر أضافة الي تحديد قيمة التركيز المثبط الأدني (M I C) .

تبين من خلال الفحص المجهري لقطاع في ساق النبات احتوائه علي خلايا برانشيمية كبيرة الحجم ومتباينة وخلايا البشرة الداخلية غير واضحة، وجود الحزم الوعائية و خلايا مركز منطقة النخاع تظهرشفافة، بينما كشف الفحص المجهري لمسحوق النبات علي أحتوائه علي الكثير من حبوب النشا، وجود الأوعية الخشبية كدلك خلايا الفلين وحبوب اُللقاح وعدم وجود أي نوع من البلورات. كشف التحليل الكيميائي علي بعض المركبات الفعالة تضمنت التانينات والصابونيات و الفلافونويدات والتربينودات والفينولات و الجليكوسيدات القلبية وكانت النسبة المئوية لمحتوي البروتين الخام والدهن الخام والألياف ومحتوي الرماد والرطوبة و محتوي الكربوهيدرات (9.633±0.205 % ، 1.656 ±0.004% 3.073 ± 0.12%،5.276 ± 0.016 %، 10.513 ± 0.026%، 69.849 ± 0.07%) علي التوالي،

فيما كانت القيمة السعرية (1.152 ± 332.834 كيلو سعرة/ جرام). واظهرت نتائج تحليل العناصر أن النبات يحتوي علي مستويات عالية من الكالسيوم والصوديوم والبوتاسيوم (53 ± 19940 ،20 ±2273 ،53 ± 13840 ميكروجرام/جرام) علي التوالي، ومستويات منخفضة من المنجيز والحديد والنحاس والزنك و الكوبلت (4.8 ± 156 ، 7.7±124 ،3.7±54 ،2.8±112 ،2.4 ± 20 ميكروجرام /جرام) علي التوالي، وأظهر المستخلص الميثانولي نشاطا جيد ضد الفطريات *C. albicans*, *C. neoformens* واعطي منطقة مثبطا لنمو تراوحت مابين (1.2±10.6–0.8±17 ملم) في كل التركيزات فيما كانت قيمة التركيز المثبط الأدني 125 ، 250 ملغم/ مل علي التوالي. اما بالنسبة للبكتريا فقد أظهر مستخلص الميثانول تأثير علي *P. aeruginosa, S. aureus* وأعطي منطقة مثبطا لنمو تروحت مابين (0.8±12 – 0.4± 25.3 ملم) في كل التركيزات فيما كانت قيمة التركيز المثبط الأدني 125 ، 62.5 ملغم /مل علي التوالي. ولم يظهر المستخلص اي تأثير علي الفطريات *A. niger*, *A. flavus*, والبكتريا *E. coli*, *K. pneumonia* . نتائج تحليل المكونات الاساسية والعناصر المعدنية تعطي لنبات أهمية غذائية ويدل وجود المركبات الكيميائية الفعالة الي أهميته كمصدر لعقاقير مفيدة.

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LIST OF ABBREVIATIONS

AOAC	Association of the Official Analytical Chemists Method
ATCC	American Types Culture Collection
B.C	Before Christ
C.	*Candida*
C.	*Cryptococcus*
C.	*Cynomorium*
CFU/ml	Colony Forming Units/ Milliliter
DMSO	Dimethyl Sulfoxide
E.	*Escherichia*
LM	Light Microscope
K.	*Klebsiella*
MC	Moisture Content
MIC	Minimum Inhibitor Concentration
NCTC	National Collection of Type Cultures
S.	*Staphylococcus*
P.	*Pseudomonas*
T.S	Transverse Section
W	Weight
UV	Ultra Violet

1. INTRODUCTION

1. INTRODUCTION

1.1 Medicinal plants

Medicinal plants have always an important place in human therapeutics in various parts of the world from ancient times. Writings indicate that the therapeutic use of plants goes back to the Sumerian and Akkadian civilizations, in approximately the third millennium B.C. Many ancient traditional systems such as Chinese, Indian, and Egyptian have been using terrestrial plants as medicines (Sarker and Nahar, 2007).

Reports by the World Health Organization (WHO) state that approximately 80% of the population living in developing countries rely almost exclusively on traditional medicine for fulfilling their health-care needs, which implies that more than 3.3 billion people in these countries utilize medicinal plants on a regular basis (Ahvazi *et al.,* 2012). Although synthetic drugs have revolutionized the management of various diseases, many of them are too expensive for millions of people living in these countries (Prakash and Jain, 2011; Siju *et al.,* 2014).

In modern medicine, plants remain an important source for drugs and an impressive number of modern drugs have been derived from them, such as the anticancer drug, vincristine, from *Vinca rosea*; narcotic analgesic morphine from *Papaver somniferum*; the antimalarial drug, artemisinin, from *Artemisia annua* (Sarker and Nahar, 2007).

Plants produce an enormous array of secondary metabolites, known as phytochemicals, natural products or plant constituents; these compounds are the end products of primary metabolites such as carbohydrates, amino acids and lipids, that naturally occur in plant's leaves, stems, bark, fruits and roots; and possess various benefits, including antimicrobial properties (Hayek *et al*., 2013;

Bargah, 2015). Natural antimicrobial compounds derived from plants have been traditionally used for centuries but only scientifically validated in the last 30 years (Gyawali and Ibrahim, 2012).

Major chemical components present in plants with antimicrobial effects include carotenoids, glycosides, terpenoids, flavonoids, phenolic compounds, alkaloids, tannins, lignins and saponins (Wendakoon *et al.,* 2012; Al-Mussawi, 2014). However, the efficaciousness of these components depends on the chemical structure of active components, and their concentration in the plants (Tiwari *et al.,* 2009).

The wide variety of chemical components in plant extracts makes it severely challenging for microorganisms to develop resistance mechanisms against them; hence, investigation and screening of these plants for chemical compounds with considerable antimicrobial activity has been a continuous process (Alothyqi *et al*., 2016).

Besides having pharmacologically important phytochemicals. Medicinal plants have its own nutrient composition. Nutrients such as carbohydrates, fats, proteins, and dietary fibre, these nutrients are essential for the biological and physiological processes of the human body (Dastagir, 2013). Therefore, evaluation of their nutritional significance can provide a better understanding of the worth of these species of plants (Isitua *et al.,* 2015).

Mineral elements are also of great physiological significance, though they usually form a small amounts of the total composition of most plants (Hannah and Krishnakumari, 2015). These elements are present in different concentrations in various parts of the plants (Lokhande *et al*., 2009). It has been established that many mineral elements play a vital role in ensuring the general well-being as well as in the curing of diseases (Hannah and

Krishnakumari, 2015). Some minerals like calcium, magnesium, phosphorus, sodium, potassium, sulfur and chlorine, required in our diets in amounts greater than 100 mg per day are called "minerals". Those that are required in amounts less than 100 mg per day are called "trace elements" including iron, iodine, copper, manganese, zinc, molybdenum, selenium, chromium and so on (Bahadur *et al.,* 2011). The estimation of mineral elements in plants is very important since the quality of many foods and medicines depends on the concentration and type of minerals (Bahadur *et al*., 2011).

1.2 Status of medicinal plants in Libya

Libya occupies an area of about 1.7 million km^2, of which more than 90% is a desert. The climate is typically Mediterranean, with erratic rainfall. Libya has a number of advantages in medicinal plants production including low production costs, suitable climatic conditions, and large areas of wild medicinal species (Louhaichi *et al.,* 2011). Medicinal plants are distributed all over the country, and about 450 species are reported to be of medicinal value; more than 100 species are utilized by local people in folk medicine; these plants have been widely used as drinks or chewed fresh or dry. They are also used to cure diseases and fungal, viral or bacterial infections (Auz, 2003). Some important families containing medicinal plants are Apiaceae, Asteraceae, Lamiaceae, Poaceae, Fabaceae and Brassicaceae. Scientific studies of the Libyan flora started about 200 years ago when the Swedish scientist Joran Rothman (1773-1776) collected numerous plant samples from western parts of Libya and stored them at the Riks Museum in Sweden. The Libyan Medicinal and Aromatic Plant Research programme were initiated in January 2001 at the Faculty of Pharmacy, University of Tripoli, Libya. The programme focuses on the chemical and biological evaluation of Libyan medicinal and aromatic plants (Auzi, 2003).

1.3 The family Cynomoriaceae

1.3.1 Botanical characteristics of family Cynomoriaceae

The family Cynomoriaceae has only one genus with two species. Plants are fleshy, with holoparasitic roots and no leaves, no chlorophyll. They parasites host plants' roots, via the haustorium; they are highly specialized parasites with some fungus-like properties (Hao-Cong *et al*., 2013).

The flowers are minute and dense, unisexual and bisexual, male flowers: perianth (-1) 3-5(-8) linear or narrowly subspatulate, whitish to reddish-tipped; stamen mature exserted, with anther ovoid-oblong, 4-loculed interorse and versatile pistillode shorter than the stamen, broad style-like, flattened, thickened and reddish in colour. Female flowers: smaller than male; perianth 1-5, similar as in male but have whitish; ovary 1-celled, globose with 1 pendulous 1-integumented ovule; style terminal, thickened but not flattened, seed solitary in fruit cavity, fruit nut-like (Jafri, 1977).

The place of the family in the taxonomic system has for long been uncertain. Most people place *Cynomorium* with the family Balanophoraceae due to the similarity in the general morphological features of the inflorescence (Heide-Jorgensen, 2008). But phylogenetic studies have indicated *Cynomorium* is not related with the Balanophoraceae (Nikvent *et al*., 2005).

1.3.2 The genus *Cynomorium*

There are two known species belong to the genus *Cynomorium* in the world: *Cynomorium songaricum* Rupr. and *Cynomorium coccineum* L. In Libya the genus is represented by one species, *C. coccineum* L (Jafri, 1977).

1.3.3 *Cynomorium coccineum* L

1.3.3.1 Taxonomic classification of *C. coccineum* L

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Santalales

Family: Cynomoriaceae

Genus: *Cynomorium*

Species: *coccineum* (Heywood, 1978).

1.3.3.2 Morphology of *C. coccineum* L

C. coccineum is a perennial, angiosperm, blackish-red leafless parasitic, the plant appears deep red when young and becomes blackish-purple when flowering, stem reach up to 40(-80) cm tall, 2-3.5 (-8) cm thick, erect, stout, arising from a branched underground rhizome bearing numerous remote 3-4 mm long, triangular lanceolate. Flowers only red colored, small that they can be seen individually, male flower 0.5 mm long, female flower 0.3(-4) mm long, stamen 0.5 mm long, ovary 0.1 mm in diameter and half inferior or inferior with 0.2 mm long terminal style, fruit orbicular (Figure1.1; A,B) (Jafri, 1977; Daoud, 1985).

Flowering is March to May each year and Pollinated by insects mainly ants and flies (Cui *et al.,* 2013). Species often parasites *Inula, Suaeda, Limonium*, and *Tamarix* (Heide-Jorgensen, 2008).

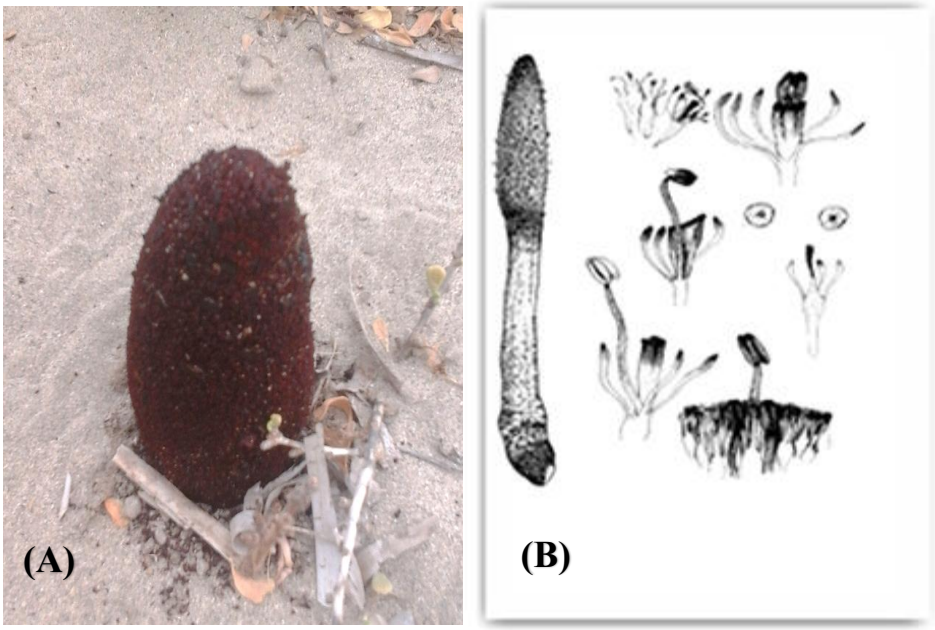

Figure 1.1:(A) *Cynomorium coccineum* L. growing in Zwara an area western Tripoli, Libya (2016); (B) *Cynomorium coccineum* L. from flora of Libya, Jafri (1977).

1.3.3.3 Geographical distribution and ecology of *C. coccineum* L

The species is distributed mainly in the Mediterranean region; it grows in dry rock or sandy soil. The plant emerges from the sand following the rains in winter for only a brief period each year. It is found in the southern parts of Italy, Spain, and also in Sicily, Malta and Crete (Lebling, 2003; Zucca *et al*., 2013). It is found in Libya, in Tobruk, Nalut, Ghade, Wadi Al-athal, Ghadames and Zwara. Moreover, it is found in the desert areas of Tunisia, Algeria, Morocco, Egypt, Syria, Iraq and Iran (Jafri, 1977). The plant is known by different vernacular names depending on the country, the most common being "Maltese mushroom" (Goncalves *et al.,* 2015). It is called a mushroom despite being a plant, most probably because of its outward appearance, lack of chlorophyll and underground growth for most of the year.

In Libya and Arabic countries, the popular name is “tarthuth”; sometimes, it is called “desert thumb” or “red thumb” (Zucca *et al*., 2013; Lebling, 2003).

1.3.3.4 History of traditional uses of *C. coccenium* L

Among the several thousand parasitic plants, which grow in an extensive range of ecosystems, few of them hold a special position either for their scientific purposes or economic significance. *C. coccineum* was one of these parasitic plants. Both Arabs and the Europeans were familiar with this species from the early middle ages. It was used as food, especially during periods of famine (Lebling, 2003).

Arab physicians recognized the significance of *C. coccineum* (the treasure of drugs) because it had a plethora of traditional therapeutic uses. Al-Razi (865−925), one of the most influential Islamic physicians, prescribed the plant as a remedy for hemorrhoids and nasal and uterine bleeding (Lebling, 2003).

C. coccineum has also known for a wide range of medicinal properties and thus used to treat dysentery, and bleeding during pregnancy as well as preventing infection. Moreover, it was a favored treatment for apoplexy and venereal diseases and was also used as a tonic, laxative, and astringent. It was prescribed in Malta to treat high blood pressure and irregular menstrual periods (Lebling, 2003). The dried mature spike of *C. coccineum* has been used to treat colic and stomach ulcers. In addition, in North Africa, consumption of three cups of a decoction of the plant's aerial parts before meals is used to treat hemorrhoids and diarrhea (Cui *et al*., 2013).

1.4 Objectives

The choice of the plant in this study was because there is no published study in Libya that deals with this particular plant; therefore, the overall objective of the study was to investigate/assess the nutritive and medicinal values of *C. coccineum* L. growing in Libya.

The specific objectives are:

- Microscopic examination to evaluate the plant key elements
- Determine preliminary phytochemical screening of the plant
- Carry out the proximate and minerals analysis of the plant
- Investigate the antifungal and antibacterial activities of methanolic extract of *C. coccineum* L.

2. LITERATURE REVIEW

2. LITERATURE REVIEW

The therapeutic properties of *C. coccineum* L. have been recorded in many cultures. In recent years, extensive research has been carried out to investigate the bioactive constituents and pharmacological effects of the plant (Cui *et al*., 2013).

2.1 Chemical studies of *C. coccineum* L

Phytochemical investigation is imperative for understanding the pharmacological activity and mechanism of action of the species. Several pharmacologically active compounds were isolated in the most recent research on *C. songarcuim* the stems of which include catechin and procyanidin B3, in addition to the triterpenes: including ursolic acid, acetyl ursolic acid, and malonyl ursolic acid hemiester. Palmitic acid and sucrose were also isolated from the aerial parts, as well as β-sitosterol palmitate, β-sitosterol, and β-sitosterol glucoside. Naringenin and epicatechin were obtained from a 50% alcohol extract of *C. songaricum*. Moreover, volatile components, trace elements, inorganic ions, condensed tannins and amino acids have been obtained from the plant (Cui *et al*., 2013).

In comparison, fewer chemical composition investigations have been conducted on *C. coccineum.* Harraz *et al*. (1996) isolated two anthocyanin pigments from the flowering tops of *C. coccineum* (Figure 2.1). Cyanidin3-*O*-6-rhamnosylglucoside was identified as the major pigment at 92% while cyanidin3-*O*-glucoside was identified as the minor pigment at 8 %.

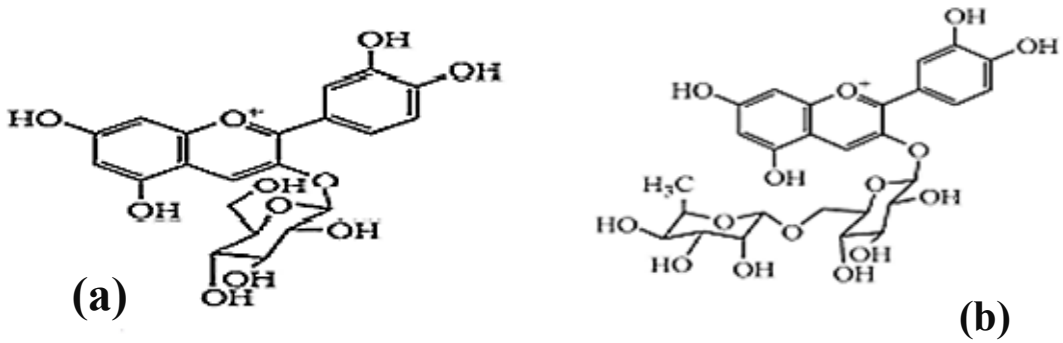

Figure 2.1: (a) Cyanidin3-*O*-glucoside, (b) Cyanidin 3-*O*-6-rhamnosylglucoside.

El-Tantawy (2002) carried out an investigation of mineral ion composition, amino acids, carbohydrate content, fat, fibre, moisture, ash and protein in the powdered stems of *C. coccineum* grown in Kuwait. This study evidenced that the hydrolyzate of the plant contains 12 amino acids (lysine, histidine, arginine, serine, isoleucine, leucine, methionine, valine, phosphoserine, phosphoethanolamine, tyrosine and phenylalanine). The content was found to be high in protein (28.67%), with a large amount of crude fat (9.35%) and moisture (78.66%), while carbohydrate, fibre and ash were found at levels of 10.93 %, 2.63% and 14.19 % respectively. The study also reported a mineral composition of sodium (Na) (23.7 mg/g), potassium (K) (19.5 mg/g), calcium (Ca) (01.5 mg/g), magnesium (Mg) (3.06 mg/g), iron (Fe) (0.068 mg/g), copper (Cu) (0.004 mg/g), zinc (Zn) (0.004 mg/g) manganese (Mn) (0.016 mg/g) and cobalt (Co) (0.012 mg/g).

In their study, Rached *et al.* (2010) used the thin layer chromatography technique (TLC) to screen phytochemical constituents from the ethanolic extract of wild *C. coccineum* grown in Algeria. The results revealed the presence of flavonoids, phenols, cuamarins, terpenoids, cardiotonic glycosides, lignins and sesquiterpenes.

For their part, Rosa *et al.* (2012) studied the chemical composition of the fixed oil isolated from *C. coccineum* collected from the island of Sardinia in Italy. The oil was obtained by supercritical fluid extraction with carbon dioxide (SFE-CO_2). The fixed oil was composed mainly of triacylglycerols and their derivatives, and the main fatty acids were oleic (18:1n-9), linoleic (18:2n-6), palmitic (16:0) and α-linolenic (18:3n-3).

Kiamanesh *et al.* (2015) identified the active ingredients in the ethyl acetate extract of *C. coccineum* grown in northeast Iran by using column chromatography. Their results revealed the presence of β-sitosterol (Figure 2.2).

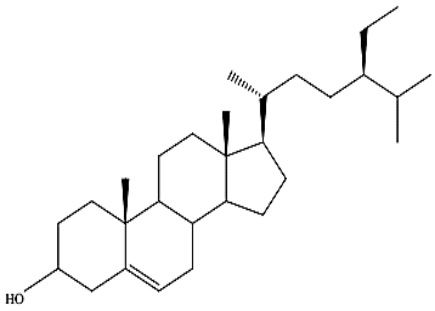

Figure 2.2: β −sitosterol

A preliminary phytochemical study was also conducted by Al-Hamaidia (2016) on the stem extract of *C. coccineum* grown in Saudi Arabia. The stem extracts were prepared in different solvents such as methanol, water, butanol and *n*-hexane. It was observed that in the methanolic extract, there was presence of glycosides, anthraquinones, flavonoids, saponins, tannins, phenols and terpenoids, while alkaloids were absent in the methanolic extract. Glycosides, anthraquinones, saponins and tannins were detected in the water extract. In the butanol extract, there was presence of glycosides, anthraquinones, flavonoids,

saponins, tannins, alkaloids, phenols and terpenoids, while the *n*-hexane extract contained flavonoids, saponins, phenols and terpenoids.

In a study carried out by Zucca *et al*. (2016), it was reported that the protein, carbohydrate, lipid, fiber and sodium, in content the dried stems of *C. coccineum* collected from island of Sardinia in Italy were 9%, 50%, 1% and 27% respectively, while the sodium content was 765mg/100g.

2.2 Biological studies of *C. coccineum* L

2.2.1 Blood pressure reduction

Ikram *et al*. (1978) found that the *C. coccineum* possesses significant blood pressure-lowering activity. This study mainly used the fresh plant juice on dogs, dried powder was also tested. While the fresh plant juice exhibited marked blood pressure-lowering characteristics, the dried plant powder did not.

2.2.2 Reproductive system effects

Many studies of *C. songaricum* have shown that its extracts affect the reproductive system (Cui *et al*., 2013). However, few studies of the effects of *C. coccineum* on the reproductive system have appeared in the literature. Abdel-Rehman *et al*. (1999) reported that the *C. coccineum* aqueous extract, using stomach tube to 10 mature male Wistar rats, at a dose of 47 mg/100 kg body weight/ day for14 consecutive days. This study showed an increase in spermatogenesis in the full sperm somniferous tubules after the extract was administered.

Al-Qarawi *et al.* (2000) studied the effects of *C. coccineum* water extract on ovarian follicular development and serum levels of FSH and LH in immature 17-day-old and 25-day-old Wistar rats. The plant extract elicited significant

changes in gonadotrophin levels coupled with significant increases in ovarian weight during folliculogenesis.

Abdel-Magied *et al.* (2001) investigated the effects of lyophilized *C. coccineum* aqueous extract on testicular development and on the serum levels of testosterone. ICSH and FSH were studied. The plant extract had a direct spermatogenic influence on the seminiferous tubules of immature Wistar rats, presumably by exerting a testosterone-like effect.

2.2.3 Antioxidant activity

A paper by Rached *et al*. (2010) demonstrated the antioxidant potential of *C. coccineum* grown in Algeria using two complementary assays, namely the1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals, and β-carotene bleaching, with a total content of phenolic compounds and flavonoids. The extract showed an antioxidant capacity, with high phenolic and flavonoids content in the plant.

Zucca *et al*. (2013) performed similar work on fresh specimens of *C. coccineum* collected from the island of Sardinia in Italy. Aqueous and methanolic extracts were tested using multiple assay systems 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals, Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and Oxygen Radical Absorbance Capacity-Pyrogallol Red (ORAC-PYR). Both aqueous and methanolic extracts showed antioxidant capacities, with a few differences between them. The ORAC-PYR assay produced the highest antioxidant value in both cases and had a protective effect in various oxidative stress bioassays *in vitro*.

Al-Humaidi (2016) reported the total phenolic and antioxidant properties of methanolic, aqueous methanolic, butanol and hexane extracts of *C. coccineum*

grown wild in Saudi Arabia, using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals and 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) methods. This study showed high phenolic content in the polar extract and the strongest antioxidant activity in both, the DPPH and ABTS methods.

2.2.4 Anticancer activity

Fats and oils play important roles in human nutrition and in the pharmaceutical industry. There is increasing interest in evaluating the chemical compositions and biological activities of nonconventional vegetable oils. Rosa *et al.* (2012) investigated fixed oil obtained from dried stems of *C. coccineum* grown in Italy by supercritical fractioned extraction with CO_2, examining its composition, lipid profile and effect on intestinal Caco-2 cell viability. The oil displayed a significant inhibitory effect *in vitro* on the growth of undifferentiated Caco-2 colon cancer cells and no toxic effect on colon epithelial cells. The plant also increased the amount of essential fatty acids in normal intestinal epithelial cells.

Similarly, a research report by Rosa *et al*. (2015) examined the potential anticancer properties on B16 F10 melanoma of fixed oil obtained from dried *C. coccineum* stems. The oil isolation was performed by supercritical fractioned extraction with CO_2. The fixed oil showed a significant inhibitory effect on the growth of B16 F10 melanoma.

2.2.5 Inhibition of diabetes related enzymes

Alpha-glucosidase and alpha-amylase are the important enzymes involved in the digestion of carbohydrates, maintaining the postprandial blood sugar level. The inhibition of these enzymes could effectively slow down the increase in blood glucose levels after a meal. Thus, α-glucosidase and α- amylase are regarded as effective targets for the development of anti-diabetes drugs. Plant

foods rich in polyphenols have been reported to affect the utilization of glucose similarly to insulin and to act as good inhibitors of key enzymes such as α-amylase and α-glucosidase associated with type 2 diabetes (Nair *et al.,* 2013).

Phoboo *et al.* (2015) evaluated the aqueous extract of *C. coccineum* grown in Qatar as an inhibitor of α-glucosidase and α-amylase *in vitro*. The *C. coccineum* aqueous extract demonstrated relatively high inhibitory activity against α-glucosidase, but only moderate inhibitory activity against α-amylase**.** This study determined that an aqueous extract of *C. coccineum* contained soluble phenolic compounds and exhibited relatively high levels of antioxidant activity associated with α- glucosidase and α-amylase.

2.2.6 Antimicrobial activity

C. coccineum extracts have demonstrated antimicrobial activity against pathogenic micro-organisms; including both Gram-positive, and Gram-negative bacteria and fungi, but their antimicrobial activity appears moderate compared to that of other, highly antimicrobial plant extracts.

Almussawi (2014) examined the antibacterial activity an ethanolic extract of *C. coccineum* grown in Iraq against five bacterial species; *Escherichia coli*, *Klebsiella pneumonia*e, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The agar well-diffusion method was used to analyse the extract's zone of inhibition. The extract showed stronger antibacterial activity against, *E. coli* and *S. aureus*, than against the other bacterial species.

Using the macr-odilution method, Goncalves *et al*. (2015) assessed the antifungal activity of a methanolic extract of *C. coccineum* grown in Italy against *Candida* spp, *Cryptococcus neoformans* and dermatophyte strains. This study found that the extract was very active against *C. neoformans*, *C. guilliermondii* and

C. krusei, with minimal inhibitory concentration (MIC) values of 0.025 mg/mL. This extract was more active than fluconazole against *C. krusei*.

According to Muhaisen *et al*. (2016), aqueous and methanolic stem extracts of wild *C. coccineum* grown in Libya were evaluated for antibacterial activity against four major human pathogenic bacteria; *Escherichia coli, Salmonella* spp, *Staphylococcus aureus*, and *Bacillus subtilis* by the agar well-diffusion method. The aqueous stem extract showed mild antibacterial activity against all tested organisms, whereas the methanolic stem extract exhibited moderate antibacterial activity against *S. aureus* and *B. subtilis* and did not show any activity against *E.coli* and *Salmonella* spp.

Zucca *et al*. (2016) investigated water extracts from whole plant (WP) external layer (EL) and peeled plant (PP) on several Gram-positive and Gram-negative bacterial strains, including; *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae,* using the disc diffusion method. The (WP) and (EL) water extract of *C. coccineum* showed a moderate effect on *Staphylococcus* genera, and the extract did not substantially inhibit the growth of Gram-negative strains, while only *A. baumannii* was inhibited by the (WP) and (EL) extract.

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

3.1.1.1 Plant collection and identification

Aerial parts of *C. coccineum* L. were collected from Zwara (200 Km west of Tripoli, Libya) in March 2016. The plant was identified and classified by Dr. Mohammed Abu-Hadra, a plant taxonomist, at the Department of Botany, Faculty of Sciences, University of Tripoli. The voucher sample (D681711) was deposited at the herbarium, Department of Botany,University of Tripoli.

3.1.2 Apparatuses

The following apparatuses were used in this study:

1. Atomic absorption spectrophotometer (AAS-Perkin Elmer, Model Analyst 800, Massachusetts, USA)
2. Desiccator
3. Flame photometer (BWB Technologies, Newbury, UK)
4. Hot plate
5. Hemocytometer slide (Superior, Germany)
6. Light microscope (LEICA, CME, Wetzlar, Germany)
7. Kjeltec 2200 Analyzer unit (Foss Analytical, Hoganas, Sweden)
8. Muffle furnace (N11, Nobertherm, Germany)
9. Raw fibre extractor (VELP Scientifica, Usmate Velat. Italy)
10. Soxhlet apparatus (Electothermal, England)
11. Soxtherm apparatus (Gerhardt, Konigswinter, Germany)

12. Oven (Memmert, Germany)

13. Ultra violet (UV) lamp (366 nm)

3.1.3 Reagents and chemicals

Mayer's reagent, Wagner's reagent, Dragendorff's reagent, 97% methanol, 40% sodium hydroxide, sulphuric acid, chloroform, ethyl acetate, ammonia solution, ferric chloride, glacial acetic acid, sodium sulphate, copper sulphate, selenium, 4% boric acid, methyl red, petroleum ether (40−60 °C), hydrochloric acid, 70% nitric acid, 30% hydrogen peroxide, 20% dimethyl sulfoxide (DMSO), 0.9% sterile saline, 70% alcohol formalin, acetic acid, safranin stain, iodine solution, potassium hydroxide and chloral hydrate.

3.1.4 Tested microorganisms

The following fungi and bacteria were used for the experiment. Fungi: *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 204092) were obtained from the Department of Microbiology, Faculty of Medicine (University of Tripoli); *Aspergillus niger* and *Aspergillus flavus* were provided by Dr. Najat Elghariany, Department of Plant Protection, Faculty of Agriculture (University of Tripoli). Bacteria: Gram-negative *Escherichia coli* (ATCC25922), *Klebsiella pneumonia* (ATCC13883), *Pseudomonas aeruginosa* (NCTC6749) and Gram-positive *Staphylococcus aureus* (ATCC29213). All the mentioned bacteria strians were obtained from the Department of Microbiology, Faculty of Medicine (University of Tripoli). Antibacterial standard Ciprofloxacine 10μ/disc (Oxoid, England) and antifungal standard Amphotericin B 10μ/disc (A-4888.Sigma-Aldrich, USA) were used.

3.1.5 Media

Sabouraud Dextrose Agar (CM-ASD197, Rapid Labs, UK), Sabouraud Dextrose Broth (CM0147,Oxoid, England,UK), Nutrient Agar (CM3, Oxoid, England, UK), Muller Hinton Agar (DM170D, Mast England,UK), Muller Hinton Broth (CM0405,Oxoid, England, UK).

3.2 Methods

3.2.1 Drying of plant material

The plant was gently cleaned using a brush to remove any soil residue. The plant was cut into small slices and dried under shade at room temperature, and then ground into a powder state using the grinder.

3.2.2 Microscopic examination

3.2.2.1 Transverse section (T.S) of stem

The fresh stem of the plant was fixed in 5ml of formalin, 5ml of acetic acid and 9 ml of 70% alcohol (F.A.A) for subsequent anatomical study. The plant sample was soaked in water overnight under room temperature to remove the preservative. A clear section was prepared, stained with safranin and observed under the light microscope (LM) (Khan *et al.*, 2009).

3.2.2.2 Powder of stem

The powder so obtained was used for the preparation of various temporary slides with water, potassium hydroxide, chloral hydrate, iodine solution and hydrochloric acid to study various anatomical features. The slides were observed under (LM) according to the method of Evans (2002).

3.2.3 Preparation of extract (hot extraction)

Extraction of the dried plant material was performed using a Soxhlet apparatus, a method described by Handa (2008); 300g of powdered plant material was placed into a thimble of Soxhlet, then 1600 ml of 97% methanol (70 °C) was added to the flask of Soxhlet, and the material was extracted for about 24 hrs. The filtrate was collected in a glass jar. The jar was left open and placed away from direct sunlight to dry the sample in order to yield a crude extract. The residual extract was preserved in a sterile glass bottle until it was used for phytochemical analysis, antifungal and antibacterial testing.

3.2.4 Preliminary phytochemical screening

Preliminary phytochemical screening of the methanolic extract obtained from *C. coccineum* was performed to identify for the presence of various chemical constituents according to the procedure described by Tiwari *et al.* (2011). Detection of the phytochemical was based on visual observation following either a colour change or the formation of a precipitate after the addition of specific reagents.

3.2.4.1 Test for alkaloids

Two ml of extract was added to 1 ml of hydrochloric acid. The solution was then heated in a water bath for 10 min. 1 ml of the solution was treated with few drops of the following reagents separately; Dragendorff's / Wagner's / Mayer's. The formation of an orange precipitate, brownish-red precipitate, cream precipitate respectively, indicated the presence of alkaloids.

3.2.4.2 Test for flavonoids (Alkaline reagent test)

Two ml of extract was treated with a few drops of 20 % sodium hydroxide solution. The formation of an intense yellow colour indicated the presence of flavonoids.

3.2.4.3 Test for terpenoids (Salkowski test)

To 0.1 g of the extract, 2 ml of chloroform was added, after which 1 ml of concentrated sulphuric acid was carefully added to form a layer. Reddish brown colouration of the interface indicated the presence of terpenoids.

3.2.4.4 Test for saponins (Foam test)

About 0.1 g of extract was added to 2 ml of distilled water in a test tube. The solution was shaken vigorously and observed; the formation stable and persistent foam confirmed the presence of saponins.

3.2.4.5 Test for tannins (Braymer's test)

About 0.1 g of the extract was boiled in 2 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added; a brownish green or a blue-black colouration confirmed the presence of tannins.

3.2.4.6 Test for phenols (Ferric chloride test)

About 0.5 g of the extract was boiled with 10 ml of distilled water for 5 min and then filtered while hot. Then, 1 ml of ferric chloride solution was added. The formation of blue-black colouration indicated the presence of phenol.

3.2.4.7 Test for anthraquinones (Borentrager's test)

About 0.5 g of the extract was boiled with 1 ml of sulphuric acid, and then filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was transferred to another test tube, and 1 ml of dilute

ammonia was added. The resulting solution was observed for red colour changes.

3.2.4.8 Test for coumarins (Fluorescence test)

The extract was dissolved in sodium hydroxide spotted in Wattman's No.1 filter paper, and then examined under a UV lamp (366 nm). The appearance of blue fluorescence indicated the presence of coumarins.

3.2.4.9 Test for cardiac glycosides (Keller- Kiliani's test)

Three ml of the extract was treated with 2 ml of glacial acetic acid containing ferric chloride; then sulphuric acid was added to the wall of the test tube. The formation of a red ring at the interface indicated the presence of cardiac glycosides.

3.2.5 Determination of nutritional value

3.2.5.1 Proximate analysis

A powdered sample of *C. coccineum* was subjected to proximate analysis to determinate it of the major nutritional components. This method partitioned nutrients in the plant stem into six components: crude protein, crude fat, crude fiber, moisture (dry matter), ash and carbohydrate content as described by Association of the Official Analytical Chemists (AOAC) (2002). The proximate analysis were performed in triplicate. All the proximate values were reported as percentages.

3.2.5.1.1 Determination of crude protein

The crude protein in the powdered sample was determined by measuring the nitrogen content, and multiplying it by a factor of 6.25. This factor is based on the fact that most protein contains 16 % nitrogen. Crude protein was determined

using micro-kjeldahl method, which involves three major steps: digestion, distillation and titration.

Digestion process, 0.700 g of the sample were placed in a Kjeldahl digestion tube, and 12 ml concentrated sulphuric acid, 4.8 g of sodium sulphate, 0.07 g of copper sulphate and 0.05g of selenium were added. Then, the digestion tube was placed on the rack in the digester unit. The sample was digested at 420°C for approximately 1 hr, until the sample had a clear pale green appearance.

Distillation process, 23ml of 4% boric acid solution were transferred to a receiving flask, and 5 drops of methyl red indicator were added. The flask was placed under the Kjeldahl condenser. Then 80 ml of distilled water and 50 ml of 40% sodium hydroxide solution were added. Finally, distillation was started.

Titration process, 0.1 N hydrochloric acid was added to a burette, and the contents of the flask were titrated. A blank was also titrated under the same conditions. The protein percentage was calculated based on the following formula:

$$\text{Nitrogen (\%)} = \frac{(\text{sample titre} - \text{blank titre}) \times 14}{\text{Sample weight}} \times 100$$

$$\text{Crude protein (\%)} = \text{nitrogen (\%)} \times 6.25$$

3.2.5.1.2 Determination of crude fat

To determinate the crude fat, 5 g of the sample were placed in an extraction thimble and covered with absorbent cotton. Then, 50 ml solvent (petroleum ether 40−60 °C) were added to a pre-weighed cup. Both the thimble and cup were attached to the extraction unit of a Soxtherm apparatus. The sample was subjected to extraction with the solvent for 30 min, followed by rinsing for 1.5

hrs. The solvent was evaporated from the cup into the condensing column. The extracted fat was dried in an oven at 110 °C for 1 hr, and after cooling, the crude fat was calculated using the following formula:

$$\text{Crude fat (\%)} = (\text{Extracted/Sample weight}) \times 100$$

3.2.5.1.3 Determination of crude fibre

To determinate the crude fibre, 5 g of the sample were placed in a glass crucible and attached to the raw fibre extractor. Then, 150ml of a boiling 1.25% sulphuric acid solution were added. The sample was digested for 30 min. Then the acid was drained and the sample was washed with boiling distilled water. After this, 150 ml of 1.25 % sodium hydroxide solution were added. The sample was digested for 30 min, and then, the alkali was drained the sample was washed with boiling distilled water. Finally, the crucible was removed from the extraction unit and oven dried overnight at 110°C. The sample was allowed to cool in a desiccator and weighed (W1). The sample was then ashed at 550°C in a muffle furnace for 2 hrs, cooled in a desiccator and reweighed (W2). The extracted fibre was expressed as a percentage of the original undefeated sample and calculated based on the following formula:

$$\text{Crude fibre (\%)} = \frac{\text{(digested sample (W1) - ashed sample (W2)}}{\text{Weight of sample.}} \times 100$$

3.2.5.1.4 Determination of moisture content

Approximately 5 g of dried *C. coccineum* powder were placed in a well-dried dish and the dish was put in the oven at 130 °C for 1.5 hrs. Then, the dish was placed in a desiccator until cooling reweight it. The moisture content percentage was calculated using the following formula.

$$\%MC = \frac{(\text{W of dish+ powder before drying g})-(\text{W of dish +powder after drying})}{\text{Weight of sample}} \times 100$$

3.2.5.1.5 Determination of total ash

To calculate the total ash, 5 g of the sample were placed into a pre-weighed porcelain crucible and incinerated overnight in a muffle furnace at 550°C. The crucible was removed from the muffle furnace, cooled in a desiccator and weighed. The ash content was calculated using the following formula:

$$\text{Ash (\%)} = (\text{ash weight/sample weight}) \times 100$$

3.2.5.1.6 Carbohydrate content

The total carbohydrates were calculated using the following formula:

Carbohydrate % = 100 - (crude protein (%) + crude fat (%) + crude fibre (%) + moisture content (%) + total ash (%)).

3.2.5.1.7 Energy value

The calorific energy value was calculated as per the formula used by Nile and Khobragade (2009). This was done by multiplying the mean value, carbohydrate, protein and fat by the factors of 4, 4 and 9 respectively.

3.2.5.2 Mineral elements analysis

To conduct mineral elements analysis, 1 g of the dried plant material was placed in a test tube. Then, 5 ml of concentrated nitric acid (NH_3) were added. The mixture was allowed to stand at least 1 hr to prevent frothing when the heat was applied, and 5 ml of NH_3 were also added to an empty tube which served as blank. The mixture was placed on a hot plate, and the temperature was

maintained at 120 °C. After the mixture was heated for 1 hr, while the tube was still on the hot plate, 1 ml of 30% hydrogen peroxide was added carefully. The process repeated two times until a total of 3 ml of hydrogen peroxide were added to the mixture. The solution was heated until turned clear. The tube was removed from the hot plate and allowed to cool. The solution was filtered through Whatman's No.1 filter paper to remove the insoluble particles. The final volume was adjusted to 50 ml using distilled water in a calibrated tube. An appropriate dilution was made for the sample before analysis. Potassium, calcium and sodium were determined using a Flame Photometer while the other mineral elements were determined using an Atomic Absorption Spectrophotometer. The resulting solutions were analyzed using the levels of the metals extrapolated from calibration graphs, which were generated using standard metal solutions (Kalra, 1998).

3.2.6 *In vitro* antifungal and antibacterial activities

3.2.6.1 Preparation of inoculum

Inoculum suspensions were prepared from recent cultures of selected bacteria/yeast and the bacteria was sub-cultured on Nutrient Agar, while the yeast was sub-cultured on Sabouraud Dextrose Agar, 4 or 5 pure colonies were then selected with an inoculating needle, transferred to a tube of 0.9% sterile saline and vortexed. The turbidity was corrected by adding sterile saline until the 0.5 McFarland turbidity standards of 1-2x 10^8 CFU/ml for bacteria and 1-5 x10^6 CFU/ml for yeast were achieved. The filamentous fungi were grown on Sabouraud Dextrose Agar at 28°C for 7- 14 days. The growth was then scraped aseptically, crushed and macerated thoroughly in 0.9% sterile saline. The inoculum size was adjusted to 0.4-5x10^5 spores/ml by microscopic enumeration with a cell-counting hemocytometer (Chandrasekaran and Venkatesalu, 2004).

3.2.6.2 Agar well diffusion method

The antifungal and antibacterial activities of the stem extract were evaluated using the agar well diffusion method. Petri plates were prepared by adding Sabouraud Dextrose Agar (for fungi) and Mueller-Hinton Agar (for bacteria), which were allowed to solidify. Plates were dried, and then 100µl of standardized inoculum suspension was poured in and spread uniformly. The excess inoculum was drained, and the plates were allowed to dry for 5 min. Wells (6 mm diameter) were punched in the agar plate using a sterile cork borer and then inoculated with 50µl of the plant extract at different concentrations (100 mg/ml, 300 mg/ml and 500 mg /ml). Antifungal standard Amphotericin B (10μ/disc) and antibacterial standard Ciprofloxacin (10μ/disc) were used as a positive control, and 20% DMSO was used as a negative control. The plates were incubated at 28°C for 48hrs (for yeast), 28°C for 3-5 days (for filamentous fungi) and 37 °C for 24 hrs (for bacteria). The zone of inhibition was measured in millimetres (mm). Tests were performed in triplicate (Balouiri *et al*., 2016).

3.2.6.3 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was found using the macro-dilution method. The test extract was dissolved in 20% DMSO to obtain a stock solution. Two-fold serial dilutions of the methanolic extract yielding concentrations of 500, 250, 125, 62.5, 31.25 and 15.6 mg/ml respectively were prepared in test tubes containing suitable growth media (1ml Sabouraud Dextrose Broth for fungi and Mueller-Hinton Broth for bacteria). Then, 0.1ml of bacterial suspension (10^8 CFU/ml), yeast suspension (10^6 CFU/ml) and filamentous fungi suspension (10^5 spores/ml) were transferred to each tube. A negative control test tube containing only the test extract, and a further test tube containing both broth and microorganism were taken as a positive control. The

tubes were incubated at 28 °C for 48hrs (for yeast), 28 °C for 3-5 days (for filamentous fungi) and 37 °C for 24 hrs (for bacteria). The tubes were examined for visual turbidity. The MIC values were taken as the lowest concentration that inhibited the visible growth of the sample. All the tests were performed in triplicate (Chandrasekaran and Venkatesalu, 2004).

3.2.7 Statistical analysis

The experimental measurements were carried out in triplicate are expressed as means ± standard deviation. Data were analyzed by Student's t-test using the Statview® version 5.0.1 software package (SAS Institute Inc, Abacus Concept, *Inc*., Berkeley, CA, USA). A p value of < 0.05 was considered significant.

4. RESULTS AND DISCUSSIONS

4. RESULTES AND DISCUSSIONS

4.1 Microscopic examination

Anatomic investigation of the stem showed the following features

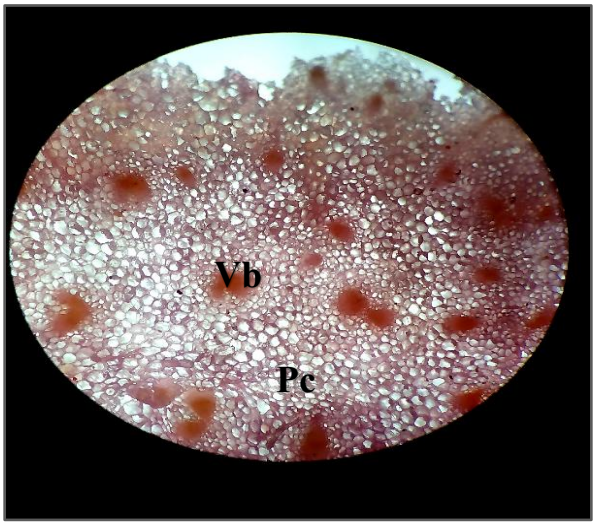

Figure 4.1: Transvers section of *Cynomorium coccineum* L. stem, Vb; Vascular bundles,Pc; Parenchyma cells (Magnification 10x)

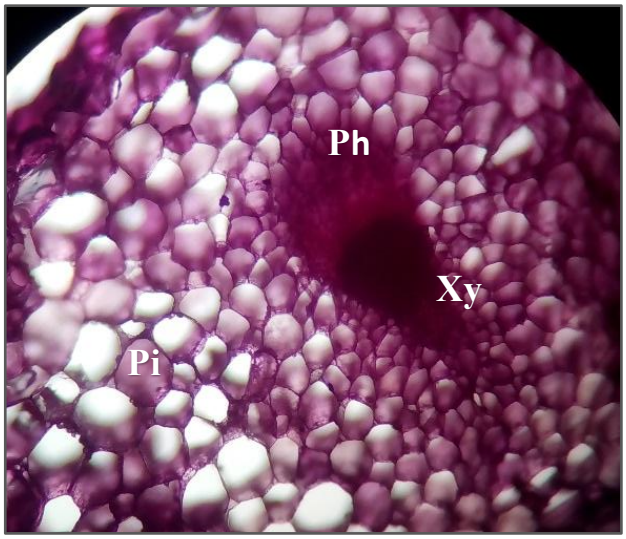

Figure 4.2: Transvers section of *Cynomorium coccineum* L. stem, Ph; Phloem, Xy; Xylem, Pi; Pith cells (Magnification 40x)

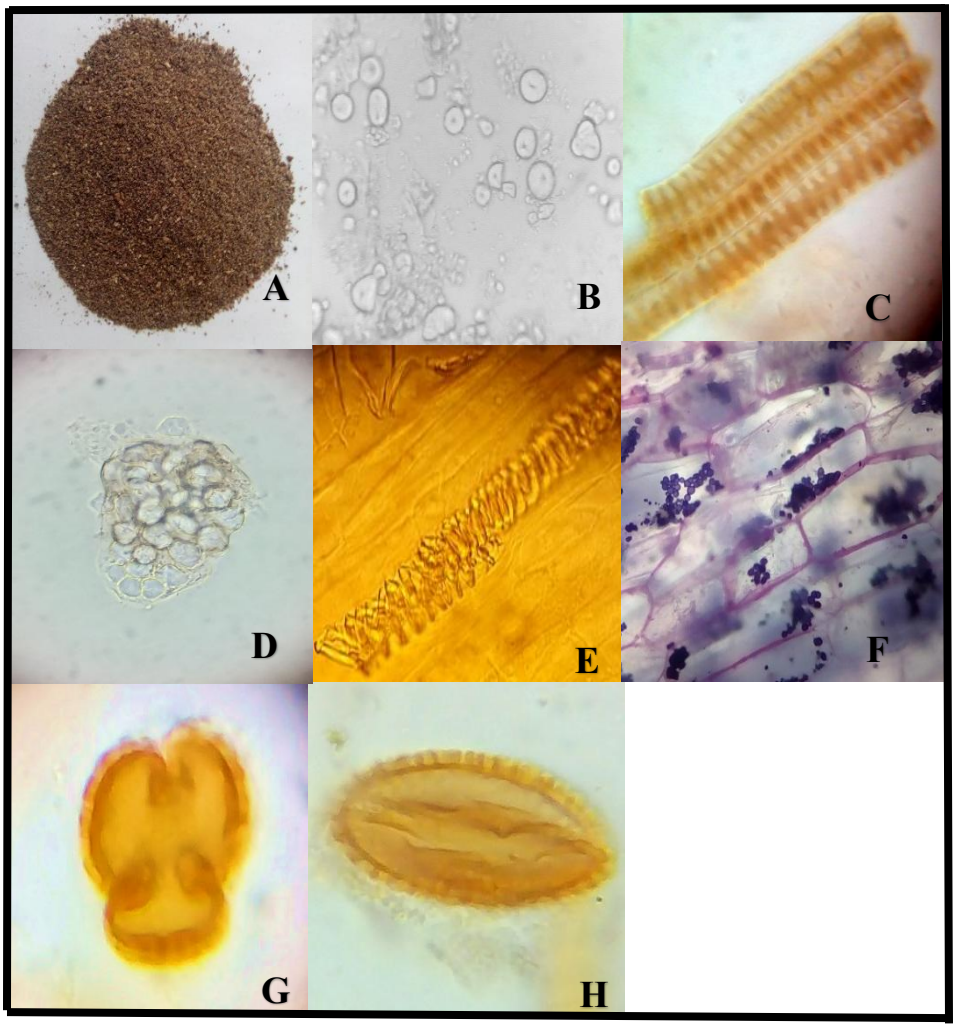

Figure 4.3: Microscopic characters of the powdered stem of *Cynomorium coccineum* L; (A) powder stem; (B) Starch grains; (C) Lignified xylem vessels; (D) Cork cells; (E) Spirel xylem vessels; (F) Starch grains in parenchyma cells; (G) Polar view of pollen grain; (H) Equatorial view of pollen grain (Magnification, 40x, 100x).

In the present study, the microscopic characteristics the stem of *C. coccineum* were served as a diagnostic character and helpful in the differentiation of species. The T.S. of the stem shows large parenchyma cells with various shapes, as well as vascular bundles consisting of phloem and xylem. There is no definite endodermis, and the central part of the pith consists of light coloured parenchyma cells (Figure

4.1, 4.2). A crude drug study of the aerial of *C. coccineum* was reported for the first time; the brownish-red powder contains many starch grains that found simple in form (Figure 4.3 A, B). In addition, lignified xylem vessels, cork cells, and spiral xylem vessels are visible (Figure 4.3 C, D, E). The parenchyma cells containing starch grains, when stained with iodine, showed blue to violet in colour (Figure 4.3, F), and the crystals were absent.

Pollen grains were observed from the same powder under a LM at (100x magnification). The morphology of these pollen grains was semi-rounded in a polar view and sub-prolate in an equatorial view, and the aperture was tricoloporate with a reticulate surface (as shown in Figure 4.3 G, H), which is consistent with the morphological description of pollen grains of *C. coccineum* given by Erdtman (1986). The different stem properties obtained from powder microscopy can be used as a taxonomic tool for identifying the plant species.

4.2 Extraction of dried plant material

The yield of extraction of *C. coccineum* 300g by Soxhlet apparatus (hot extraction) for 24 hours in 1600 ml of methanol solvent is given in Table 4.1 below.

Table 4.1: Physical properties of methanolic extract.

Extract yield (gm)	Extract yield (%)	Colour	Odour	Physical
135gm	42%w/w	Dark brown	Characteristic odour	Semi-solid

4.3 Preliminary phytochemical screening

The presence or absence of phytochemicals was determined using qualitative tests and the results are shown in Table 4.2 below.

Table 4.2: Phytochemical screening of methanolic extract of *Cynomorium coccineum* L. stem

Phytochemicals	Specific test	Observation	Result
Alkaloids	Dragendorff's reagent Wagner's reagent Mayery's reagent	No orange precipitate No brownish precipitate No cream precipitate	- - -
Flavonoids	Alkaline reagent	Formation of intense yellow colour	+
Terpenoids	Salkowski test	reddish brown coloration of the interface	+
Saponins	Foam test	Formation of persistent foam	+
Tannins	Braymer's test	Formation of a brownish green colour	+
Phenols	Ferric chloride test	Formation of blue-black colour	+
Anthraquinones	Borentrager's test	No change of ammonia layer to red colour	-
Coumarians	Fluorescence test	Not appearance of blue fluorescence under long UV lamp (366nm).	-
Cardiac glycosides	Keller- Kiliani's test	Formation red ring of the interface	+

Key: (-) not detected (+) detected.

Usually, phytochemical screening is the first step in research focusing on the isolation and purification of natural compounds. This step may provide information about the potential use of the plant material for medical and pharmaceutical purposes (Oikeh *et al.,* 2013).

In the present study, tannins, terpenoids, saponins, phenols, flavonoids and cardiac glycosides were detected; this result was in agreement with previous work reported by Al-Hamaidia (2016) on the methanolic stem extracts of *C. coccineum* collected in Saudi Arabia. In the present study, alkaloids, anthraquinones and coumarins were absent in the methanol extract. The absence of alkaloids also correlated with previous work (Al-Hamaidia, 2016). The results for terpenoids, phenols, cardiac glycosides and flavonoids in this research were in agreement with the findings reported by Rached *et al*. (2010) and Zucca *et al*. (2013). However, the absence of coumarins was not in agreement with the findings reported by Rached *et al*. (2010).

Tannins have astringent properties; the presence of tannins suggests a plant's ability to be an anti-diarrheal and anti-haemorrhoidal agent (Pacome *et al*., 2014). This may be why the plant has been used as an astringent to treat haemorrhoids and diarrhoea (Cui *et al*., 2013). The presence of saponins in *C. coccineum* justifies the use of this plant to stop bleeding (Lebling, 2003), because saponins have the property of precipitating and coagulating red blood cells. Other properties of saponins include formation in aqueous solution, haemolytic activity and cholesterol binding properties (Gogoi and Islam, 2012).

Flavonoids are potent water soluble, super antioxidant and free radical scavengers that prevent oxidative damage; they have anti-cancer and anti-inflammatory properties (Udu-Ibiam *et al*., 2014). Perhaps this explains why *C. coccineum* is used to treat ulcers in herbal medicine. Rached *et al.* (2010), Zuccu *et al*. (2013) and Al-Humaidi (2016) have reported a strong relationship between phenol content and antioxidant activity. Thus, the presence of phenol in a plant extract adds value to its nutritional and health potential.

4.4 Nutritional value

4.4.1 Proximate analysis.

Table 4.3: Proximate analysis of *Cynomorium coccineum* L. stem

Component	Mean value (%)
Crude protein	9.633 ± 0.205
Crude fat	1.656 ± 0.004
Crude fibre	3.073 ± 0.12
Total ash	5.276 ± 0.016
Moisture content	10.513 ± 0.026
Carbohydrate content	69.849 ± 0.074
Energy value	332.834±1.152 Kcal/g

Value are means ± Standard deviation of triplicate determinations.

Few studies have focused on the nutritional value of *C. coccineum*. However many previous studies have demonstrated that *C. coccineum* has biological activities as an anti-oxidant, anti-cancer and anti-diabetic agent (Zucca *et al*., 2013; Rosa *et al.,* 2015; Phoboo *et al.,* 2015), suggesting that this plant may be used to meet human nutritional needs.

The results obtained in the present study quantified the nutritive value of the *C. coccineum* sample. The experiment was performed under standard laboratory conditions using standard protocols, and the results are presented in Table 4.3.

A one g sample of protein gives 4.0 kcal of energy. *C. coccineum* contains a significant portion of protein (9.633±0.205 %); the obtained value was similar to the protein content (9%) of the same plant in Sardinia, Italy, as reported by

Zucca *et al.* (2016), while current data showed that the crude protein was lower than (28%) that reported by El-Tantawy (2002). This difference may be due to the fact that the protein content varies depending climatic and habitat conditions (Hameed and Hussain, 2015). Foods that contain protein are essential for the human body because they aid in building cells and tissue and they help repair tissues (Nehete *et al*., 2013).

In the present study, only a small amount of crude fat (1.656± 0.004 %) was detected in the plant extract sample. In the literature, the crude fat concentration in wild *C. coccineum* samples has been reported to be 1.0 % in Sardinia (Zucca *et al.*, 2016) and 9.359 % in Kuwait (El-Tantawy, 2002).

The crude fibre content (3.073±0.12 %) result obtained in the present study is in disagreement with that reported by Zucca *et al.* (2016) (27.7 %). However, the crude fibre content (2.163%) that El-Tantawy (2002) reported for wild *C. coccineum* in Kuwait was nearly similar to that reported in the present study.

Fibre is the part of the food that is not digested by humans. Therefore, the presence of high fibre levels in the diet can cause intestinal irritation; hwoever, normal functioning of the intestinal tract depends on the presence of suitable amount of fibre (Ojinnaka *et al.,* 2016).

Carbohydrate were the principal component (69.849±0.074%) in the sample used in the present study. In general, *C. coccineum* grown in Libya has a somewhat higher carbohydrate content than the carbohydrate content (45.5%) reported in the plant from Sardinia (Zucca *et al.*, 2016) and in the plant from Kuwait (10.933 %) (El-Tantawy, 2002). Carbohydrates play a critical role in living organisms. They can be oxidized to yield energy, and their polymers serve as energy storage molecules. Carbohydrate derivatives occur in a number of biological molecules such as coenzymes and nucleic acids (Hasan *et al*.,

2011). The different climatic conditions might account for the variations in the fat, fibre, and carbohydrate content; the variations may also occur due to the different life stages in the plant (Gupta *et al*., 2005).

Total ash content (5.276±0.016 %) was also obtained for *C. coccineum*. This result differs from the total ash content (14.191%) reported by El-Tantawy (2002). The ash content is generally taken to be a measure of the mineral content of the original food (Isitua *et al*., 2015).

A high quantity of moisture in nutrients makes them susceptible to microbial attack, and hence, spoilage. Moisture content is employed as a measure of stability and susceptible to microbial contamination, and also as an indication of amount of water in the plant (Bolanle *et al.,* 2014). In the current study, the moisture content was (10.513±0.026 %). This means that the plant will most likely have a long shelf life due to its low moisture content. However, this result was significantly lower than (78. 667 %) that reported by El-Tantawy (2002) for the plants grown in Kuwait.

The energy value was collected by multiplying the mean values of crude protein, crude fat and total carbohydrate by factors of 4.9.4 respectively. The energy value was (332.834±1.152 Kcal) this value was higher than (281kcal) that reported by Zucca *et al.*(2016),but the difference was not statistically significant. This energy value indicates that the plant can serve as a good source of energy for the human body. The nutritional value of *C. coccineum* appears to be compatible with human consumption, as described in traditional medicine (Lebling, 2003).

4.4.2 Mineral elements analysis

Table 4.4: Mineral elements of *Cynomorium coccineum* L. stem

Mineral	Concentration(μg/g)
Calcium (Ca)	19940±53
Potassium (K)	13840±53
Sodium (Na)	2273±20
Manganese (Mn)	156±4.8
Iron (Fe)	124±7.7
Copper (Cu)	112±2.8
Zinc (Zn)	54±3.7
Cobalt (Co)	20±2.4

Value are means ± Standard deviation of triplicate determinations.

Many researchers have investigated the mineral elements of several types of medicinal plants used in developing countries around the world. The mineral elements present in medicinal plants are very important in the formation of chemical constituents, because minerals are involved in plant metabolism and the chemical constituents of medicinal plants are the metabolic products of plant cells (Abdalla and Dafalla, 2014; Bahadur *et al.,* 2011; Kolasani *et al*., 2011).

In the present study, a total of eight elements calcium, potassium, sodium, manganese, iron, zinc, copper and cobalt were determined in the powdered sample of *C. coccineum*. The mean concentration of various metals in the plant sample is presented in Table 4.4.

The calcium content of (19940±53 μg/g) was found in this plant. The calcium content in this study is generally higher than that reported by El-Tantawy

(2002). Calcium is one of the minerals required for growth and the maintenance of bones, teeth and muscles (Ogbe and George, 2012).

The mean concentration of potassium in this study is (13840±53 μg/g); this value differed from the previous result by El-Tantawy (2002). Absorption of potassium in the plant generally depends upon the type and characteristics of the soil (Imelouane *et al.,* 2011).

The mean concentration of sodium was (2273±20 μg/g). The sodium content was lower than that reported by El-Tantawy (2002) and Zucca *et al.* (2016). Besides maintaining the acid-base balance, sodium also contributes to the regulation of plasma volume, nerve impulses and muscle contraction (Akpanyung, 2005).

In the human body, a large number of elements are required in small quantities to facilitate a wide range of functions; some these elements are manganese, iron, copper, zinc and cobalt. In this study, the mean concentration of manganese was (156±4.8 μg/g). Manganese helps to uphold the immune system and energy production. In addition, it works with B-complex vitamin to regulate the effects of stress (Muhammad *et al*., 2011).

The level of iron in this plant was (124±7.7 μg/g). Iron is essential for the formation of hemoglobin in the red blood cells to carry oxygen around the body (Kumar *et al*., 2013).

The mean concentration of copper was (112±2.8 μg/g). Copper is a vital component of many enzyme systems, such as cytochrome oxidase (Kumar *et al*., 2013). Copper may be toxic to humans when its level exceeds safe limits (Abdolgader *et al*., 2016). The recommended daily allowance (RDA) of copper

required for children and adults 0.7 or 1.1 mg/day respectively (Unuofin *at al.,* 2017). The copper content in *C. coccineum* is however below the RDA.

The mean concentration of zinc was determined to be (54±3.7 μg/g). Zinc facilitates different reactions in the body that help to construct and preserve DNA required for the growth and repair of body tissues (Kumar *et al*., 2013). Finally, the lowest mean concentration in this plant was recorded for the cobalt element (20±2.4 μg/g).

In the current study, the mean concentration of trace elements manganese, iron, copper, zinc and cobalt disagreed with the previous results reported by El-Tantawy (2002) for wild Kuwait *C. coccineum.*

The difference may be due to the fact that the content of elements and their various concentrations in the plants vary according to climatic conditions and soil fertility, as well as the selectivity and absorbability of plants for the uptake of these elements. Hence, the variations in the concentrations of the elements are attributed to the character of the plants, additionally as its surroundings (Bahadur *et al.,* 2011; Hannah, and Krishnakumari, 2015).

4.5 *In vitro* antifungal and antibacterial activities

4.5.1 Agar well diffusion method

Table 4.5: Antifungal activity by agar well diffusion method

Fungi species	Number of strains	Zone of inhibition in diameter (mm)				
		Concentration of methanolic extract (mg/ml)			Amphotericin B (10μ/disc)	DMSO
		100	300	500		
Candida albicans	ATCC10231	10.6±1.2	14.3±1.2	17±0.8	15.3±0.47	-
Cryptococcus neoformans	ATCC204092	12.5±1.2	14.6±0.9	15±1.2	18±1.2	-
Aspergillus niger	1 (isolate)	-	-	-	18±1.0	-
Aspergillus flavus	1 (isolate)	-	-	-	19.04±0.6	-

Mean zone of inhibition of three assays, ±: Standard deviation, (-) means no growth inhibition zone, Amphotericin B as a positive control, dimethyl sulfoxide (DMSO) as a negative control.

Table 4.6: Antibacterial activity by agar well diffusion method

Bacteria species	Number of strains	Zone of inhibition in diameter (mm)				
		Concentration of methanolic extract (mg/ml)			Ciprofloxacin (10μ/disc)	DMSO
		100	300	500		
Staphylococcus aureus	ATCC29213	12±0.8	13±1.6	15±0.8	23.6±0.9	-
Pseudomonas aeruginosa	NCTC6749	14.3±0.9	21.3±1.8	25.3±0.4	30.3±0.47	-
Klebsiella pneumonia	ATCC13883	-	-	-	33.3±0.9	-
Escherichia coli	ACCT25922	-	-	-	31.6±1.2	-

Mean zone of inhibition of three assays, ±: Standard deviation, (-) means no growth inhibition zone, Ciprofloxacin as a positive control, dimethyl sulfoxide (DMSO) as a negative control.

4.5.2 Minimum inhibitory concentration (MIC)

MIC was taken as the least concentration of the methanolic extract that showed a zone of inhibition in Tables 4.5, 4.6.

Table 4.7: Minimum inhibitory concentration (MIC) of methanolic extract of *Cynomorium coccineum* L. against fungi.

Fungi species	Number of strains	Concentration of methanolic extract (mg/ml)						
		500	250	125	62.5	31.25	15.6	MIC
Candida albicans	ATCC10321	-	-	-	+	+	+	125
Cryptococcus neoformans	ATCC204092	-	-	+	+	+	+	250

(+) Growth, (-) No growth.

Table 4.8: Minimum inhibitory concentration (MIC) of methanolic extract of *Cynomorium coccineum* L. against bacteria

Bacteria species	Number of strains	Concentration of methanolic extract (mg/ml)						
		500	250	125	62.5	31.25	15.6	MIC
Staphylococcus aureus	NCTC6749	-	-	-	+	+	+	125
Pseudomonas aeruginosa	ATCC29213	-	-	-	-	+	+	62.5

(+) Growth, (-) No growth.

Antimicrobial activity is typically investigated to complement phytochemical study; a variety of laboratory methods can be used to evaluate the *in vitro* antimicrobial activity of plant extracts. The best known and most basic methods are the wells, disk diffusion, and broth or agar dilution methods (Balouiri *et al*., 2016). In the present study, antifungal and antibacterial activities of the

C. coccineum methanol extract were investigated using the agar well diffusion and macro-dilution methods. The results are summarized in Tables 4.5, 4.6, 4.7 and 4.8.

For antifungal activity, the methanolic extract of *C. coccineum* was tested against *C. albicans*, *C. neoformans, A. niger* and *A. flavus*. The results showed that the extract had an inhibiting effect on the growth of *C. albicans* and *C. neoformans* (the range of the mean zone of inhibition was between $10.6\pm1.2-17\pm0.8$ mm) that no a significant difference to positive control; Amphotericin B ($p<0.05$), whereas *A. niger* and *A. flavus* were totally resistant to all concentrations (100, 300 and 500 mg/ml) of the plant extract. Moreover, the results indicated that DMSO had no effect on the fungi as there was no zone of inhibition.

Hence, an experiment conducted subsequently to determine the MIC against the most susceptible fungal strains (*C. albicans* and *C. neoformans*), the methanol extract showed MIC value of 125mg/ml against *C. albicans* and MIC value of 250mg/ml against *C. neoformans*. A similar investigation was carried out by Goncalves *et al*. (2015) on the antifungal activity of *C. coccineum* growing in Sardinia against, *C. albicans*, *C. neoformans* and dermatophytes using broth dilution assays. They also found evidence of *C. coccineum* activity against *C. albicans* and *C. neoformans,* in line with the result of the present study.

For antibacterial activity, the extract was tested against one strain of Gram-positive bacteria (*S. aureus*) and three strains of Gram-negative bacteria (*P. aeruginosa*, *K. pneumoniae* and *E. coli*). The results showed that the methanolic extract had an inhibiting effect on the growth of *S. aureus* and *P. aeruginosa*, (the range of the mean zone of inhibition was between $12\pm0.8-25.3\pm0.4$ mm). At a concentration of 500mg/ml, the extract had a zone

of inhibition that no significant difference to positive control; Ciprofloxacin. Moreover, the concentrations of 100 and 300 mg/ml the methanolic extract showed a lower zone of inhibition against *S. aureus* and *P. aeruginosa* than the positive control; ciprofloxacin ($p<0.05$). MIC values of 125mg/ml and 62.5mg/ml were found against *S. aureus* and *P. aeruginosa* respectively. In comparison, *E. coli* and *K. pneumoniae* were not susceptible to all the concentrations of the plant extract. In addition, the results indicated that DMSO had no effect on the bacteria as there was no zone of inhibition.

In line with these findings, previous studies, have also reported growth inhibition of *S. aureus* by the extract of *C. coccineum* (Almussawi, 2014; Muhaisen *et al.,* 2016; Zucca *et al.,* 2016). *S. aureus* one of a diarrhoea-causing bacterium, which explains the use of *C. coccineum* in folk medicine as a treatment for diarrhoea (Cui *et al*., 2013).

The most sensitive bacterium of those tested was *P. aeruginosa*. In this case, the results of the present study is disagree with the findings reported by (Muhaisen *et al.* (2016) and Zucca *et al.* (2016). This discrepancy may be attributed to differences in the solvents used for extraction and in the methods followed for testing antibacterial activity; the geographical location the plant samples were collected from may also be a factor (Lulekal *et al.,* 2014).

The aforementioned researchers (Zucca *et al.,* 2016; Muhaisen *et al.,* 2016) also showed that *E. coli* and *K. pneumonia* were not susceptible to extract of *C. coccineum*, a finding that matches the results of the present study but disagrees with the result reported by Almussawi (2014).

The activity methanolic extract of *C. coccineum* against *C. albicans, C. neoformans, S. aureus* and *P. aeruginosa* may be due to an interaction of different chemical compounds (such as flavonoids, terpenoids, and phenols) with

enzymes and proteins in the microbial cell membrane causing dispersal of protons towards the cell exterior. This, in turn, may induce cell death or inhibit enzymes necessary for biosynthesis of amino acids (Mostafa *et al.,* 2017).

5. CONCLUSION AND RECOMMENDATIONS

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5.1 Conclusion

The general analysis of Libyan plant, *Cynomorium coccineum* L., revealed valuable results after microscopic examination, phytochemical investigation, proximate analysis, mineral elements analysis, as well as the study of antifungal and antibacterial activities of samples grown in Libya.

The microscopic examination provided a description of the anatomic features of the *C. coccineum* stem and revealed the presence of large parenchyma cells, vascular bundle, and starch and pollen grains. The results obtained can help in the identification of the species in future.

The phytochemical screening of the methanolic extract of the *C. coccineum* stem revealed the presence of flavonoids, terpenoids, saponins, tannins, phenols and cardiac glycosides. In addition, the methanolic extract of *C. coccineum* possesses growth inhibitory activity against *C. albicans, C. neoformans, S. aureus* and *P. aeruginosa*. These results could explain why this plant has been used in traditional medicine for the treatment of infections.

Information from the proximate and mineral analysis revealed that the *C. coccineum* stem has a long shelf life because of its low moisture content. Its high carbohydrate content makes it a good source of energy. It is also a good source of calcium, potassium and sodium. Therefore, this work provides information on the nutritional value of *C. coccineum* and its potential for use in nutraceutical formulations.

5.2 Recommendations

1- Further research needs to isolate and identify the chemical constituents responsible for antimicrobial treatment.
2- The extract should be tested on other micro-organisms to ascertain their effect on other disease-causing agents.
3- It is necessary to determine the toxicity of bioactive constituents and their side effects.

6. RFERENCES

6. REFERENCES

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