**ANTIBACTERIAL ACTIVITY OF *HELIANTHUS ANNUUS* SEEDS EXTRACT ON**

***SALMONELLA* SPECIES AND *ESCHERICHIA COLI***

# BY

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# A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF TECHNOLOGY (MTech) IN PHARMACEUTICAL MICROBIOLOGY

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# ABSTRACT

Medicinal plants contain active ingredients with therapeutic values. *Helianthus annuus* L (Asteraceae) commonly referred to as “sunflower” has been reportedly used in managing and treating some ailments over the years. This study investigated the *in vitro* and *in vivo* antibacterial effects of the crude extracts of *Helianthus annuus* seeds on *Salmonella spp* and *Eschericha coli.* The phytochemical screening of the methanolic and ethanolic crude extracts revealed the presence of alkaloids, saponins, tannins, flavonoids and phenols. The effect of the crude extracts against the test organisms ranges from 7.33±0.67 mm to 25.67±0.33 mm at concentrations ranging from 60 mg/ml to 480 mg/ml. The minimum inhibitory concentration of the extracts ranges from 0.07±0.03 mg/ml to 2.40±0.06 mg/ml. The minimum bactericidal concentration of the extracts ranges from 0.48±0.01 mg/ml to 2.40±0.12 mg/ml. The acute toxicity study revealed LD50>2000 mg/kg. The sub acute toxicity study showed that there was a significant decrease in Alanine transaminase, Aspartate transaminase and Alanine phosphatase levels in most of the treated groups compared to the control group. Total cholesterol, triglyceride and low density lipoprotein cholesterol were significantly reduced compared to the control while a significant increase in high density lipoprotein was observed in the treatment groups. The serum biochemical protein components were comparable to the control. The result of the haematological study revealed that mean cell volume, haemoglobin and packed cell volume levels were significantly increased in most of the treated groups compared to the control group. There were no features of acute or chronic damage by the extracts on the hepatic and renal tissues. The in vivo antibacterial effect of the extracts revealed decrease in total bacteria load in treated rats. Therefore, *H. annus* seeds extracts possess antibacterial effects against *Salmonella spp and E.coli* and is considered to be relatively safe. Further studies should be carried out to identify the exact active compound or compounds that possess bactericidal activity. There is need to establish the validity and mechanism of action of the hepato-protective effect of *H.annuus* seed extracts inferred in this study.

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# CHAPTER ONE

* 1. **INTRODUCTION**

# Background to the Study

Medicinal plants contain active ingredients in one or more of its parts with therapeutic value or play the role of precursors for chemo-pharmaceutical synthesis (Bassam, 2012). Over the years, several medicinal plant parts have been utilized in managing and treating certain disease conditions, however only a few have been successfully evaluated and scientifically validated for the claimed effects (Saravanakumar *et al.,* 2010). Among the plants used in traditional medicine is *Helianthus annuus.*

Sunflower (*Helianthus annuus* L) is majorly cultivated in certain areas in the world for its seed, which is an important source of edible oil (Eze *et al.,* 2015). It is a carrier oil and also used in the production of biodiesel and margarine, as it is less expensive compared to olive oil. Sunflowers have the potential to play vital role inx phytoremediation asx it can be usedx to extractx toxic chemicals like heavy metals, such as lead, arsenic and uranium from soil, as well as engaged inx rhizofiltration to neutralize radionuclides and other toxic compounds and materials as well as harmful bacteria from water (Adler, 1996). Extracts from the leave as well as other preparations made from different parts of plant, are usedx in the treatmentx of high fevers, as a poultice on sores, swellings, snakebites and spider bites, in the treatmentx of malaria, lung ailments, diabetes (Saini and Sharma, 2011).

*Helianthus annuus* seed oil, and herbal tincture have been reported to have anti- inflammatory, antioxidant, antitumor, antipyretic, antihypoglycemic, cathartic, diuretic and antimicrobial activity (Aboki *et al.,* 2012). It has shown antimicrobial properties againstx diverse microorganism such as *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* among others. Thex effect of polar oil extract from the seeds of sunflower

(*Helianthus annuus*) in Napkinx dermatitis and its antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli* and *Proteus vulgaris* were studied (Ahmad *et al.,* 2015). Sunflower oil is highly absorbable throughx the dermal tissues, providingx significant nourishmentx andx moisturizing effects. As ax resultx of this, it is therefore, famous as an over-the-counter ingredient as well as ingredients used in the manufacturing of homemade beauty products suchx as lotions, creams andx massage oils. Oil extracted from sunflower plants may also possessx a protective barrier property which resists infections in developingx infants. Infants administeredx thisx oil subcutaneously onx ax daily basis were 69 % more likely not to develop infections in thex hospital (Ahmad *et al.,* 2015).

# Statement of the Research Problem

Antimicrobial resistance (AMR)x is a complex and growingx international public health problemx (WHO, 2015). Antimicrobial resistance is estimated tox account for more than 700,000 deaths annually. Antimicrobial resistance is projectedx tox increase to 10 millionx deaths with estimated cost of about US$100 trillionx by the year 2050 (O’Neill, 2016). Antimicrobial resistance affects every country irrespective of income and development (O’Neill, 2016). The rapid spread of multidrug resistant pathogens is of global threat. Drug-resistant pathogens accounts for 214,000x neonatal sepsis leading to deaths annually (Laxminarayan, 2016). Antimicrobial resistance isx not restrictedx to communicable diseases, it also has potential consequences onx non-communicable diseases (NCDs). Antimicrobial resistance poses threat tox surgical interventions, cancer treatment and organ transplant (Laxminarayan, 2013). About 50 %x of pathogensx implicatedx in surgical site infections are resistant to standard prophylactic antibiotics (Teillant *et al.,* 2015). Cancer patients whox have undergone chemotherapy also suffer infections caused by pathogens which are resistant to commonly used antibiotics (Teillant *et al.,* 2015).

Treating drug-resistant infections is expensive andx takes longer period andx has lower success rate than treating drug-susceptible infections. Therefore, AMRx negatively impacts on national economic performance (Smith *et al.,* 2015). The challengesx of AMRx and its magnitude within the African Region includingx Nigeria have been monitored by surveillance of drug resistance. In the past decades, the widespread of antibiotic usedx againstx microbial pathogens of humanx and animal origin has led to resistance to many antimicrobial agents which constitute a major health threatx around the globe (WHO, 2013).

One of the factors contributingx tox AMR is misuse of antibiotics. However, lackx of tests for other infections in patients who test negative for malariax hasx led to indiscriminate use of antibiotics (Dox *et al.,* 2016). According to Leopold *et al.* (2014), there is high level of resistance to commonly used antibiotics in the sub-Saharan African region. For example, 90%x of Gramx negatives were resistant to chloramphenicol, a commonly used antibiotic while resistance to third-generationx cephalosporins (ceftriaxone) was lessx common (Leopoldx *et al.,* 2014).

In Nigeria, resistance to Ampicillin increasedx from 70 to 90 %, Co-trimoxazole from 77 to 85 %, Chloramphenicol from 71 to 77 % and Streptomycin from 71 to 79 % between 1990x and 2000x in findingx the root cause of antimicrobial resistance (Ifeanyi, 2012). Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycinx Resistant *Staphylococcus aureus* (VRSA), carbapenem resistant Enterobacteriaceae, Multi drugx and extensive-drugx resistant *Mycobacterium tuberculosis* (MDR-TB and XDR-TB) have also been reportedx (Nannini, 2013).

The transfer of geneticx elements such as plasmids, transposons, and integrons has contributed to the rapidx spread of AMR among bacterial species. According to Harbottle

(2011), AMR genes have been found on mobile elements, leading to multidrug resistance which can be transferred to a susceptible recipientx via ax single genetic event. The emergence of multidrug-resistant bacteria can only be detected through systematic screeningx in quality assuredx microbiology laboratories ( Xavier *et al.,* 2010; Liu *et al.,* 2016). The healthx and economic consequences of AMRx has led to collaborative global action to improve access tox antimicrobial medicines and to promote availability of new product (Mendelson, 2015). Therefore, the use of herbal traditional medicines has emerged as effective alternatives.

# Justification for the Study

*Helianthus annuus* has been found to possess broad spectrum of *in vitro* antimicrobial activity against pathogenic microorganisms such as *Staphylococcus aureus, Salmonella typhi, Escherichia coli, Candida albican, Aspergillus fumigatus* and *Rhizopus stolonifera* (Subashini and Rakshitha, 2012; Eze *et al.,* 2015). As evident from the antibacterial study of *H. annuus* seeds by Rubab *et al.* (2016), there are claims that it may contain important chemical substances that confer this plant as medicinal agent possessing antibacterial activity. According to Sharma (2014), *H.annuus* contains various alkaloids, flavonoids, volatile oils and terpenoids which are essential for various antimicrobial activities, antitumor activity and antioxidant activity.

*H. annuus* seeds are cheap, readily available, accessible and nutritious which possess promising role in variety of infections, inflammations, cancers and cardiac diseases (Ruchika, 2014). Previous studies by Aboki *et al.* (2012) used Soxhlet extraction method and n-hexane (as solvent of extraction) at 60-65˚C, to determine antimicrobial activity of

*H. annuus* extracts. In this study, cold maceration method was employed. Several studies

have proved that the methanolic crude extracts of *H. annuus* have antimicrobial activity against wide range of pathogenic microorganisms.

# Aim and Objectives of the Study

The study investigated the antibacterial effect of crude extracts of *Helianthus annuus*

seeds on *Salmonella spp* and *Escherichia coli*. The objectives of the study were to determine the:

* + 1. phytochemical constituents of the crude extracts of *Helianthus annuus* seeds.
    2. *in vitro* antibacterial effect of the crude extracts of *Helianthus annuus* seeds on

*Salmonella* spp. and *E.coli*

* + 1. minimum inhibitory concentration (MIC) and minumum bactericidal concentration (MBC) of the crude extracts of *Helianthus annuus* seeds.
    2. toxicity of the crude extracts of *Helianthus annuus* seeds.

# CHAPTER TWO

# LITERATURE REVIEW

# History of Medicinal Plant Use

Medicinal plants have undoubtedly been considered by human beings since ancient times. It can be said that before history, the early humans recognized and exploited the plants around them for use as fuel, clothing, shelter and food, they became aware of their properties (Jamshidi-Kia *et al*., 2018). Medicinal plants have been transformed into one of the oldest sciences in countries such as China, Greece, Egypt and India.

The oldest written evidence of medicinal plants’ usage for preparation of drugs has been found on a Sumerian clay slab from Nagpur, approximately 5000 years old. It comprised 12 recipes for drug preparation referring to over 250 various plants, some of them alkaloid such as poppy, henbane, and mandrake (Kelly, 2009). The Chinese book on roots and grasses “Pen T’Sao,” written by Emperor Shen Nung circa 2500 BC, treats 365 drugs (dried parts of medicinal plants), many of which are used even nowadays such as the following: *Rhei rhisoma*, camphor, *Theae folium*, Podophyllum, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra (Wiart, 2006).

The Indian holy books Vedas mention treatment with plants, which are abundant in that country. Numerous spice plants used even today originate from India: nutmeg, pepper, clove, etc. The Ebers Papyrus, written circa 1550 BC, represents a collection of 800 prescriptions referring to 700 plant species and drugs used for therapy such as pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander, juniper, common centaury (Jamshidi-Kia *et al*., 2018).

In Homer’s epics The Iliad and The Odysseys, created circa 800 BC, 63 plant species from the Minoan, Mycenaean, and Egyptian Assyrian pharmacotherapy were referred to.

Some of them were given the names after mythological characters from these epics; for instance, Elecampane (*Inula helenium* L. Asteraceae) was named in honor of Elena, who was the centre of the Trojan War. As regards the plants from the genus Artemisia, which were believed to restore strength and protect health, their name was derived from the Greek word artemis, meaning “healthy.” (Toplak, 2005; Jamshidi-Kia *et al*., 2018). Herodotus (500 BC) referred to castor oil plant, Orpheus to the fragrant hellebore and garlic, and Pythagoras to the sea onion (*Scilla maritima*), mustard, and cabbage. The works of Hippocrates (459– 370 BC) contain 300 medicinal plants classified by physiological action: Wormwood and common centaury (*Centaurium umbellatum* Gilib) were applied against fever; garlic against intestine parasites; opium, henbane, deadly nightshade, and mandrake were used as narcotics; fragrant hellebore and haselwort as emetics; sea onion, celery, parsley, asparagus, and garlic as diuretics; oak and pomegranate as astringents (Gorunovic and Lukic, 2001; Jamshidi-Kia*et al*., 2018).

There are several other historical reports on the usage of medicinal plants as therapy in managing and treating ailments, not withstanding, today, they are still being used. Medicinal plants have gained international popularity due to its natural source, availability in local communities, affordability and ease of administration. Herbal medicine may be used as alternative treatment in case of adverse effects and drug resistance (Sasidharan *et al.,* 2011; Pandey and Tripathi, 2014; Azwanida, 2015; Ingle *et al.,* 2017).

* 1. **Sunflower (*Helianthus annuus* L.) Plant**

# History of sunflower usage and domestication

Sunflower was domesticated by Native Americans in the Eastern United States in about 3000 BC (Smith, 2006). They used the seeds directly as food and crudely extracted the oil. Native Americans had selected a tall, single-headed variety by the time European

explorers reached North America in the sixteenth century. While sunflower was not the staple that the ‘three sisters’ (maize, beans, and squash) were, it nonetheless was cultivated by many tribes from eastern North America through the Midwest and as far as northern Mexico. The Native Americans also used sunflower hulls as a source of dye, leaves for herbal medicines, and pollen in religious ceremonies (Seiler *et al*., 2017).

Historical records indicate that the Spanish were the first to introduce sunflower to Europe in the early 1500s (Marek, 2019). Sunflower was initially grown as an ornamental plant. Early English and French explorers also introduced it to their respective countries. From Western Europe, sunflower spread along the trade routes to Egypt, Afghanistan, India, China, and Russia. By the early 1700s, sunflower seeds were eaten as a snack, and in 1716, the first patent for the use of sunflower oil (for industrial purposes) was filed in England. The most significant boost for sunflower as a crop, however, came from the Russian Orthodox Church. Lenten regulations prohibited the consumption of many oily foods, but since sunflower was not specifically listed, the seed and oil became a staple diet item in Russia (Marek, 2019).

Efforts by Russian scientists led to significant crop improvements with oil contents soon exceeding 40 %. While sunflower was grown throughout Western and Eastern Europe, Russia historically was the largest producer with excess of 3 million hectares in the early twentieth century, compared to one-half million hectares for the rest of Europe. Russian immigrants are credited with introducing sunflower to North America. In fact, the open- pollinated variety ‘Russian Mammoth’ still sold by garden seed firms traces its lineage back to the same-named variety initially introduced in the 1880s. Early cultivation of sunflower in North America was primarily for livestock silage and seed for poultry. By the second half of the twentieth century, improved Russian varieties with oil levels of 45–55 % were available (Putt, 1997). Increased US production of these high-oil

sunflower varieties spurred interest by oil crushers, which led to expanded US production, especially in the Northern Great Plains (North Dakota and Minnesota). The discovery of cytoplasmic male sterility (CMS) by French scientists laid the foundation for the development of sunflower hybrids in the early 1970s. Hybrid sunflower, with higher yields and oil content, more uniformity, and disease resistance, in comparison with open-pollinated varieties, provided the last great impetus in establishing sunflower as a worldwide crop (Fick and Swallers, 1972; Marek, 2019)

# Botanical description of the plant

*Helianthus annuus* L. is a coarse, stout and erect annual plant, up to 1-3 meters high. The roots of the seedling are initially tap rooted, with maturing, it develops into a large fibrous and lateral root. Their stem are 1-6.5 ft. (30-200 cm) tall, hispid, round, branched. The branching are simple to highly branched, each terminating with a composite head (capitulum).

It possesses a large inflorescence (flowering head), and its name is derived from the flower's shape and image, which is often used to depict the sun (Plate I). The flowering head has large, composite heads, solitary at terminal end of peduncle or terminal on a branch, or axillary; composite disk usually 0.8-3.2 in. (2-8 cm) wide or more including rays; peduncles 0.8-8 in. (2-20 cm) long, densely hispid-scabrous. The receptacle are low-convex and chaffy (Khaleghizadeh, 2011).The heads consist of many individual flowers. The ray flowers are sterile with length ranging from 0.6-1.6 inch (1.5-4 cm) long, ligules are yellow in colour. Disc flowers are perfect, corolla lobes are about five in number and are 0.2- 0.3 inch. (5-8 mm) long, tubular, purple-brown to yellow in colour with each floret subtended by a small firm.



**Figure 1.0: *Helianthus annuus* L. Flower**

Source: (Seiler *et al*., 2017).

*Helianthus annuus* L lower leaves mostly opposite along stem, upper leaves mostly alternate along stem. Leaf blades narrowly to usually broadly deltoid-ovate, lower ones often cordate, to sub-truncate to broadly cuneate at base, 1.5-8 inch (4-20 cm) long or more, 1.2-6 inch (3-15 cm) wide or more, entire to margins minutely to coarsely serrate, apex acute to abruptly auminate.



**Figure 1.1: *Helianthus annuus* L. Leaf**

Source: (Seiler *et al*., 2017).



# Figure 1.2: Sunflower Field in Bloom

Source: (Seiler *et al*., 2017).

The seeds are in the form of Achenes and are between 0.1-0.3 inches (3-6 mm) long or more, they are narrowly obovate to ovate in shape, more or less four angled and somewhat compressed (Figure 1.3)



**Figure 1.3: *Helianthus annuus* L. Seeds**

Source: (Seiler *et al*., 2017).

# Taxonomy

**Table 1: Scientific classification of *Helianthus annuus* L*.***

|  |  |
| --- | --- |
| **Classification Taxonomy** | |
| Kingdom | Plantae |
| Division | Tracheophyta |
| Class | Magnoliopsida |
| Order | Asterales |
| Family | Asteraceae |
| Genus | *Helianthus* |
| Specie | *Annuus* |

Source: (Fabian *et al.,* 2014)

* + 1. **Species of *Helianthus***

There are about 70 species of *Helianthus* which include *H. annuus*L., *H anomalus* Blake,

*H. argophyllus* T. & G., *H. bolanderi* A. Gray, *H. debilis* Nutt., *H. deserticola* Heiser, *H. exilis* A. Gray, *H. neglectus* Heiser, *H. niveus* (Benth.) Brandegee, *H. paradoxus* Heiser,

*H. petiolaris* Nutt., *H. praecox* Engelm and A. Gray, *H. agrestis* Pollard, *H. arizonensis*

R. Jackson, *H. ciliaris* DC., *H. laciniatus* A. Gray, *H. cusickii* A. Gray, *H. gracilentus*

A. Gray, *H. pumilus* Nutt., *Atrorubens Corona-solis H. californicus* DC., *H. decapetalus* L., *H. divaricatus* L., *H. eggertii* Small, *H. giganteus* L., *H. grosseserratus* Martens, *H. hirsutus* Raf., *H. maximiliani* Schrader, *H. mollis* Lam., *H. nuttallii* T. & G., *H. resinosus* Small, *H. salicifolius* Dietr., *H. schweinitzii* T. & G., *H. strumosus* L., *H. tuberosus* L.,

*H. glaucophyllus* Smith, *H. laevigatus* T. & G., *H. microcephalus* T. & G., *H. smithii* Heiser, *H. atrorubens* L., *H. occidentalis* Riddell, *H. pauciflorus* Nutt. (synonym *H. rigidus* Cass.), *H. silphioides* Nutt., *H. angustifolius* L., *H. carnosus* Small, *H. floridanus* A. Gray ex Chapman, *H. heterophyllus* Nutt., *H. longifolius* Pursh, *H. radula*

(Pursh) T. & G. and *H. simulans* E.E. Wats. Howerver, few of these species are of medicinal importance which are *Helianthus annuus L*. and *Helianthus tuberosus* (Al- Snafi, 2018).

* + 1. **Common names of *Helianthus annuus* L.**

Arabic: Dawar El Shams, Zahrat El Shams; English: Sunflower; French: Grand soleil, Tournesol; German: Sonnenblume; Hindi: Surajmukhi; Italian: Girasole; Japanese: himawari; Korean: Haebaragi; Portuguese: Girassol; Spanish: Girasol; Swedish: solros (Al-Snafi, 2018).

* + 1. **Biological and chemical composition of *Helianthus annuus* L.**

*Helianthus annuus* seeds are described as achene, a specific type of indehiscent fruit. Individual seeds are approximately 10 to 15 mm long (Muhammad and Muhammad, 2012). The seeds are used as food and poultry feed worldwide. Its oil is used for frying and cooking. The iron-rich sunflower seeds are, by weight, 47 % fat and 24 % protein (Muhammad and Muhammad, 2012). Sunflower seed proteins are characterised by a moderately low level of albumin and high level of globulin proteins. The globulins represent 55 to 60 %, albumins 17-23 %, glutelins 11 to 17 %, Prolamines, 1 to 4 % and the combined non-protein nitrogen and insoluble residue is less than 11 % of the total nitrogen in the meal (Muhammad and Muhammad, 2012).

The phytochemical constituent of methanolic extract of *Helianthus annuus* seeds have been reported to contain carbohydrates, flavanoids, tannins, alkaloids, saponins, phytosterols, steroids and fixed oils (Subashini and Rakshitha, 2012). Allelochemicals in leaves, stems and roots of *Helianthus annuus* were determined using thin layer chromatography for alkaloids and spectrophotometry for phenols and flavonoids. These revealed high content of allelochemicals in leaves compared to roots and stems (Kamal

*et al.,* 2011). The aerial part of *Helianthus annuus* was found to contain an ent-kaurane glycoside named helikauranoside A together with three known ent-kaurane-type diterpenoids: (−)-kaur-16-en-19-oic acid, grandifloric acid, and paniculoside IV (Macias *et al*., 2008)

There are four tocopherol (α, β, γ and δ) isomers present in *Helianthus annuus* seed oil Fiska *et al.,* 2006). Proteins which were light colored were also isolated which contains helianthinin as globulin (Pickardt *et al.,* 2011). A bioflavonoid called Nevadensin, which has significant biological activities including hypotensive, anti-tubercular, antimicrobial, anti-inflammatory, anti-tumour and anti-cancer activities was isolated from *Helianthus annuus* (Pickardt *et al.,* 2011).

# Uses of sunflower

# Uses of sunflower as edible oil

Commercially available sunflower varieties contain from 39 to 49 % oil in the seed. In 1985-86, sunflower seed was the third largest source of vegetable oil worldwide, following soybean and palm (Pilorge, 2020). The growth of sunflower as an oilseed crop has rivaled that of soybean, with both increasing production over 6-fold since the 1930s. Sunflower accounts for about 14 % of the world production of seed oils (6.9 million metric tons in 1985-86) and about 7 % of the oil cake and meal produced from oil seeds. Europe and the USSR produce over 60 % of the world's sunflowers (Dafalla, 2012).

The oil accounts for 80 % of the value of the sunflower crop, as contrastedx with soybean which derives most of its value from the meal. Sunflower oil is generally considered a premium oil because of itsx lightx color, high level of unsaturated fatty acids andx lackx of linolenicx acid, bland flavor and high smoke points (Hamedx *et al*., 2012). The primary fatty acids in the oil are oleic and linoleic (typically 90 % unsaturatedx fatty acids), with

the remainder consisting of palmitic and stearic saturatedx fatty acids. The primary use is asx a saladx and cookingx oil or in margarine. In the USA, sunflower oils accountx for 8 % or lessx of these markets, but in many sunflower-producing countries, sunflower is the preferred andx thex most commonly used oil (Gupta, 2002).

High oleic sunflower oil (over 80 % oleic acid) was developed commercially in 1985 and has higher oxidated stability than conventional oil (Purdy, 1985). It has expanded the application of sunflower oils for frying purposes, tends to enhance shelf life of snacks, and could be used as an ingredient of infant formulas requiring stability.

# Uses of sunflower as meal

Non-dehulled or partly dehulled sunflower meal has been substituted successfully for soybean meal in isonitrogenous (equal protein) diets for ruminant animals, as well as for swine and poultry feeding. Sunflower meal is higher in fiber, has a lower energy value and is lower in lysine but higher in methionine than soybean meal. Protein percentage of sunflower meal ranges from 28 % for non-dehulled seeds to 42 % for completely dehulled seeds. The color of the meal ranges from grey to black, depending upon extraction processes and degree of dehulling (Ogello *et al*., 2017).

# Industrial applications of sunflower

The price of sunflower oil usually prohibits its widespread use in industry, but there are several applications that have been explored. It has been used in certain paints, varnishes and plastics because of good semi drying properties without color modification associated with oils high in linolenic acid (Gunduz, 2015). In Eastern Europe and the USSR where sunflower oil is plentiful, sunflower oil is used commonly in the manufacture of soaps and detergents. The use of sunflower oil (and other vegetable oils) as a pesticide carrier, and in the production of agrochemicals, surfactants, adhesives,

plastics, fabric softeners, lubricants and coatings has been explored. The utility of these applications is usually contingent upon petrochemical feedstock prices.

Sunflower oil contains 93 % of the energy of US Number 2 diesel fuel (octane rating of 37), and considerable work has been done to explore the potential of sunflower as an alternate fuel source in diesel engines. Blends of sunflower oil and diesel fuel are expected to have greater potential than the burning of pure vegetable oil (Ilkilic, 2008).

# Uses of sunflower as non-oil seed

The use of sunflower seed for birdfeed or in human diets as a snack has grown consistently over the past 15 years (Putnam *et al*., 1990). Varieties used for non-oil seed purposes are characterized by a larger seed size and require slightly different management practices. During processing, seed is divided into

1. larger seed for in-shell roasting,
2. medium for dehulling, and
3. small for birdseed. However, the standards for different uses vary.

# Use of sunflower as forage

Sunflower can also be used as a silage crop. It can be used as a double crop after early harvested small grains or vegetables, an emergency crop, or in areas with a season too short to produce mature corn for silage (Putnam *et al*., 1990). Forage yields of sunflower are generally less than corn when a full growing season is available. In one study, sunflower dry matter yields ranged from 2.0 to 3.0 ton/acre compared with 3.1 to 3.8 ton/acre for corn. Moisture content of sunflower at maturity is usually high (80 to 90 %) and would require wilting before ensiling (Heuze *et al.,* 2015).

Nutritional quality of sunflower silage is often higher than corn but lower than alfalfa hay. Crude protein level of sunflower silage is similar to grass hay and higher than corn silage. Generally, crude protein of sunflower decreases and lignin percentage increases after the flowering stage. High plant populations increases fiber and lignin percentage. Seed size does not seem to affect yield or quality (Heuze *et al.,* 2015).

* + 1. **Medicinal uses *Helianthus annuus* (sunflower) plant**

*Helianthus annuus* has been grown widely due to its traditional use as anti-inflammatory, antimalarial, anti-asthmatic, anti-oxidant, anti-tumor and antimicrobial agent (Saini and Sharma, 2011). The Seeds of *Helianthus annuus* is of great medicinal importance. The seeds and flowers of *Helianthus annuus* have been used as excellent source of protein and Vitamins B, D, E and K and also used in the treatment of pulmonary disorders (Saini and Sharma, 2011). Oil extracted from *Helianthus annuus* seeds have been used in treatment of dysentery, dysuria, hemorrhoids, fever, menorrhagia, pleuritis, Inflammation and bronchitis (Sharma *et al.,* 2009).

A decoction made from the leaves of *Helianthus annuus* leaves is used as astringent, diuretic and expectorant (Arshad and Amjad, 2012). The crushed leaves were used as a poultice on sores, swellings, snakebites and spider bites. A decoction of the roots was used as a warm wash on rheumatic aches and pains (Aboki *et al.,* 2012).

The seeds can be made into infusions and used in the treatment of whooping cough (Dwivedi and Sharma, 2014). Seeds are also used as diuretic, expectorant and in treatment of other lung ailments (Kunduraci *et al.,* 2010). The flowers and seeds were used in Venezuela in folk remedies for the treatment of cancer (Dwivedi and Sharma, 2014). The seed oil, and herbal tincture was used as anti-inflammatory, antioxidant,

antitumor, antipyretic, antihypoglycemic, cathartic, diuretic and antimicrobial (Aboki *et al.,* 2012). The following are the specific medicinal properties of *H. annuus*:

* + - 1. **Antimicrobial activity of *Helianthus annuus***

The antimicrobial activity of methanolic extract of seeds from *Helianthus annuus* was evaluated. The results of antibacterial activity showed high sensitivity to *Salmonella typhi*, moderate sensitivity to *Staphyllococcus aureus* and *Vibrio cholera* and less sensitivity to *Bacillus subtilis* (Subashini *et al.,* 2012). The results of antifungal activity of the extract of *Helianthus annuus* showed high sensitivity to *Rhizopus stolonifer* and *Aspergillus fumigates,* moderate sensitivity to *Candida albicans* and resistant to *Fusarium oxysporum* (Subashini *et al.,* 2012).

Antimicrobial activity of oil of *Helianthus annuus* seed was investigated against four bacterial isolates which include *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and one fungal isolate, *Candida albicans (*Aboki *et al.,* 2012). The tested microorganisms were cultured on Nutrient agar (for bacteria at 37 ˚C for 24 h) for the bacteria isolates and on Potatoes Dextrose Agar for the fungal isolates (for fungus at 30 ˚C for 48-72 h). *Bacillus subtillus* had the least mean zone of inhibition of 8 mm, the mean zone of inhibition observed with oil of sunflower seed (at low concentration of 0.2, 0.4, 0.6 mL) of up to 23 mm against C albicans, 25 mm against S aureus, 15 mm with *P. aurenosa* justified that oil of *Helianthus annuus* seed could be used as an antimicrobial agent *(*Aboki *et al.,* 2012).

The antibacterial activity of *Helianthus annuus* leaves was tested on some bacterial pathogens (*Escherichia coli, Salmonella* spp., *Shigella* spp. andx *Staphylococcus aureus*. The extracts were prepared usingx chloroform, N-hexane andx methanol. The antibacterial susceptibility testing of the extracts were carried out using the Agar well diffusion

methodx (Eze *et al.,* 2015). The antibacterial screening showed that the testx organisms were all susceptible to the extract. *S. aureus* showed the highestx susceptibility to the extracts while *Shigella* spp. showed least susceptibility. The minimum inhibitory concentration of the methanolic fraction of the extractx was determinedx tox be 125 μg/ml on all the test organisms which was lower than the MIC of gentamycin (280 μg/ml) (Eze *et al.,* 2015).

The *in vitro* antibacterial activity of the methanol, ethyl acetate and petroleum extracts obtained from the seeds of *H. annuus* was determined against three pathogenic bacteria (*Salmonella typhi*, *Pseudomonas aeruginosa* and *Vibrio cholerea*) (Rubab *et al.,* 2016). The antimicrobial activity was determined by using the standardized disc diffusion method (Zavala *et al.*, 1997; Hossain *et al.,* 2012). The extract concentration able to inhibit bacterial growth was observed through inhibition zone of 8 mm. The methanolic and ethyl acetate extract of *H. annuus* seeds showed high sensitivity to *Salmonella typhi* and moderate sensitivity to *Pseudomonous aeuregenosa*. The petroleum ether extract revealed high sensitivity to *P. aeuregenosa (*Rubab *et al.,* 2016*).*

* + - 1. **Antiasthmatic effect of *Helianthus annuus***

The effects of *Helianthus annuus* aqueous seed extract was examined on an in vivo anti- asthmatic model of ovalbumin induced mice and their lungs were assessed by hematoxylin and eosin staining. These revealed that the extract has potential in reducing asthma (Heo *et al*., 2008).

* + - 1. **Antioxidant effect of *Helianthus annuus***

The antioxidant capacity of the stripedx *Helianthus annuus* seed cotyledon extracts was obtained by extraction with different polarities of solvents by three different in vitro methods which are, ferric reducing/antioxidant power, 2.2-diphenyl-1-picrylhydrazyl radical andx oxygen radical absorbance capacity assays. In the three methods, the aqueous extract withx a dose 30 μg/ml showed a higher antioxidant capacity value than the ethanolic extract (Giada and Mancini-Filho, 2009). When compared with the synthetic antioxidant butylated hydroxyl toluene, the antioxidant capacity of the aqueous extract variedx from 45% tox 66% (Giada and Mancini-Filho, 2009). The high antioxidant capacity observed for the aqueous extract of *Helianthus annuus* seed suggests that the intake of this seedx may prevent cancer and other oxidative reaction relatedx diseases (Giadax and Mancini-Filho, 2009).

The antitumor andx antioxidant activity of *Helianthus annuus* was determined*.* Antioxidant activity results were measured using DPPH and fixed oil of *Helianthus annuus* showed moderate antioxidant activity. Fixed oil of this plant showed no activity when using Iron Chelating Assay (Abushama *et al*., 2014). The antioxidant activity of methanol, chloroform and N-hexane leaves extract was determined using the 2, 2 - diphenyl-1-picrylhydrazyl (DPPH) photometric assay (Eze *et al.,* 2015). The extracts produced a concentration dependentx increase in antioxidantx activity. The chloroform extract showedx greater antioxidantx activity while the methanolic extractx was leastx when compared to the ascorbic acid (standard) (Eze *et al.,* 2015).

Based on a study by Rubabx *et al.* ( 2016), the in vitro antioxidant activity of methanolic seed extract of *H. annuus* was determined by DPPH free radical scavengingx assay, whichx demonstrated very significant antioxidant potential compared to standard antioxidantx (ascorbic acid). Methanolicx extract of *H. annuus* showed 51.57 % DPPH scavengingx

activity and the half maximumx inhibitory concentration (IC50) was 0.814 whereasx 46.66

%x DPPH scarvenging activity and half maximum inhibitory concentration (IC50) of 1.0 in case of ascorbic acid (Rubab *et al.,* 2016).

# Research Microorganisms

## Salmonella spp

*Salmonella* infection is one of the most common food-borne infections worldwide. *Salmonella* belongs to the family Enterobacteriaceae. It is a Gram-negative, non-spore- forming, rod-shaped and facultative anaerobic bacterium. Some sub species of *Salmonella* ferment lactose. *Salmonella* are hydrogen sulfite producers, they are oxidase- negative and catalase-positive. It hydrolyzes urea, utilizes citrate and decarboxylates lysine as its sole carbon source (Feasey *et al.,* 2012; Andino and Hanning, 2015).

* + - 1. **Epidemiology of *Salmonella* infection**

There is high global burden of morbidity and mortality from human enteric pathogenic bacteria such as *Salmonella* species, despite the presence of antibiotic drugs (Petri *et al.,* 2008; Kirk, *et al.,* 2010; Dixon and Hall, 2015). *Salmonella* infection causes about 2.8 billion cases of diarrhoea annually worldwide. *Salmonella* enterica serovar Typhi (*S. typhi*) which is the causative agent of typhoid fever, is reported to cause 16–33 million infectious cases, with an estimated 500 000 to 600 000 deaths, while nontyphoidal *Salmonella* (NTS) infections account for 90 million cases and 155 000 deaths worldwide annually (Bula-Rodas *et al.,* 2015). The high prevalence of human immunodeficiency virus (HIV) infections in Africa, has led to exercebation of *Salmonella* infections and it has been reported that there are 2000–7500 *Salmonella* infection cases per 100 000 HIV infected adults (Feasey *et al.,* 2012). In Africa, 29.1 % of community- acquired bloodstream infections were attributed to *Salmonella* species (Nam *et al.*, 2015).

# Sources of infection and mode of transmission

*Salmonella* are commonly isolated foodborne pathogens, and are predominantly found in include fresh fruits and vegetables (Pui *et al.,* 2011). Farm animals such as swine, poultry and cattle are the prime sources of *Salmonella* infections. The slaughtering process of food animals at abattoirs is also considered one of the important sources of contamination with *Salmonella* (Gillespie *et al.* 2005). The bacterium can be transmitted through faecal–oral routes, where susceptible hosts may acquire *Salmonella* through contaminated foods and water (Ford *et al.,* 2016). The major dissemination is through consumption of uncooked animal food products.

* + - 1. **Pathogenicity of *Salmonella* infection**

When contaminated food or water is ingested, *Salmonella* colonizes the distal ileum and proximal colon (Hocking, 2012; Lonnermark *et al.,* 2015). The infective dose for salmonellosis that is capable of establishing infection in the mucosa of the small intestine ranges from 105 to 106 cells (Lonnermark *et al.,* 2015). Flagella serve as means of locomotion as well as chemotaxis to target cells, the enterocytes. *Salmonella* cells use type I fimbriae such as long polar fimbriae (Lpf) and thin aggregative fimbriae (Tafi), to adhere to enterocytes. Type IV pili are used by *S. typhi* to attach to host cells (Wagner and Hansel, 2011). Once *Salmonella* has adhered to the host cells on the apical side of M cells or enterocytes, it uses *Salmonella* pathogenicity islands (SPIs) encoded by type III secretion systems (T3SSs) to be phagocytized into the receptive macrophages (Wagner and Hansel, 2011).

*Salmonella* cells are exocytosed into the interstitial spaces of the lamina propria, where they are randomly picked by macrophages, dendritic cells and polymorphonuclear cells and distributed to the host efferent lymph in the mesenteric lymph nodes and then transported to the spleen and liver through the bloodstream (Velg *et al.,* 2012). The

attachment of *Salmonella* to the receptive epithelial cells and internalization into lamina propria causes inflammatory responses such as, release of pro-inflammatory cytokines which lead to diarrhoea, ulceration and the destruction of the mucosa cells (Velg *et al.,* 2012). Some species of *Salmonella* release enterotoxin and cytotoxin which can cause diarrhoea.

* + - 1. **Diagnosis of *Salmonella* infection**

The widal tube agglutination test has been used for decades. It involves detection of *Salmonella* serovar Typhi and Paratyphi A, with serum that measures agglutinating antibodies to the LPS (O) and flagellar (H) antigens. The efficacy of the Widal test is controversial, due to poor sensitivity and cross-reactivity with other *Salmonella* serovars, resulting in a low predictive value for typhoid fever (Ley *et al.,* 2010; Baker *et al.,* 2010). Non-*Salmonella* infections including malaria and brucellosis were also shown to lead to cross-reactivity in regions where enteric fever is endemic (Baker *et al.,* 2010). Due to low cost and simplicity, Widal test is still commonly used as a diagnostic test in regions that lack advanced laboratory infrastructure (Thriemer *et al.,* 2013). The laboratory diagnosis of typhoid fever is dependent on the detection of bacteria in the blood by PCR or culture. Microbiological culture has excellent specificity with low sensitivity and often requires 24 to 72 h of incubation. Hence, molecular approaches for *Salmonella* identification characterized by high sensitivity and a short time have been developed. PCR-based assays have been clinically validated for diagnosis of gastrointestinal Non Typhoidal *Salmonella* infection (Lin *et al.,* 2011), invasive Non Typhoidal *Salmonella* infection (Tennant *et al.,* 2010) and typhoidal *Salmonella* infection in the blood of patients with enteric fever.

* + - 1. **Treatment of *Salmonella* infection**

Antibiotics are the mainstay in the treatment of infectious diseases and improve healthx related quality of life, in addition tox reducing the mortality associated with bacterial infections. The selectivity of antibiotic drugs against invading bacteria ensuresx minimal harm to the patients andx at the same time ensures maximumx eradication of the target bacteria (Namx *et al.,* 2015). Non typhoidal Salmonellosis infections are associated withx complications such as meningitis and septicaemia therefore, require treatment withx antibiotic drugs, including ciprofloxacin, ceftriaxone and ampicillin (WHO, 2003; Medalla *et al.,* 2012). Infections caused by *S. typhi* and *S. paratyphi* may involve serious complications and require treatment with antibioticsx such as cefixime, chloramphenicol, amoxicillin, azithromycin, aztreonam, cefotaxime or ceftriaxone (Kumar and Kumr, 2017).

* + - 1. **Antimicrobialx resistance to *Salmonella***

Antimicrobial drug resistance of non-typhoidal *Salmonella* organisms has been discoveredx inx developedx countries as a result of inevitable consequence of the use of antimicrobial drugs in food producing animals. Such drugs may be used either therapeutically or prophylactically, or for growth promotionx (feed additives). Thex first line treatment choices for enteric and non- typhoidal salmonellosis disease were co- trimoxazole, ampicillin or chloramphenicol. However, from the late 1980s, there was an increase in prevalence of resistance to these commonly usedx antibiotics.

Resistance to commonly used antimicrobials in Typhi and Paratyphi A isx a widespread problemx in endemic areas and returningx travellers (Wain *et al.,* 2015). This led tox use of broad spectrum cephalosporins and fluoroquinolones whichx replaced older agents in the management of *Salmonella* disease (Crump *et al.,* 2011; Msefulax *et al.,* 2012)**.** Studies by Wong *et al.* (2014)x reported that specific *Salmonella* isolates ST313 were discovered to

be resistant to cephalosporins, tetracyclines, Co-trimoxazole, chloramphenicol and Aminoglycoside such as Streptomicin.

* + 1. ***E. coli* infection**

*Escherichia coli* are a groupx of gram negative bacteria normally foundx inx the flora of human andx animal digestive tractsx and as symbionts participating in digestion. *E. coli* are involvedx inx urinary tract infection (UTI), hospital acquired pneumonia (HAP), sepsis, surgical site infection (SSI), gastrointestinal tract infections and hemolytic-uremicx syndrome (HUS) (Alkeskas *et al.,* 2015). *Escherichia coli* is the major areobic organism residing in the intestine. It is also foundx inx soil and water as a result of faecal contamination (Tenaillon *et al.,* 2010). Some pathotypes of *E. coli* are known to cause infection of the gastrointestinal system (intestinal pathogenic *E. coli*) while others cause infection outside the gastrointestional system (extraintestinal pathogenic *E. coli*) (Croxen and Finlay, 2010).

* + - 1. **Epidemiology of *E. coli***

*Escherichia coli* is the most common cause of urinary tractx infectionx (UTIs) in humans. And is the leading cause of entericx infection and systemic infectionsx (Kim, 2012). The systemic infections include bacteremia, nosocomial pneumonia, cholecystitis, cholangitis, peritonitis, cellulitis, osteomelytis and infectious arthritis. *E. coli* is also the leading cause of neonatal meningitis (Kim, 2012). *E. coli* has been associatedx with increased morbidity and mortality in recent years (Barton *et al.,* 2011).

Enterotoxigenic *E. coli* (ETEC) is reported to be the most common bacterial enteropathogen isolated in children less than 5 yrs of age in developing countries accounting for about ten thousandx deaths each year (Barton *et al.,* 2011). It is also the most common cause of travelers’ diarrhea accounting for 10–60 % of infections

dependingx on the region visited (Barton *et al.,* 2011). Enteroaggregative *E. coli* (EAEC) isx the secondx most common cause of travelers’ diarrhea after ETEC. It causes persistentx diarrhea in children in developingx countries and has been implicated asx an important enteric pathogen affecting AIDS patientsx (Cappello *et al.,* 2011).

# Sources of infection and mode of transmission

*Escherichia coli* are the most commonx member of the Enterobacteriaceae whichx accounts for 75 - 90 % of Urinary tract infetions in humans (Barton *et al.,* 2011). *E. coli* can be isolated from contaminated food obtained from animal origin like milk (Disassa *et al.,* 2017). Humanx infection occurs through consumption of contaminatedx food productsx such as uncoooked meat or water contaminated withx animal or humanx wastes. It could be through directx person-person spread from poor hygeine (Berger *et al.,* 2010). The potential source of pathogenic *E. coli* isx hostx ownx intestinal flora, but the infection can also be transmittedx through the fecal–oral route or through sexual contact (Terlizzi *et al.,* 2017).

* + - 1. **Pathogenicity of *E. coli* infection**

The factors responsible for the virulence of pathogenic *E.coli* include adhesins, toxins (e.g. alpha-hemolysin, cytotoxic necrotizing factor 1, autotransporter toxins), iron/heme acquisition systems, and iron ion transport. P, S and type 1 fimbriae which are responsible for adhesion tox epithelial cells of intestines, kidneys, or lower urinary tract and for stimulatingx cytokine production by T cells. *E. coli* has the ability to multiply intracelullarly (Baldy-Chudzik *et al.,* 2015). The pathology of *E. coli* (ETEC) occurs through secretion of heat stable toxins STa/STIx andx STb/STII. STa/STI mimics the intestinal hormone guanylin, bindingx to and activating intestinal borders guanylate cyclase C receptor, increasing intracellular messenger cyclicx GMP. This activates cyclic GMP-dependent proteinx kinase II leading to phosphorylation of cysticx fibrosis

transmembrane regulator and deregulated ion absorption and secretion hence diarrhoea occurs (Baldy-Chudzik *et al.,* 2015).

* + - 1. **Diagnosis of *E. coli* infections**

*Escherichia coli* grow readily on simple culture media with minimal nutrientx of glucose or glycerol. *E. coli* is identified as lactose fermenting gram negative rod. It can grow areobically or anaerobically at 37 ˚C andx can be motile or non motile. Itx is oxidase negative and produces indole. Itx does notx ferment citrate (Croxen and Finlay, 2010). *E. coli* isx typed accordingx to their somatic lipopolysaccharide (O), capsular (K) and Flagella

(K) antigens. The O:H combination is referred to as serotype. *E. coli* O15; H7 is one of the major serotypes implicated in enterohaemorrhagic *E. coli* infections (Debroy *et al.,* 2011).

* + - 1. **Treatment of *E. coli* infection**

Antimicrobial agents such as β-lactams, fluoroquinolones, aminoglycosides and sulfamethoxazole- trimethoprim are used to treat *E. coli* infectionsx (Pitout, 2012).

* + - 1. **Antibacterialx resistance to *E.coli***

*Escherichia coli* have become resistant to β-lactam antibiotics due to its outer membrane barrier. The production of β-lactamase isx the mostx importantx mediator of resistance to broad spectrum of β-lactamsx (Johnsonx *et al.,* 2013). β-lactamases constitute different class of enzymes, which are often encoded on plasmids andx are most commonly produced by *Enterobacteriaceae.* β-lactamases confer resistance to penicillinsx and cephalosporins andx are the major cause of multidrug resistance in Gram-negative bacteria (Johnson *et al.,* 2013).

The up-regulation of efflux pumps and plasmidx mediated resistance mechanisms reduce fluoroquinolone susceptibilities in *E. coli*. Resistance to fluoroquinolones is characterised

by 1-2 point mutationx withinx the quinolone resistant determining regions of genes encodingx for DNA gyrase andx topoisomerase (Johnson *et al.,* 2013).

# Antibacterial Resistance to infections

Antibiotic drug-resistant bacteria refers to bacteria with the ability to grow or survive in a concentration of antibiotic drug thatx is normally sufficient to be bactericidal or bacteriostatic (Sabtu *et al.,* 2015). Antibiotic drug resistance may be innate or acquiredx through exposure of the bacteria to the antibiotic. Conjugation, transduction andx transformation are the geneticx mechanismsx used by bacteria to acquire antibiotic-resistant genes (Sabtu *et al.,* 2015). Resistant bacteria from animals can infect humans by direct contact or through foodx products of animal origin. There is an increase in multi-resistantx strains due to the spread of genes suchx as plasmids, integrons and transposons which combine with chromosomally encoded resistance genes. Bacteria that have been exposedx to low doses of these antibiotics in tissues and products from animals may be less susceptible to drugs therefore, when such bacteria invadesx the human body throughx consumptionx of contaminatedx foods, they may cause infections that are resistant tox many antibioticsx (Clauben *et al.,* 2013).

The emergence of major foodx borne pathogens such as *Salmonella* and *Escherichia coli* have persistedx as a major public health concernsx andx provide evidence of persistence of foodx borne pathogens despite considerable efforts aimed at prevention and control (Newell *et al.,* 2010). Thex resistant strains of *Salmonella and E. coli* is mainly promotedx by the use of antibiotics in animal feed to promote the growth of food animals, and in veterinary medicine to treat bacterial infections in those animals (Hyeon *et al.,* 2011). The irrational use of antibiotics in food producingx animals could resultx into antibiotic residues in edible tissues and products (Darwish *et al.,* 2013). The rise inx antibiotic resistant

pathogens has led to the developmentx of medicinal plants as an alternative methodx tox control pathogenicx microorganisms.

# x Phytochemical Components

Phytochemicals is obtained (from the Greek word phyto, meaningx plant). These are biologically active, naturally occurring chemical compounds found inx plants, which provide health benefits for man (Hasler andx Blumberg, 1999). They also protect plant cells from environmental hazards such asx pollution, stress, drought, UV exposure andx pathogenic attack. These phytochemicals contribute to the plant’sx color, aroma and flavor (Gibson *et al*., 1998). A wide range of dietary phytochemicals are found in fruits, vegetables, legumes, wholex grains, nuts, seeds, fungi, herbs and spices (Mathai, 2000). Phytochemicals are foundx inx differentx parts of thex plants, such as in the roots, stems, leaves, flowers, fruits and seedsx (Kochie *et al*., 2010).

Phytochemicals are also known as secondary plant metabolites which possses biological properties such as antioxidant activity and antimicrobial effect. Current researches have shown that many phytochemicals are used to preventx certain diseases in humans (Kochie *et al*., 2010). Phytochemicals are classified as primary or secondary constituents, dependingx onx their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines andx pyrimidinesx of nucleic acids andx chlorophyll’s. Secondary constituentsx are plant substances such as alkaloids, terpenes, flavonoids, plant steroids, saponins, phenolics, and glucosides (Ramawat *et. al.,* 2009). The following are classes of phytochemical compounds.

# Classification of phytochemicals

# Phenolic contents

Phenolicx compounds are large and complex group of chemical constituentsx found in plants (Dai and Mumper, 2010). Phenolics posses several beneficial properties to humans and its antioxidantx properties serve as protective role against free radical-mediatedx disease processes. There are three (3) most importantx groups of dietary phenolicsx which are flavonoids, phenolic acids, andx polyphenols.

# Phenolic acids

These are phenolic compounds that contain one carboxylic acid functional group. Naturally occurringx phenolic acids containx two diferentx carbon frameworks which are the hydroxycinnamic and hydroxybenzoic structures. Hydroxycinnamic acid compounds are produced as simple esters with glucose or hydroxy carboxylic acids. Plantx phenolic compounds are different in molecular structure, and are characterizedx by hydroxylated aromatic rings (Mandal *et al*., 2010).

# Flavonoids

Flavonoids are polyphenolic compounds that are abundant inx nature. About 4,000 flavonoids have been knownx to occur in nature and are found inx vegetables, fruits and beverages like tea, coffee and fruit drinksx (Piero *et al*., 2015). Flavoinoids are common in parts of plants normally consumed by humans whichx include approximately 650 flavones and 1030 flavanols (Piero *et al*., 2015).

Most flavonoids occur naturally in combination with sugar moiety which can be characterized as monoglycosidic or diglycosidic. The glycosidicx linkage is usually located at positionx 3 or 7 and the carbohydrate unitx can be L-rhamnose, Dglucose, glucorhamnose, galactose or arabinose (Nyanmai *et al*., 2015).

# Tannin contents

Tannins are naturally occuringx substances that containx diverse oligomers andx polymers (Harborne, 1999). They are heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible andx irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin,), alkaloids, nucleic acids andx minerals (Kar, 2007). Based on their structural characteristics, tannins are dividedx into four (4) major groups: Gallotannins, ellagitannins, complex tannins, and condensedx tannins (Kar, 2007).

1. Gallotannins are all those tannins in which galloyl units or their meta-depsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units.
2. Ellagitannins are those tannins inx which at least two galloyl unitsx are C–C coupled to each other, and do not contain a glycosidically linked catechin unit.
3. Complex tannins are tannins in which a catechin unitx is boundx glycosidically to a gallotannin or an ellagitannin unit.
4. Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin.

# Alkaloid contents

Alkaloids are natural product which contains heterocyclic nitrogen atomsx thatx are basic inx character. The name of alkaloidsx is derived from thex word “alkaline” and it is used to describe nitrogen-containingx base. The basicity of alkaloids varies depending on the structure of the molecule, presence and location of the functional groups (Sarker & Nahar, 2007). Alkaloids are grouped based on the type of heterocyclic ring system present in the molecule .Alkaloids are named based on the plants they are derivedx fromx or from their characteristic physiological activity. Mostx alkaloids are readily soluble in alcohol, although they are sparingly soluble in water, their salts of are usually soluble

(Firn, 2010). There are variousx classes of alkaloids according to heterocyclicx ringx system. They are; pyrrolidine alkaloids, pyridine alkaloids, pyrrolidine-pyridine alkaloids, pyridine-piperidine alkaloids, quinoline alkaloids and isoquinoline alkaloids.

# x Terpenoid contents

Terpenoids are a class of natural products derivedx from five-carbon isoprene units. Most of the terpenoidsx have multi cyclic structures that differ from one another by their functional groupsx and basic carbon skeletons. They are found in living things, and therefore considered as thex largest group of natural productsx (Firn, 2010). Terpenoidsx are usedx as flavours and fragrances inx foods andx cosmetics examples menthol and sclareol. Terpenes are present in plants as constituents of essential oils. Their building blockx isx the hydrocarbon isoprene, CH2=C (CH3)-CH=CH2. Terpene hydrocarbons therefore have molecular formula (C5H8) n and they are classified according to the number of isoprene units (Martinez *et al*., 2008). They are hemiterpenoids, monoterpenoids: sesquiterpenes, diterpenes, Triterpenes and Tetraterpenoids.

# Saponin contents

Saponinsx are a group of secondary metabolites foundx inx plantx kingdom. The term saponin isx derived from Saponaria vaccaria (Quillaja saponaria), a plant, whichx is abundantx in saponins and was once used as soapx (Kar, 2007). Saponins therefore produce foam in water. On hydrolysis, an aglycone isx produced, which is called sapogenin. There are two types of sapogenin: steroidal andx triterpenoidal. Thex sugar moiety is attached to C-3 in saponins, because in most sapogenins there is a hydroxylgroup at C-3. There are two major groups of saponins which are; steroidx saponins and triterpene saponins. Saponins are soluble in water and insoluble in ether, and like glycosides on hydrolysis, they yieldx aglycones. They are mostly amorphous in nature, soluble in alcohol and water, but insoluble inx non-polar organic solvents like benzene andx n-hexane (Kar, 2007).

# Medicinal properties of phytochemicals Phenolics

Phenolic compounds have been subjected to Agricultural, biological, chemical and medical studies. Results have shown that phenolic compound posses antioxidant activities which can be used in processed foods as antioxidants. Therefore, the biological activity of these compounds is related to their antioxidant property (Lafay and Gil- Izquierdo, 2008). Phenolics acid have been found to possesses diverse biological activities such as antiulcer, anti- inflammatory, antioxidant (Silva *et al*., 2007), cytotoxic and antitumor, antispasmodic, and antidepressant activities (Ghasemzadeh *et al*., 2010). Flavonoids are group of phenolics with various therapeutic values. Flavonoids have been reported to posses antihyperglycemic effect (Muriithi *et al*., 2015). Flavonoids have been recognised due to their broad biological and pharmacological activities including anti- microbial, cytotoxic, anti-inflammatory and anti-tumor activities. The most prominent property of the flavonoid family is antioxidant property (Shirsat *et. al.,* 2012; Teiten *et. al.,* 2013) which protects humans from oxidative stress damage.

# Tannins

In Asian medicine, plants containing tannins are used as astringents, diuretics and in treating diarrhoea, as well as stomach and duodenal tumours (Dolara *et al*., 2005). They are also used as anti- inflammatory, antiseptic and antioxidant (Dolara *et al*., 2005).

# Alkaloids

Alkaloids are important in protection and survival of plants. They ensure protection against micro-organisms (such as bacteria and fungi), insects and herbivores (feeding deterrens) and also against other plants by production of allelopathic chemicals (Madziga *et al*., 2010). Alkaloids have many pharmacological activities including antihypertensive effects (many indole alkaloids), antiarrhythmic effect (quinidine), antimalarial activity

(quinine), and anticancer actions (vincristine, vinblastine). Some alkaloids are used as CNS stimulant such as caffeine and nicotine, they also posses analgesic activity as seen in morphine (Madziga *et al*., 2010).

# Terpenoids

Teroenoids have medicinal properties such as anticarcinogenic (example, taxol), antimalarial (example, artemisinin). Sesquiterpene lactones have been isolated and found to posses antimicrobial (particularly antiprotozoal) and neurotoxic action (Degenhardt, 2003).

# Saponins

Saponinsx are important for therapeutic purposes and have shown hypolipidemic and anticancer activity (Sarker and Nahar, 2007). The twox major types of steroidal sapogenin are diosgenin and hecogenin. Steroidal saponins arex used in production of sex hormones for clinical use such as progesterone is derived from diosgenin. The most abundant startingx material for the synthesis of progesterone is diosgenin isolatedx from Dioscorea species. Steroidal hormones, such as cortisone and hydrocortisone, can be prepared from the starting material hecogenin, whichx is isolated fromx Sisal leaves (Sarker and Nahar, 2007).

# Extraction of Plant Materials

Extractionx refers to the separationx of medicinally activex portionsx of plant or animal tissues from the inactive or inert components by using selective solvents using standardx extraction procedures (Azwanida, 2015). The main purpose of extraction isx to separate the soluble plant metabolites, leaving behind the insoluble cellular marc (residue). The crude extractsx obtained contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids and flavonoids (Sasidharan *et al*., 2011).

Some of these crude extracts may be ready for use asx medicinal agents in the form of tinctures and fluidx extracts but some need to be further processed andx incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities (Rungsung *et al*., 2015). Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug. The commonx extraction methods include;

# Types of extraction methods

# Maceration method

Maceration involved soaking plant materials (coarse or powdered) inx a stoppered container with a solvent and allowedx to stand at room temperature for a periodx of minimum 3 days withx frequentx agitationx (Bimakr, 2010). This process is intended to soften and break the plant’s cell wall to release the soluble phytochemicals. After 3 days, the mixture is pressed or strainedx by filtration. This method is best suitable for use in case of the thermolabile drugs (Bimakr, 2010).

# Infusion method

This is an extraction process similar to maceration. The drug material is grinded into fine powder, and then placed in ax container. The extraction solvent hot or cold is then poured onx the drug material, soaked, and kept for ax shortx period of time (Pandey and Tripathi, 2014; Azwanida, 2015; Majekodunmi, 2015; Ingle *et al*., 2017). This method is suitable for extraction of bioactive constituentsx that are readily soluble. This method is employed for preparation of fresh extract before use. The solvent to sample ratio is usually 4:1 or 16:1 depending on the intendedx use (Pandey and Tripathi, 2014; Azwanida, 2015; Majekodunmi, 2015; Ingle *et al*., 2017).

# Digestion method

This extraction methodx involves the use of moderate heat during extraction. The solvent used for the extraction is poured into a clean container followed by powdered drugx material. The mixture isx placedx over water bath or inx an oven atx ax temperature about 50 ºC (Pandey and Tripathi, 2014; Majekodunmi, 2015; Ingle *et al*., 2017). Heat is required throughout the extractionx process to decrease the viscosity of extraction solvent andx enhance the removal of secondary metabolites. This method is suitable for plant materials that are readily soluble (Pandey and Tripathi, 2014; Majekodunmi, 2015; Ingle *et al*., 2017).

# Decoction method

This process involves continuous hot extraction using specified volume of water as a solvent. A dried, grinded, and powdered plant material is placed intox a clean container. Water is then poured and stirred. Heat isx then applied throughout the processx tox hasten the extractionx (Pandey and Tripathi, 2014; Azwanida, 2015; Majekodunmi, 2015; Ingle *et al*., 2017). The process isx lastedx for ax shortx duration usually about 15min. The ratio of solventx to crude drugx is usually 4:1 or 16:1. It is used for extraction of water soluble and heat stable plant material (Pandey and Tripathi, 2014; Azwanida, 2015; Majekodunmi, 2015; Ingle *et al*., 2017).

# Percolation method

This involves use of a percolator which is a narrow-cone-shaped glass vessel with opening at both ends. A dried, grinded, and finely powdered plant material is moistened with the solvent for extraction. Solventx is then added, and the mixture is keptx for a periodx of 4h. Subsequently, the content is then transferred into the percolator with the lower endx closed andx allow to stand for a periodx of 24 hx (Azwanida, 2015; Pandey andx Tripathi, 2014; Majekodunmi, 2015). The solvent of extraction is then poured from the top until

the drug material is completely saturated. The lower partx of the percolator is then opened, and the liquid allowedx to drop slowly. A little quantity of solvent is added continuously, and the extraction takes place by gravitational force, pushing the solvent through the drugx material downward (Azwanida, 2015; Pandey and Tripathi, 2014; Majekodunmi, 2015). The addition of solvent isx stopped whenx the volume of solvent added reaches 75 % of the intended quantity of the entire preparations. The extract is separated by filtration followedx by decantation. The marc is then expressed and final amount of solventx added to get requiredx volume (Azwanida, 2015; Pandey and Tripathi, 2014; Majekodunmi, 2015).

# Soxhlet extraction or hot continuous extraction method

This method involves the use of apparatus called Soxhlet extractor made up of glass. It consists of a round bottom flask, extraction chamber, siphon tube, and condenser at the top. A dried, grinded, and finely powdered plant material is placed inside porous bag (thimble) made up of a clean cloth or strong filter paper and tightly closed (Doughari, 2012; Hossain *et al*., 2014). The extraction solvent is poured into the bottom flask, followed by the thimble into the extraction chamber. The solvent is then heated from the bottom flask, evaporates, and passes through the condenser where it condenses and flow down to the extraction chamber and extracts the drug. When the level of solvent in the extraction chamber reaches the top of the siphon, the solvent and the extracted plant material flow back to the flask (Doughari, 2012; Hossain *et al*., 2014). The entire process continues repeatedly until the drug is completely extracted, a point when a solvent flowing from extraction chamber does not leave any residue behind. This method is suitable for plant material which is partially soluble in the chosen solvent and for plant materials with insoluble impurities. However, it is not a suitable method for thermolabile plant materials.

Advantages. Large amountx of drug can be extractedx with smaller amountx of solvent. It is also applicable to plant materials that are heat stable. No filtration isx required, and high amount of heat could be applied (Doughari, 2012; Hossain *et al*., 2014). Disadvantage; This methodx is not suitable for thermolabile materials. Itx leads tox exposure to hazardous liquidx organic solvents, withx potential toxic emissions duringx extraction (Doughari, 2012; Hossain *et al*., 2014).

# Microwave-assisted extraction method

This is an advanced extraction procedure in preparation of medicinal plants. This technique uses mechanism of dipole rotation and ionicx transfer by displacementx of charged ions present inx the solvent and drug material. This method is suitable for extractionx of flavonoids. It involves thex application of electromagnetic radiation inx frequencies between 300 MHz and 300 GHz and wavelengthx between 1cmx and 1 m (Doughari, 2012; Altemimi *et al*., 2017; Ingle *et al* ., 2017). The microwavesx applied at frequency of 2450 Hz yield energy between 600 and 700 W. The technique uses microwave radiation to bombard an object, which can absorb electromagneticx energy and convert it intox heat. The heat produced facilitates movement of solvent into the drug matrix (Doughari, 2012; Altemimi *et al*., 2017; Ingle *et al.,* 2017). When polar solvent is used, dipole rotation and migration of ions occur, leading to increase solvent penetration and thisx assist extractionx process. However, when nonpolar solvent is used, the microwave radiation released will produce only ax little heatx hence, this method does notx favor use of nonpolar solvents (Doughari, 2012; Altemimi *et al*., 2017; Ingle *et al*., 2017). Advantages; Itx minimizes solvent and time of extraction as well as increase in the outcome (Doughari, 2012; Altemimi *et al*., 2017; Bhan, 2017; Ingle *et al*., 2017). Disadvantages; This method is suitable only for phenolic compoundsx and flavonoids.

Compoundsx such as tanninsx and anthocyanins may be degradedx due to high temperature (Doughari, 2012; Altemimi *et al*., 2017; Ingle *et al*., 2017).

# Ultrasound-assisted extraction method

This process involvesx applicationx of sound energy at a very highx frequency greater than 20x KHz to disrupt plant cell all and increase the drugx surface area for solvent penetration leading to release of secondary metabolites. Plant materials are dried, grinded into fine power, andx sieved properly. The prepared sample is then mixed with and appropriate solvent of extraction and packed into the ultrasonic extractor (Pandey and Tripathi, 2014; Azwanida, 2015; Altemimi *et al*., 2017).The high sound energy applied hasten the extractionx process by reducing the heat. Advantages; Ultrasound-assisted extraction is applicable to small sample, it reduces the time of extraction andx amount of solvent used, and maximizes the yield (Pandey and Tripathi, 2014; Azwanida, 2015; Altemimi *et al*., 2017). Disadvantages. Thisx method is difficult to be reproduced; also, high amount of energy applied may degrade the phytochemical by producing free radical (Pandey and Tripathi, 2014; Azwanida, 2015; Altemimi *et al*., 2017).

# Factors to be considered in choosing extraction method

(a) Stability to heat. Heat-stable plant material are extracted using Soxhlet extraction or microwave-assisted extraction, whereas plant materials thatx are not heat stable are extractedx using maceration or percolationx (Azwanida, 2015; Majekodunmi, 2015). (b) Nature of solvent. If the solvent of extractionx is water, maceration is a suitable methodx but for volatile solvent percolation and Soxhlet extraction are more appropriate (Azwanida, 2015; Majekodunmi, 2015). (c)x Costx of the drug. Cheap drugs are extracted usingx maceration, whereas costly drugs are preferably extracted usingx percolation (Azwanida, 2015; Majekodunmi, 2015). (d) Durationx of extraction. Maceration is suitable for plant material requiringx longx exposure to the menstruum, whereas techniques such as

microwave- or ultrasound-assisted extractionx are used for a shorter duration (Azwanida, 2015; Majekodunmi, 2015). (e) Final volume required. Large volume products such as tinctures are prepared by maceration, whereas concentrated productsx are produced by percolation or Soxhlet extraction (Azwanida, 2015; Majekodunmi, 2015). (f) Intended use. Extracts intended for consumptionx by human are usually prepared by maceration, whereas products intended for experimental testing are prepared using other methods in addition to maceration (Azwanida, 2015; Majekodunmi, 2015)

# Solvents used in extraction

The solvent used for the extraction of medicinal plants is known as the menstruum. The choice of solvent depends onx factors such as, the type of plant, part of plant to be extracted, nature of the bioactive compounds, and the availability of solvent. Polar solvents such asx water, methanol, andx ethanol are used inx extraction of polar compound, whereas nonpolar solvents such as hexane andx dichloromethane are used in extraction of nonpolar compounds (Sasidharan *et al*., 2011; Pandey andx Tripathy, 2014; Altemimi *et al*., 2017). Duringx liquid–liquid extraction, the conventional method isx to select two miscible solvents such as water dichloromethane, water–ether, and water–hexane. In all the combinations, water isx present because of its high polarity and miscibility with organic solvent. The compound tox be extractedx using liquid–liquid extractionx should be soluble inx organic solvent but notx in water to ease separation (Majekodunmi, 2015). Solvents usedx in extraction are classified according to their polarity, from n-hexane which isx the least polar to water the most polar (Sasidharan *et al*., 2011; Pandey and Tripathy, 2014; Altemimi *et al*., 2017).

Fractionationx is a process of separation of plant extracts into various fractions. It further segregates the fractions into portions comprising a number of compounds. The process continues until pure compound is isolated (Doughari, 2012; Banu and Lugas, 2015).

During fractionation, the selected solvent is added according to the order of increasing polarity, startingx fromx n-hexane, the leastx polar to water with the highest polarity ( Pandey and Tripathy, 2014).

# Factors to be considered in selecting solvents of extraction

i) Selectivity; the ability of a chosen solvent to extract the active constituent and leave the inertx material. (ii) Safety; ideal solvent of extraction shouldx be nontoxic andx nonflammable. (iii) Cost; it should be as cheap as possible. (iv)x Reactivity; suitable solvent of extraction should not react with the extract. (v) Recovery; the solvent of extractionx should be quickly recovered and separated from the extract. (vi) Viscosity; itx shouldx be of low viscosity tox allow ease of penetration. (vii) Boiling temperature; solvent boilingx temperature shouldx be as low as possible to prevent degradation by heat (Sasidharan *et al*., 2017; Pandey and Tripathy, 2014).

# Toxicity Studies

Toxicity studies are carried out for the purpose of evaluation of toxic effects of therapeutic agents or potential toxicants that could pose threatx to the lives of humans andx animals. The traditional methods of determiningx toxic effects of chemicals and drugs include acute toxicity study which is carried out to determine the short term toxic effect of a toxicant from (1day to 2 weeks), subacute toxicity study is carried out to know the relative longx term effect of a toxicantx (4 weeks- 6 months)x and Chronic toxicity studies are carried out to know the longx term effect of a toxicantx (1–1½ years) (Saganuwan, 2012).

# Acute Systemic Toxicity

Acute systemic toxicity isx used to evaluate the adverse effects that occur following exposure of organisms to a single or multiple doses of a test substance within 24 hoursx by

a knownx route (oral, dermal or inhalation) (Saganuwa, 2016). After administration, the test substance is absorbed and distrib¬uted to various parts of the body before it elicits systemic adverse effect. The regulatory body requires the acute toxicity test report for labeling and classification of substances for humanx use ( Peers *et al*., 2012). The LD50 test was introduced by Trevan in 1927 and used to estimate the dose of a testx substance thatx produces 50 % death in a given species of animals.

This is the firstx testx conducted for every chemical before further toxicity tests are carried out. It is used to estimate the potential hazards of chemicalsx on humans. Although its major endpointx is death, non-lethal acute effectx may occur as signs of toxicity dependingx onx the chemical beingx tested (Maheshwari and Shaikh, 2016). Acute toxicity determines adverse effects that might occur due tox accidental or deliberate short-termx exposure (Clemedsonx *et al*., 2000).

Therefore, substances with LD50 below 5 mg/ kg are classified to be highly toxic while substances with LD50 above 15,000 mg/kg are termedx relatively harmless (Loomis andx Hayes, 1996).

# Lorkes method of acute toxicity

This method was introduced inx 1983 andx it involvesx the use of thirteen animals inx 2 phases. In the first phase, nine animals are dividedx into threex groups of three animals each and are administered 10, 100 and 1,000 mg/kg body weight of the test substance in order to establishx the dose range producing any toxic effect. The number of deaths in each group is recorded after 24 hours. In the second phase, four doses of the test substance are selected basedx on the result of phase 1 and are administered to four (4) groups of animals (one animal per group). After 24 hours, the number of deaths is recorded and the LD50 is

calculated as the geometricx mean of the highest non-lethal dose (a) and the least toxicx dose (b). LD50 = √a×b (Lorke, 1983; Enegide *et al*., 2013).

# Limit Test for acutex toxicity

The Limit testx is a sequential test that uses a maximum of 5 animals. A testx dose of 2000, or exceptionally 5000 mg/kg, may be used. The procedures for testing at 2000 and 5000 mg/kg are slightly different. For limit testx at 2000 mg/kg, one animal is dosed at 2000 mg/kg and observedx for mortality, if animal dies, the main test is then carried out. If the animal survives, four additional animals will be dosed sequentially so that a total of five animals are tested. However, if three animals die, the limitx test is terminated and the main test isx performed. The LD50 is therefore, greater than 2000 mg/kg if three or more animals survive andx the LD50 is less than the test dose (2000 mg/kg) when three or more animals die (OECD, 2001). For limitx test at 5000 mg/kg, a dose range of 2000-5000 mg/kg is used exceptionally and only when justified by specific regulatory needs and this shouldx only be considered when there is a strong likelihoodx that results of suchx ax test have a direct relevance for protectingx human or animal health or the environment. Onex animal isx dosedx at 5000 mg/kg, if the animal dies, the main testx is conductedx to determinex the LD50. If the animal survives, two additional animals arex dosed. If both animals survive, the LD50 is greater thanx the limit dose and the test is terminated (OECD, 2001).

# Subacute toxicity

The subacute toxicity study is conducted for 28 days to examinex the toxicity onx organs (OECD, 2001). This isx carried out in order to choose dosage levels to be used in sub- chronic andx chronic toxicity studies. animals are grouped based on experimental design and variousx concentrations of the test substance is administered. Control groups are included Group 1 could served as control (normal saline) while Groupsx 2 to 6 can be

administreredx 400, 600, 800, 1000 and 1200 mg/kg body weight of the test substance respectively. The animals are thenx observedx every 2 hours for toxic symptoms, signs of poisoning and mortality over a period of 30x days. Animals are then euthanised and organs are assessedx (Yeo *et al*., 2012).

# Subchronic toxicity Studies

The test isx conducted for a period of 90 days (3 months). In thisx type of study, subchronicity factor gives an indication of the cumulative effects of poisons. Itx is the ratio of acute LD50 to 90 days LD50. A compound though may have low acute toxicity, but has the tendency to accumulate inx the body tissues and can cause subacute or chronic toxicity. Suchx toxicants are termedx as cumulative poisons such as lead and fluoride (Saganuwan, 2012). A satellite group may be included inx the study protocol, and this group has both a control group and a high dose group (Muralidhara *et al*., 2010). Tissuesx are collected for gross pathology and histopathology.

# Chronicx toxicity studies

It is a long term toxicity study that lasts for a long period of time usually 1–2 years. Rodents such as mice and rats are usually used. Chronicity factor gives an indication of the cumulative effects of poisons. It is the ratio of acute LD50 to 1–2 years LD50. This type of test can be conducted on drugs developed for terminal diseases such as cancers andx AIDs (Saganuwan, 2012). A satellite group may be included in the study protocol. This group has both a control group and a high-dose group. The animals are observedx for normal and abnormal body functions and biochemical parametersx should be measured. Tissue are collected for gross andx histopathology (Jaijoy *et al*., 2010). Carcinogenicity testingx is under chronic toxicity testing in which both rodent and non-rodent species of animals are used. The testx can be terminated after 1½x years inx case of mice andx hamsters and after 2 years inx case of rats. Haematological analysis is performed in healthy animals

after 1 and 1½ years inx mice andx rats respectively and the study is terminated. The animals are euthanised for gross pathology and histopathology (Saganuwan, 2012).

# CHAPTER THREE

# METHODS

# Collection of plant material

The *Helianthus annuus* seeds were collected from its habitat in Kwara State. It was then transported to Biological Sciences Department, Federal University of Technology Minna for identification and authentication by a Herbarium.

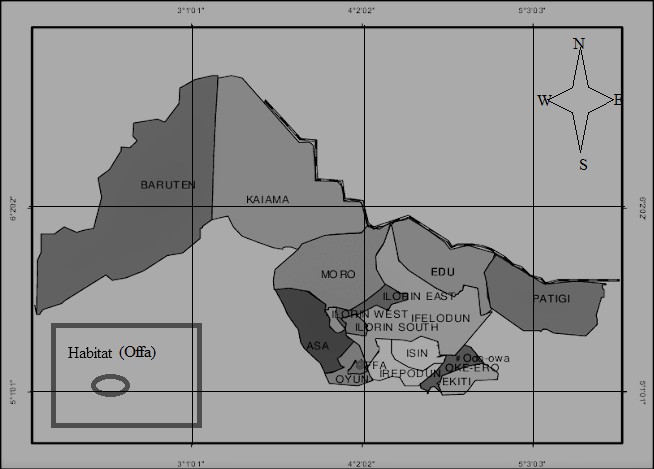


Figure 5: Map of Kwara state showing Offa as the Habitat Source: (Oye, 2008).

# Experimental animals and management

Male rats with body weight between (108-190 g) were obtained from a commercial breeder in Jos and transported. Animals were housed in the animal house of Federal University of Technology Minna in a temperature-controlled room under 12 hours light and 12 hours dark cycle. Animals had free access to food and water and were acclimatized for a week prior to experiments.

# Sources of Microorganisms

The clinical bacteria isolates used in this study were *Escherichia coli* and *Salmonella spp.* These isolates were obtained from Generel Hospital Minna after acquiring ethical approval from the hospital. Nutrient Broth was used to confirm the organisms by sub- culturing at 37 °C for 18 hours. The organisms were maintained on Nutrient Agar (NA) slant and refrigerated at 4 ˚C (Igbinosa *et al.,* 2009).

# Identification of the Isolates

* + 1. **Gram staining**

A clean glass slide was picked, a drop of normal saline was dropped on the clean glass slide, a loop full of colonies was picked from the nutrients agar culture and was emulsified with the normal saline to form a smear. The smear was dried by using heat fixing method, crystal violet reagent was added to the smear and was allowed for one minute and then, the crystal violet was rinsed with water. Grams iodine solution was added to the smear for a minute and rinsed off with water. Alcohol was added in drops for 25 seconds which serves as decolourizer and was rinsed with water. Safranin solution was added to the smear which serves as counter stain and was allowed for one minute, the safranin solution was rinsed and the smear was allowed to dry (Smith and Hussey, 2016).

# Microscopic Examination

During the microscopical viewing, the colony that appeareddark purple in colour were considered as Gram positive organism while those that appears pale to dark red in colour were considered as Gram negative organism, also their cellular morphological shape was noted (Smith and Hussey, 2016).

# Biochemical test

* + - 1. **Indole test**

A loop full of the colonies from the Nutrient Agar plate was picked and inoculated into a pepton water broth. The broth was incubated for five days at 37 0C. After the incubation period, three drops of kovacs reagent was added into the broth and was shook gently. The presence of the red or pinkish ring top layer indicates the organism to be Indole positive while the absence of it indicates the organism to be indole negative

# Methyl Red (MR) test

A loop full of the colonies from the Nutrient Agar plate was picked and inoculated into the MR-VP broth. The broth was incubated for five days at 37 0C. After the incubation periods a methyl-red reagent was added to the tube and it was observed for change in colour. When the culture medium turns red after addition of methyl red the organism is MR Positive whilst when the culture medium remains yellow the organism is MR Negative

# Catalase test

A drop of hydrogen peroxide was dropped in a clean glass slide a colony was picked from Nutrient agar and was emulsify on the slide, It was observed for bubble formation within 5 seconds. Formation of bubbles within five seconds indicates the organism to be catalase positive whilst absent of the bubbles indicate the organism to be catalase negative

# Oxidase test

A piece of filter paper was placed on a clean a clean petri dish and a 2-3 drops of oxidase reagent (tetramethyl-p- phenylenediamine dihydrochloride). The colonies were picked with a sterile stick and emulsified on the surface of the filter paper. Development of a blue-purple colour within a few seconds ranges from 10-30 seconds indicate the organism to be oxidase positive as whilst absent of such show the organism to be oxidase negative

# Motility test

Semi agar medium was produced using nutrient broth and agar in a test tube. The organism was stabilized at the center of the media in the test tube using a sterile wire loop. The tube was incubated at 37 °C for 2-7 days within routine observation. Observation of diffused hazy growth that spread throughout the media makes the organism to be motile whist the conferment of growth within the stab line makes the organism to be non-motile.

# Urease test

Urea agar slant was prepared in a sterile bottle and the test organism was inoculated heavily on the slant surface. The cap of the bottle was left partly loose and the slant was incubated for 5-7 days at 37 °C Development of pink colour within the 7 days incubation period termed the organism to be urease positive whilst absent of pink colour within the incubation period makes the organism to be urease negative.

# Preparation of plant sample

The *Helianthus annuus* seeds were air dried at room temperature for two weeks. The dried seeds were milled to powder and air dried at room temperature for one week.

# Extraction

The method of Shankeshwari *et al.* (2018) was employed to prepare the extracts by cold maceration with slight modification. The powdered seed material 500 g was mixed with 2500 mL in the ratio (1:5 w: v) of 99 % methanol and 99 % ethanol respectively and kept in a conical flask (Hossain *et al.,* 2013). The mixture was stirred thoroughly with a glass rod. The conical flask was kept with intermittent shaking for 72 h. The mixtures were then filtered using Whatman No.1 filter paper. The methanolic and ethanolic filterate were then concentrated by using rotary evaporator and then allowed to dry at room temperature for two weeks. The extracts were then weighed and stored in closed containers at room temperature.

The method of Hossain *et al.* (2014) was employed in fractionation of the methanol crude extract. The methanol crude extract (20 g) was suspended in water (400 mL). Then it was extracted successively with different organic solvents which are hexane and ethyl acetate to obtain hexane, ethyl acetate and residual methanol aqueous fractions respectively. All extracts were filtered separately through Whatman No. 41 filter paper to remove particles. The particle free extracts were evaporated completely at room temperature to obtain dried extracts. The dried extracts were weighed and kept in sterile sample bottles and stored in the refrigerator at 4 °C for further use.

# Phytochemical Screening

Qualitative and quantitative phytochemical tests of each of the seeds extract was carried out to identify the constituents. Standard procedures were followed to detect the presence of Tannins, saponins, alkaloids, phenols and flavonoids (Trease and Evans, 1989; Sofowara, 1993).

# Qualitative phytochemical screeningof *H. annuus* extract

1. **Saponin**

Two milliliters (2mL) of water was added to 0.5 g of the extract. It was shaked and observed for persistence in the foam produced for 10 minutes.

# Tannins

Twenty milliliters (20mL) of water was added to the dried powdered sample 0.5 g in a test tube and then filtered. Few drops of 0.1% ferric chloride was added and observed for brownish green or a blue- black colour indicating the presence of tannins.

# Phenols

Ferric Chloride Test: extracts were treated with 3-4 drops of Ferric Chloride solution and observed for the formation of a bluish black colour.

# Flavonoid

Extracts were treated with few drops of sodium hydroxide solution and observed for the formation of an intense yellow colour that turned colourless on addition of diluted acid.

# Alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered, the filtrates of the extractwastreated with Mayer’s reagent (Potassium Mercuric iodide) and observed for the formation of yellow precipitate.

# Diterpenes

Copper acetate test**:** Extracts were dissolved in water and treated with 3-4 drops of Copper acetate solution and observed for the formation of an emerald green colour.

# Anthraquinones

One millilitre (1 mL) of the extract was dissolved in 10 mL of benzene in a test tube. The mixture was filtered and to the filtrate, 5 mL of 10 % Ammonia was added, shaked and observed for the appearance of a pink red/violet colour in the ammonia phase.

# Cardiac glycosides

Five millilitres (5mL) of each extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution with 1ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, whole in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

# Quantitative phytochemical screeningof *H. annuus* extract

1. **Saponin determination**

The method as described by Harbone (1999) wasx used. Twenty grams of each plant sample was weighedx intox a conical flask andx 100 cm3 of 20 % aqueous ethanol were added. The samples were heated over a hot water bath for 4 hour with continuous stirring at 55 oC. The mixture was filteredx and the residue re-extractedx withx another 200 ml 20 % ethanol. The combined extracts were reducedx tox 40ml over water bath at about 90 oC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer recovered while the ether layer was discarded. The processx was repeated. 50 ml of n-butanol extracts were washedx twice with 10 ml of 5 % aqueous sodium chloride. The remaining solution was heated on a water bath. After evaporation the samples were dried in the oven to a constant weight, the saponin content was calculated.

# Tannin

Tanninx wasx determined usingx the method outlined by Harbone (1999). Two grams of each plant sample were defatted with 100 ml of diethyl ether usingx a Soxhlet apparatus for 2 hours. The fatx free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 minutes. Five milliliter of the extract was pipetted into a 50 ml flask, and thenx 10 ml of distilledx water was added. Two milliliter of ammoniumx hydroxide solution andx 5 ml of concentratedx amyl alcohol were also added. The samples were made up to mark and left to react for 30 minutes for colour development. Thisx was measuredx at 505 nm.

# Alkaloid

Harbone’s (1999)x method of alkaloid determinationx was used. Five grams (5 g) of the samples were weighed into 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added and covered andx allowed tox stand for 4x hours. This was filteredx and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide wasx added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washedx with dilute ammoniumx hydroxide and then filtered. The residue is the alkaloid, which was dried and weighedx (Harbone, 1999).

* 1. **Antibacterial assay of seed extracts of *Helianthus annuus***

# Reconstitution of extracts

The extracts were reconstituted with DMSO and distilled water tox get extract concentrations of 60, 120, 240 and 480 mg/mL. These concentrations were preparedx by weighing 0.3, 0.6, 1.2 and 2.4 g of each extractx and dissolved in 1 ml of DMSO andx 4x mL of distilled water.

# Preparation of media

The media wasx prepared according to the manufacturer’s instructions. Twenty-eight grams of Nutrient agar standard measurement was poured into sterile conical flask, 1 Litre of distilled water wasx added. The prepared media was cork-fittedx with cotton wool, foil paper, and masking tape. This was done to avoid removal of the cork when autoclaving. The media wasx autoclaved for 15 minutes at 121 °C. Itx was thenx removed and cooled to 47 °C. The media was mixed properly andx poured into sterile petri-dishes close to the flame.

# Standardization of inoculum

The test microorganisms (*Escherichia coli* and *Salmonella spp*) three colonies were picked, inoculated into 5 mLx of sterile nutrient broth and incubated (18-24 hours) for bacterial growth. Turbidity was adjustedx to match 0.5 McFarland’s standard (Coyle, 2005).

# Preparation of disc

The paper disc diffusionx method described by Doughari *et al.* (2007); Adebayo andx Adegoke (2008) was employed.Whatsmann filter papers (6 mm) in diameter were cut into roundx pieces withx the aidx of ax puncher to obtainx disks. Fifty (50)x pieces of the disks were transferred into labelled sterile bottles and sterilized in hot air oven at 45 ˚C for three (3) days. Five (5 mL) of each of the prepared extracts was transferred into the corresponding labelledx bottles containing sterile disks. The disks were dried inx hot air oven at 45 ˚C for 1-2 hrs. The control disk was prepared by dissolving 500 mgx of ciprofloxcin in 5 mLx of distilledx water andx transfering into a sterile bottle containingx sterile disk. The disk was dried at 45 ˚C for 1-2 hrs.

# *In vitro* evaluation of antibacterial activity

The crude extracts of *Helianthus annuus* seeds (methanol and ethanol) as well as fractions of the crude extracts were evaluated in vitro for antibacterial activity against *Escherichia coli* and *Salmonella spp.* Muellax Hinton Agar (38 g) was added to 1 Litre of distilledx water in ax conical flask andx stirred. It was then autoclaved at 121 ˚C and cooled to 50 ˚C. A portion of the mediumx (20 mL) was poured into sterile Petri dishes and allowedx to solidify (Daniyan *et al.,* 2011).

A loopful colony of each test organism was sub-cultured on Nutrient broth and incubated at 37 °C for 24 hours. This broth culture was spread on the entire plate with the aid of a swab stick to ensure uniform growth. The diskx impregnated with various concentration of the extracts were placed on the surface of the medium at four points equidistant from one another. The control disk was also placed on the surface of the medium in a different plate. The plates were allowed to stand for 2 hrs and thenx incubated for 24 hours atx 37 °C. Zones of inhibitionx were observed and measured. Antimicrobialx assays were carriedx outx in triplicate and the mean diameter was calculated.

# Minimum inhibitory concentration (MIC) of extracts

The Minimum inhibitory concentrationx (MIC) of extracts againstx the test organisms were determinedx usingx the broth dilution method as described by Daniyan *et al.* ( 2011). Using test tubes, 8 mLx of the nutrientx broth wasx dispensedx into various labelled testx tubes andx autoclavedx for 15 minutes. Usingx two (2 mL) of the initial concentration of the extracts, broth dilution was carried out in 8 foldsx and a control test tubex containing 2 mL of the extract and 8 mLx of broth was setx aside. The bacteria suspension of the test organisms diluted at 106 Cfu/ml was inoculated into each tube .The test tubes were sealedx with foil paper, cotton wool, andx masking tape andx then incubated atx 37 ℃ for 18-24 hours. The

leastx concentration of the extractx that showed lessx turbidity wasx taken as thex minimumx inhibitory concentration (Abalaka *et al.,* 2011).

# Minimum bactericidal concentration (MBC) of the extracts

The 18-24 hrs broth culture in the testx tubes used for determination of minimumx inhibitory concentration were then streaked on nutrient agar plate and incubated for 24 hrs. The lowest concentration of the extract indicating a bactericidal effect after incubation was regardedx as the Minimum Bactericidal Concentration (MBC) (Aboadax *et al.*, 2006).

# Toxicity studies

# Acute toxicity (Limit test)

The acute oral toxicity of the plant extracts was determined, usingx the method describedx by Organization of Economic and Cooperative Development OECD (2000). The limitx test was used at 2000 mg/kg. Oral route of administration was used. Animals were fasted overnight for aboutx 16 hrs prior to dosing. A group of 5 ratsx per extract receivedx oral dose of 2000 mg/kg of the extract. The treated rats were observed within 24 hrs for mortality and general behaviour. The number of dead animalsx wasx recorded and the lethal dose (LD50) was calculated usingx the formula below:

LD50x √ (D0 × D100)

Where D0 ꞊ Dosage of 0% mortality, D100 ꞊ Dosage of 100%x mortality

The animals were observed closely at 0 hr, 4 h, 24 h andx 14 days for any delayed toxicx reaction such as tremors, convulsion and irritability. The number of death was recorded.

# Subacute toxicity studies

The subacute toxicity studies was carried out according to Chung-Tack *et al.* (2015) withx slight modifications. In the subacute toxicity studies, the extracts were administered once a day for twenty eight (28) days withoutx fasting. The rats were weighed on weekly basis and dosingx was based on recently measured body weight. The rats were grouped intox four groups, each group containing four rats. Group one (1) rats were administeredx distilled water which served asx the control group. Group two (2) to four (4) were administered different concentrations of the methanol crude extract at 10 mg/kg, 300 mg/kgx and 600 mg/kg respectively. This process was repeated for the groupingx of the other three extracts (methanol-nhexane fraction, ethanolcrude and ethanol hexane fraction). All the animals were observed once daily for clinical signs and twice daily for mortality and morbidity throughout the study. Onx the twenty eight day, the rats were euthanisedx andx bloodx samples were takenx for haematological and biochemical analysis (Peter and Kwiterovich, 2004). The rats were dissected and organs (liver andx kidney) were taken for histopathological examinations (Ateeq, 2015).

# Biochemical analysis

# Determination of total cholesterol

Total cholesterol level wasx estimated by cholesterol oxidase peroxidase methodology usingx a commercial kit (AGAPE, Switzerland) with the help of UV-visible spectrophotometer at wavelength of 505 nm *in vitro* quantitative determination of cholesterol in serum or plasma. The reagent is prepared by dissolving contents of reagent 2 (R2) with the amount of reagent 1 (R1) indicated on the vial label,linear up to 500 mg/dl, extended stability reconstituted reagentx stable up to 90 days whenx stored atx 2-8 ᴼC (Peter and Kwiterovich, 2004).

# Clinical Significance

It is the main lipid found in the blood, bile and brain tissues. It is also onex of the most important steroids of the body and isx a precursor of many steroid hormones. Two thirds of cholesterol present in the blood is esterified. The liver metabolizes the cholesterol and it isx transportedx in the blood stream by lipoproteins. Increased levels are found in hypercholesterolemia, hyperlipidemia, hypothyroidism, uncontrolledx diabetes nephritic syndrome and cirrhosis. Decreased levels are found in malabsorption, malnutrition, hyperthyroidism, anaemia and liver diseases (Peter and Kwiterovich, 2004).

# Principle

Enzymatic determination of total cholesterol is based on the followingx reactions. Cholesterol ester + H O Cholesterol esterase Cholesterol + fatty acids

2

Cholesterol + O Cholesterol esterase 4-cholesten-3-one + H O

2 2 2

2H O + phenol + 4-Aminoantipyrine peroxidaseRed quinine + 4H O

2 2 2

# Laboratory Procedure

1. To each tubes Add 1000 μl of Working Reagentx to Blank, Standardx and Sample (test) tubes
2. Add 10 μl of Standard reagent to the standardx test tube
3. Add 10 μl of sample (test) to the sample test tube
4. Mix, and incubate for 5 min. at 37 °C. Measure the Absorbance of sample and standard againstx reagent blank (Peter and Kwiterovich, 2004).

# Calculation

Total Cholesterol conc. (mg/dl)

= Absorbance of sample Absorbance of Standard

x 200

Normal range:150-220 mg/dl (Peter and Kwiterovich, 2004).

# Determination of serumx triglyceride (TG)

Serum triglyceride level was estimated by GPO-TOPS (glycerol-3-phosphate oxidase) methodology using a commercial kitx (AGAPE, Switzerland) with the help of UV-visible spectrophotometer at wavelength of 546 nm *in vitro* quantitative determination of triglyceride in serum or plasma. The reagent is ready to use, linear up to 1000 mg/dL, extended stability up to the expiry date stated on the label, when stored at 2-8 °C (Peter and Kwiterovich, 2004).

# Clinicalx Significance

Triglycerides are simple lipids, formedx in the liver by glycerol and fatty acids. They are transportedx by VLDL, LDLx andx constitute about 95 % of fat, stored as source of energy inx the tissue and plasma. Increased levels are found inx hyperlipidemia, diabetes, nephritic syndrome and hypothyroidism. Increased levels are risk factor for arteriosclerotic coronary disease, peripheral vascular disease, acute pancreatitis andx hyperlipoproteinaemia. Decreased levelsx are found in malnutrition and hyperthyroidism (Peter and Kwiterovich, 2004).

# Principle

Enzymatic determination of triglyceride is based on the following reactions.

TGL + H O Lipoprotein lipase glycerol + fatty acids

2

glycerol + ATP Glycerol kinase Mg2+ x glycerol-3-phosphate + ADP

glycerol-3-phosphate oxidase

Glycerol-3-Phosphate + O2--------------------------------> Dihydroacetonephosphate + 2H2O2

peroxidase

2H2O2 + 4-Aminoantipyrine + TOPS > violet coloured complex

# Laboratory Procedure

1. Tox each tubes Add 1000 μl of Workingx Reagent to Blank, Standard and Sample (test) tubes
2. Add 10 μl of Standard reagent to the standardx test tube
3. Add 10 μl of sample (test) to the sample test tube
4. Mix andx incubate for 5 min. at 37 °C. Measure the Absorbancex of sample and standardx against reagent blank.

# Calculation

Triglyceride conc. (mg/dl)x

Triglyceride conc. (mg/dl) = Absorbance of sample x 200

x

Absorbance of Standard

Normal range:60-165 mg/dl (Peter and Kwiterovich, 2004).

# Determination of serum Low Density Lipoprotein (LDL-C) Clinical Significance

Blood total cholesterol levels have long been known to be related to coronary heartx disease (CHD). In recent years, in addition to total cholesterol, low density lipoprotein cholesterol (LDL-C) hasx become an important tool used tox assess an individual risk of developing CHD since a strong negative relationship between LDL-C concentrationx and the incidence of CHD was reported. LDLx cholesterol acts as a key factor in the pathogenesis of atherosclerosis and coronary artery disease (Peter and Kwiterovich, 2004).

# Principle

The LDL-C Direct is a homogenous assay for directly measuring LDL-C levels in serum or plasma without the need for any off-line pretreatment or centrifugation. In thex first reaction, non LDL unesterifiedx is consumed by peroxidase inx the presence of 4-AAP (aminoantipyrin) to yield ax colorless product. The second reagent consists of ax detergent capable of solubilizing LDLx specifically. Cholesterol esterase and chromogenic coupler react with this solubilizex LDL-C to develop color. The intensity of color formed is directly proportional to the concentration of LDL-C (Peter and Kwiterovich, 2004).

# Laboratory Procedure Differential measurement

1. Tox each tubes Add 270 μl of Reagent, R1 to Blank, Calibrator and Sample (test) tubes
2. Add 3 μl of Calibrator reagent to the Calibrator test tube
3. Add 3 μl of sample (test) to the sample test tube
4. Mix andx incubate for 5 min at 37 °C. measure the absorbance (OD1) at 546V nm/660 nm.
5. To each tubes Add 90 μl of Reagent, R2 tox Blank, Calibrator and Sample (test) tubes
6. Mix andx incubate for 5 min at 37 °C. measure the absorbance (OD2) atx 546 nm/660 nm.

# Calculation

LDL-C conc. (mg/dl) = (OD2 – OD1) sample

x Calibrator conc (OD2 –x OD1) calibrator

# Laboratory Procedure

1. To each tubes Add 450 μl of Reagent, R1 to Blank, Calibrator andx Sample (test) tubes
2. Add 5μl of Calibrator reagent to the Calibrator test tube
3. Add 5 μl of sample (test) tox the sample test tube
4. Mix and incubate for 5 min at 37 °C
5. To each tubes Add 150 μl of Reagent, R2 tox Blank, Calibrator and Sample (test) tubes
6. Mix and incubate for 5 min at 37 °C. measure the absorbance of calibrator and sample against reagent blank at 546 nm/660 nm (Peter and Kwiterovich, 2004).

# Calculation

LDL-C conc.= Absorbance of sample

Absorbance of calibrator

x Calibrator conc

# x Determination of Total protein

Total protein level was estimated by direct Biuret methodology using a commercial kitx (AGAPE, Switzerland) with the help of UV-visible spectrophotometer atx wavelength of 546 nm *in vitro* quantitative determination of Total Proteinx in serumx or plasma. Reagent isx ready to use, linear up to 15g/dl, extended stability reagent stable up to expiry date whenx storedx at room temperature andx standard at 2-8 °C (Peter and Kwiterovich, 2004).

# Clinicalx Significance

Proteins formx thex major portion of dissolved substances in the plasma. They form the basic structural components of the body. They constitute the enzymes present in our body and also act as secondary source of energy. The other functions include distributionx of water, buffering, transport of various components, defense and coagulation of blood in our body. Increased levels are foundx in dehydrationx andx myeloma. Decreasedx levels are

foundx in the liver disorders, nephritic syndrome, malnutritionx and protein due to haemorrhage (Peter and Kwiterovich, 2004).

# Principle

Colorimetric determination of total proteinx based on the principle of the Biuret reaction (copper salt in an alkaline medium). Protein in plasmax or serum sample forms a blue coloredx complex when treated with cupric ions in alkaline solution. The intensity of the blue color is proportional ox the protein concentration (Peter and Kwiterovich, 2004).

# Laboratory Procedure

1. To each tubes Addx 1000 μl of Reagent to Blank, Standard and Sample (test) tubes
2. Add 20 μl of Standard reagent to the standard test tube
3. Add 20 μl of sample (test) to the sample test tube
4. Mix, and incubate for 10 min. at 37 °C. Measure the Absorbance of sample andx standard against reagent blank (Peter and Kwiterovich, 2004).

# Calculation:

Total protein conc. (g/dl)= Absorbance of sample x 6

Absorbance of Standard

Normal range: 6.2-8.0 mg/dl (Peter and Kwiterovich, 2004).

# Determination of Serum Bilirubin Concentration

Biosystems diagnostic kitx (Barcelona Spain) was usedx inx the determination of total bilirubin. The working reagent for total bilirubin determinationx was prepared by transferingx the content of reagent BT vial into a reagent AT bottle followed by proper mixing.One hundred (100 µl) of distil water, serum sample and standard were pipettedx

into the respective test tubes of blank, sample blank/sample and standard. One mL (1 mL) of reagent AT was pipetted into sample bank test tube followed by addition of 1ml of workingx reagent into reagent blank, sample and standard test tubes. All the mixtures were allowedx to standx for 2 mins at room temperature after thorough mixingx before the absorbance (A) of the sample blank andx the sample were taken atx 540 nm against the distilled water and reagent blank respectvely. The bilirubin concentrationx was calculatedx asx shown below:

ASample – ASamplex Blank

Bilirubin Conc. (mg/dl) = × Standard conc.

AStandard

The Bilirubin in the serum sample reacts with diazotizedx sulfanilic acid to produce azobilirubin, which has anx absorbance maximum at 560 nm inx the aqueous solution. The intensity of the colour produced is directly proportional to the amountx of direct bilirubinx concentration presentx in the sample. The total bilirubinx value represents the sum of the bilirubin glucuronide (direct) and the unconjugated (indirect) bilirubin. The colour produced measured at 560 nm is proportional to the amount of thex total bilirubinx concentration present in the sample (Peter and Kwiterovich, 2004).

# Estimation of Urea Concentration

Urea concentration was determined by usingx a randox commercial kit. The reagent R1 was prepared by addition of the content of R1a into R1bx bottle followed by gently mixingx while the contentx of R2 and R3 were mixed with 660 ml and 750 ml of distilled water respectvelly. Ten microlitres (10 µl) of each sample, distil water and standard was added to the sample, blankx and standardx tubes followed by the addition of 100 µl of R1 each of the testx tubes. The mixtures were properly mixed and incubated atx 37 °C for 10 mins after which 2.5 ml of both reagent R2 and R3 was added.The resultant mixtures were

incubated at 37 °C for 15 mins. The absorbance of the samples (ASample) and standardx (AStandard) against the blank and the concentration of urea was calculated as shown below:

Asample

Urea concentration (mg/dl) = × Standardx Conc.

AStandard

The principle involves the hydrolysis of urea in the serum into ammonia in the presence of urease enzyme. The ammonia was then measured photometrically at 546 nmx by Berthelots reaction.

Urea + H2O Urease 2NH3 + CO2

NH3 + Hypochlorite + Phenol Indophenol (blue compound)

* 1. **Preparation of Mcfarland standard and Standardization of innoculum** Macfarland standard is a chemical solution of bariumx chloride andx sulfuric acid. The chemical reaction between two chemicalsx produce a fine precipitate of barium sulfate. The turbidity of McFarland standardx isx visually comparable tox a bacteria suspension. McFarland turbidity standard (0.5 MFU) was prepared by mixingx 0.05 ml of 1 %x Bacl2 and 9.95 mls of 1 % H2SO4 tox obtain solution of 0.5 optical density. 0.5 McFarland turbidity standard provides anx optical density comparable to the density of bacterial suspension with a 1.5×108colony formingx unit (CFU/mL). Bacteria Suspension was prepared by pickingx colonies of 18-24 hrs culture of test organismsx and transferred into sterile nutrient brothx andx incubated for 2-3hrs. The turbidity was adjusted tox matchx McFarland standard which contains 1.5×108 CFU/ml.

# 3.12. *In vivo* antibacterial activity of extracts

Determination of the antibacterial activity was done as reported by Hosseinzadeh *et al*. (2007) with little modifications. The rats were grouped into eight groups of eight (8) rats each. In each group, four ratsx were infected orally with 1.0 mL (106 CFU/mL)x of

overnight broth culture of *E. coli* and the other four with 1.0 mL (106 Cfu/mL) of overnight broth culture of *Salmonella spp* andx then kept in separate cages. After Seven days, blood samples were taken from the animal’s tail and cultured on nutrient broth. The overnight blood cultures were inoculated on specific medium for *E. coli* (Eosin methylene blue agar) and *Salmonella spp* (*Salmonella Shigella* agar) respectively. Growth was observed for both *Salmonella spp* and *E. coli* and colonies were counted. Twenty-four hours (24 hrs) later, eachx groupx received different treatments. Groups were designed as follows:

# Table 3.1: Experimental Design

|  |  |
| --- | --- |
| Group | Administration |
| Group 1 (Negativex control) | 1. received 2ml of distilled water and   *Salmonella spp*   1. received 2ml of distilled water and   *Escherichia coli* |
| Group 2 (Positive Control) | 1. received 20mg/kg of ciprofloxacin and   *Salmonella spp*   1. receivedx 20mg/kg of ciprofloxacin and   *Escherichia coli* |
| Group 3 Methanol crude extract | 1. received 100mg/kg of methanol crude extract and *Salmonella Spp* 2. receivedx 100mg/kg of methanol crude extract and *Escherichia coli* |
| Group 4 Methanol crude extract | 1. received 200mg/kg of methanol crude extract and *Salmonella Spp* 2. receivedx 200mg/kg of methanol crude extract and *Escherichia coli* |
| Group 5 Methanol crude extract | 1. received 400 mg/kg of methanol crude extract and *Salmonella Spp* 2. received 400 mg/kg of methanol crude extract and *Escherichia coli* |
| Group 6 Methanol nhexane fraction | 1. received 100mg/kg of nhexane fraction of methanol and*x Salmonella Spp* 2. receivedx 100mg/kg of nhexane fraction of methanol and *Escherichia coli* |
| Group 7 Methanol nhexane fraction | 1. received 200mg/kg of nhexanex fractionx of methanol and *Salmonella Spp* 2. x received 200mg/kg of nhexane fraction of methanol and *Escherichia coli* |
| Group 8 Methanol nhexane fraction | 1. received 400mg/kg of nhexane fraction of methanol and*x Salmonella Spp* 2. received 400 mg/kgx of nhexane   fraction of methanol and *Escherichia coli* |

Treatment durationx was for fourteen (14) days. Animals were allowedx access to food andx water throughout the treatmentx period. On the 15th day, bloodx samples were collectedx from each group of animalsx tox assess the bacteria load by culturing on specificx media.

# 3.13 Data Analysis

The data collectedx were analysed using Statistical Package for the Social Sciences (SPSS) version 20. The results were evaluated using Analysis of Variance (ANOVA) andx were presented asx the mean value ± SEM (standard error of mean). Differences amongx

the means for the groups were assessed using the Duncan’s Multiple Range Testx to determine which mean values were significantly different at p<0.05.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

# Results

**Table 4.1: Qualitative Phytochemical Components of *Helianthus annuus* Seed Extracts**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Methanol extracts of *H. annuus* | | | | | Ethanol extracts of *H. annuus* | | | |
| Components | MCR | MHXN | META | MAQS | ECR | EHXN | EETA | EAQS |
| Alkaloids | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |
| Saponins | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |
| Steroids | **+** | **+** | **+** | **-** | **+** | **+** | **+** | **-** |
| Tannins | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |
| Flavonoids | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |
| Phenols | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |
| Terpennoids | **+** | **+** | **+** | **-** | **+** | **+** | **+** | **-** |
| Anthraquinones | **-** | **-** | **-** | **-** | **-** | **-** | **-** | **-** |
| Cardiac glycoside | **-** | **-** | **-** | **-** | **-** | **-** | **-** | **-** |

Note: -absent, + present;

**KEY: MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **META**= ethylacetate fraction of methanol, **MAQS**= aqueous fraction of methanol, **ECR**= ethanol crude, **EHXN**= n-hexane fraction of ethanol, **EETA**=ethylacetate fraction of ethanol, **EAQS**= aqueous fraction of ethanol

* + 1. **Phytochemical Contents of *Heliantus annuus* seeds extract**

The results in Table 4.1 show the qualitative phytochemical constituents of *Helianthus annuus* seed. The results showed that alkaloids, saponins, tannins, flavonoids and phenols were present in all of portions extracted with methanolic and ethanolic extracts while anthraquinones and cardiac glycoside were absent. Steroids and terpennoids were present in all, except in aqueous methanolic (MAQS) and ethanolic (EAQS) fractions.

**Table 4.2: Quantitative Phytochemical (mg/100 g) Components of *Helianthus annuus* Seed**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Extract | Phenols | Flavonoids | Tannins | Saponins | Alkaloids |
| MCR | 310.58±0.02d | 23.08±0.03g | 21.92±0.00e | 252.14±0.28d | 34.26±0.29f |
| MAQS | 440.16±0.03g | 16.63±0.02d | 6.94±0.01ab | 270.58±0.02f | 31.89±0.21e |
| META | 490.61±0.02h | 13.85±0.05c | 7.07±0.00b | 190.77±0.07c | 22.61±0.53b |
| MHXN | 263.22±0.37c | 12.02±0.06b | 23.12±0.01f | 386.18±0.31h | 29.01±0.21d |
| ECR | 312.37±0.33e | 20.24±0.06f | 23.17±0.05f | 255.91±0.15e | 35.92±0.42g |
| EAQS | 200.36±0.87a | 20.66±0.39f | 9.01±0.51c | 157.92±0.57a | 38.91±0.53h |
| EETA | 323.41±1.34f | 18.49±0.39e | 6.29±0.36a | 181.03±0.53b | 27.22±0.65c |
| EHXN | 214.24±0.17b | 9.39±0.28a | 19.94±0.11d | 330.48±1.19g | 17.82±0.54a |

Values are presented as Mean ± Standard Error of Mean of triplicate determinations. Values in a column with different superscripts are significantly different at p< 0.05. While **MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **META**= ethylacetate fraction of methanol, **MAQS**= aqueous fraction of methanol, **ECR**= ethanol crude, **EHXN**= nhexane fraction of ethanol, **EETA**=ethylacetate fraction of ethanol, **EAQS**= aqueous fraction of ethanol.

The results in Table 4.2 show the quantitative phytochemical components analysis of *Helianthus annuus*seeds. There was significant difference (p<0.05) among all the constituents (phenols, flavonoids, tannins, saponins and alkaloids) extracted using the various solvents. Phenols was significantly (p < 0.05) higher in the portion extracted with aqueous ethylacetate (META) and aqueous methanolic (MAQS) fractions while it was significantly lower in aqueous ethanolic (EAQS) and n-hexane (EHXN) fractions. Flavonoids was quantitatively and significantly (p < 0.05) highest in crude methanolic (MCR) extracts followed by aqueous (EAQS) and crude (ECR) ethanol. It was lowest in n-hexane (EHXN) fraction of the ethanol. Tannins was significantly (p < 0.05) higher in crude (ECR) and n-hexane methanol (MHXN) fractions and ethyacetate fraction of ethanol (EETA). Saponins was significantly (p<0.05) highest in n-hexane fraction of methanol (MHXN) followed by n-hexane fraction of ethanol (EHXN) while it was lowest in aqueous fraction of ethanol (EAQS). Alkaloids was highest in aqueous fraction of ethanol (EAQS) and lowest in n-hexane fraction of ethanol (EHXN).

# Table 4.3: Cultural growth characteristics on selective media, microscopic morphological characteristics and Gram staining Reaction

|  |  |  |  |
| --- | --- | --- | --- |
| **Organism** | **Cultural Growth**  **characteristics** | **Morphological**  **characteristics** | **Gram staining** |
| *Salmonella spp* | Colourless colonies with black center on  SSA | Rod shaped | Negative |
| *Escherichia coli* | Metallic green  sheen colonies on EMB | Rod shaped | Negative |

**Key: SSA=**Salmonella Shigellaagar, **EMB=**Eosine Methylene blue agar

# Cultural, morphological and growth characteristics of organisms

Table 4.3 shows the cultural growth characteristics, morphological characteristics and Gram stain reaction of the test organisms. *Salmonella spp* forms colourness colonies with black centre on *Salmonella Shigella* agar, its rod shaped and Gram negative while *Escherichia coli* forms metallic green sheen colonies on Eosine Methylene blue agar, its rod shaped and gram negative on staining

# Table 4.4: Biochemical characteristics of the test organisms

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Organisms | Indole | Methyl-  red | Catalase | Motility | Oxidase | Urease |
| ***Salmonella***  ***spp*** | **-** | **+** | **-** | **+** | **-** | **-** |
| ***Escherichia***  ***coli*** | **+** | **+** | **-** | **+** | **-** | **-** |

**Key; + present, -ve absent**

# Biochemical characteristics of the test Organisms

Table 4.4 shows the biochemical characteristics of the test organisms. *Salmonella spp* and *Escherichia coli* tested positive to indole, methyl red, and catalase. They were both motile while they tested negative to oxidase and urease.

**Table 4.5: Zones of inhibition (mm) of *Helianthus annuus* Seed Extracts against**

## Salmonella spp.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Extract |  | Zone of inhibition (mm) | |  |
|  | 60 mg/mL | 120 mg/mL | 240 mg/mL | 480 mg/mL |
| MCR | 18.50±0.50b | 20.33±0.33b | 23.33±0.88a | 25.67±0.33a |
| MAQS | N.I | N.I | N.I | N.I |
| META | N.I | N.I | N.I | N.I |
| MHXN | 17.33±0.67b | 19.33±0.67b | 21.33±0.67a | 23.33±1.67a |
| ECR | 12.67±0.67a | 17.33±1.45ab | 20.33±1.20a | 22.00±1.53a |
| EAQS | N.I | N.I | N.I | N.I |
| EETA | N.I | N.I | N.I | N.I |
| EHXN | 12.33±0.33a | 14.33±1.45a | 19.00±0.58a | 21.67±0.33a |
| Positive control (Ciprofloxacin) at 100 mg/mL | 32.67±2.91c | 32.67±2.91c | 32.67±2.91b | 32.67±2.91b |

Values are presented as Mean ± Standard Error of Mean of triplicate determinations. Values in a column with different superscripts are significantly different at p< 0.05. While **MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **META**= ethylacetate fraction of methanol, **MAQS**= aqueous fraction of methanol, **ECR**= ethanol crude, **EHXN**= nhexane fraction of ethanol, **EETA**=ethylacetate fraction of ethanol, **EAQS**= aqueous fraction of ethanol. **N.I = No Inhibition.**

* + 1. ***In vitro* Antibacterial study**

The results in Table 4.5 show the zone of inhibition of different solvents extraction of *Helianthus annuus* seed against *Salmonella spp.* The aqueous fraction of methanol (MAQS), ethyacetate fraction of ethanol (META), aqueous fraction of ethanol (EAQS) and ethylacetate fraction of ethanol (EETA) showed no zone of inhibition. Crude

methanolic extract (MCR), n-hexane fraction of methanol (MHXN), crude ethanol extract (ECR) n-hexane fraction of ethanol (EHXN) and Ciprofloxacin which serves as positive control showed zone of inhibition against *Salmonella spp*.

Among the four extracts that have inhibitory activities, methanol crude extract (MCR) and n-hexane fraction of methanol (MHXN) have significantly (p < 0.05) higher inhibitory activities compared to crude ethanol extract (ECR) and n-hexane fraction of ethanol (EHXN) at 60 mg/mL. Their inhibitory activities were however, lower compared to ciprofloxacin (100 mg/mL) used as standards or positive control. Similar result was obtained at 120 mg/mL, but at dose 240 mg/mL and 480 mg/mL there was no significant (p<0.05) difference among the four extracts. However, there was significant difference (p<0.05) between the inhibitory activities of those four extracts and the ciprofloxacin (100 mg/mL) used as standard or positive control. The inhibitory activities at the difference concentration increases with increase in concentration for all the extracts.

**Table 4.6: Zones of Inhibition (mm) of *Helianthus annuus* Seed Extracts against**

## Escherichia coli

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Extract |  | Zone of inhibition (mm) | |  |
|  | 60 mg/mL | 120 mg/mL | 240 mg/mL | 480 mg/mL |
| MCR | 8.50±0.50a | 10.00±0.00a | 10.67±0.67a | 11.67±0.67a |
| MAQS | N.I | N.I | N.I | N.I |
| META | N.I | N.I | N.I | N.I |
| MHXN | 7.33±0.67a | 11.00±0.58a | 15.33±0.33b | 17.00±0.58b |
| ECR | N.I | N.I | N.I | N.I |
| EAQS | N.I | N.I | N.I | N.I |
| EETA | N.I | N.I | N.I | N.I |
| EHXN | N.I | N.I | N.I | N.I |
| Positive control (Ciprofloxacin)  at 100 mg/mL | 11.67±0.88b | 11.67±0.88a | 11.67±0.88a | 11.67±0.88a |

Values are presented as Mean ± Standard Error of Mean of triplicate determinations. Values in a column with different superscripts are significantly different at p< 0.05.**KEY:MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **META**= ethylacetate fraction of methanol, **MAQS**= aqueous fraction of methanol, **ECR**= ethanol crude, **EHXN**= nhexane fraction of ethanol, **EETA**=ethylacetate fraction of ethanol, **EAQS**= aqueous fraction of ethanol, **N.I = No Inhibition**

The results in Table 4.6 showed the zone of inhibition of *Helianthus annuus* seed of the various extracts against *Escherichia coli.* The aqueous fraction of methanol (MAQS), ethyacetate fraction of ethanol (META), aqueous fraction of ethanol (EAQS), crude ethanol extract (ECR), n-hexane fraction of ethanol (EHXN) and ethylacetate fraction of ethanol (EETA) showed no zone of inhibition. Methanolic Crude extract (MCR), n- hexane fraction of methanol (MHXN) and ciprofloxacin which serves as positive control showed inhibitory activities against *Escherichia coli.*

There was no significant (p<0.05) difference between methanol crude extract (MCR) and n-hexane fraction of methanol (MHXN) at 60 mg/mL and 120 mg/mL, but the difference was significant (p<0.05) at 240 mg/mL and 480 mg/mL. Ciprofloxacin (100 mg/mL) was significantly (p<0.05) higher at 60 mg/mL compared to methanol crude extract (MCR) and n-hexane fraction of methanol (MHXN), but there was no significant (p<0.05) difference between ciprofloxacin control, methanol crude extract (MCR) and n- hexane fraction of methanol (MHXN) at 120 mg/mL. Extracts from n-hexane fraction of methanol (MHXN) had significantly (p<0.05) higher zone of inhibition at 240 mg/mL and 480 mg/mL when compared to MCR and the ciprofloxacin (standard or control) while there was no significantly difference (p<0.05) between the control (ciprofloxacin) and methanol crude extract (MCR) at those concentrations. Zone of inhibition also increased with increase in concentrations across the two extracts of *Helianthus annuus* seed that shows inhibitory activities.

**Table 4.7: MIC (mg/ml) of the *Helianthus annuus* Seed Extracts against *Salmonella spp* and *E. coli***

|  |  |  |
| --- | --- | --- |
| Extracts | *Salmonella* | *E. coli* |
| Methanol Crude (**MCR)** | 0.46±0.01b | 0.19±0.01b |
| Methanol n-hexane (**MHXN**) | 0.48±0.02b | 0.96±0.01c |
| Ethanol Crude (**ECR**) | 0.07±0.03a | \_ |
| Ethanol n-Hexane (**EHXN)** | 2.40±0.06c | \_ |
| Ciprofloxacin 20 (Standard) | 0.02±0.00a | 0.02±0.00a |

Values are presented as Mean ± Standard Error of Mean of three replicates. Values in a column with different superscripts are significantly different at P < 0.05**. KEY:MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **ECR**= ethanol crude, **EHXN**= nhexane fraction of ethanol

The results in Table 4.7 showed comparison of the minimum inhibitory concentration (MIC) of *Helianthus annuus* seed extracts on both *Salmonella spp* and *Escherichia coli.*

The minimum inhibitory concentration (MIC) of methanol crude, methanol n-hexane and ethanol crude and ethanol n-hexane extracts against *Salmonella spp.*, were significantly (p<0.05) higher than the standard drug except that of ethanol crude extract (0.07±0.03 mg/ml) which was comparable to the standard drug (0.02±0.00 mg/ml). The n-hexane fraction of ethanol (EHXN) had significantly (p<0.05) higher MIC value (2.40±0.06 mg/ml) against *Salmonella spp.* than the other extracts. The MIC of the extracts against

*E. coli* showed significantly higher values than that of the standard drug.

**Table 4.8: MBC (mg/ml) of the *Helianthus annuus* Seed Extracts against *Salmonella spp* and *E. coli***

|  |  |  |
| --- | --- | --- |
| Extract | *Salmonella* | *E. coli* |
| Methanol Crude (**MCR)** | 2.40±0.12d | 0.96±0.02b |
| Methanol n-hexane (**MHXN**) | 2.40±0.17d | 4.80±0.12c |
| Ethanol Crude (**ECR**) | 0.48±0.01b | \_ |
| Ethanol n-Hexane (**EHXN)** | 1.20±0.12c | \_ |
| Ciproloxaxin 20 | 0.18±0.02a | 0.25±0.05a |

Values are presented as Mean ± Standard Error of Mean of three replicates. Values in a column with different superscripts are significantly different at P < 0.05**KEY:MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **ECR**= ethanol crude, **EHXN**= nhexane fraction of ethanol

Table 4.8 Presents the minimum bactericidal concentration (MBC) of *Helianthus annuus* seed extracts on *Salmonella spp* and *Escherichia coli.* The MBC values of the extracts against *Salmonella spp* range from 0.18 mg/ml for the standard drug to 2.40±0.12 mg/ml for MCR and MHXN. The MBC of ethanol hexane (EHXN) was lower than that of MCR and MHXN while that of ethanol crude (ECR) was lower than EHXN but statistically different (p<0.05) from the standard (Ciprofloxacin). Similarly, none of the extracts had MBC on *E. coli*comparable (p<0.05) to the standard drug, however, Methanol Crude (MCR) and ethanol crude (ECR) had MBC significantly (p<0.05) lower

than was observed for nhexane fraction of ethanol (EHXN) (4.80±0.12 mg/ml) and nhexane fraction of methanol (MHXN) (4.80±0.12 mg/ml).

**Table 4.9: Safe dose Determination (LD50) of *Helianthus annuus* Seed Extracts**

|  |  |  |  |
| --- | --- | --- | --- |
| **Extracts** | **Number of rats** | **Concentration of extracts (mg/kgbw)** | **Number of deaths** |
| MCR | 5 | 2000 | 0/5 |
| MHXN | 5 | 2000 | 0/5 |
| ECR | 5 | 2000 | 0/5 |
| EHXN | 5 | 2000 | 0/5 |

**KEY: MCR**= methanol crude extract, **MHXN**= nhexane fraction of methanol, **ECR**= ethanol crude, **EHXN**= nhexane fraction of ethanol

# Acute Toxicity Studies

The results in Table 4.9 show the LD50 also referred to as safe dose of *Helianthus annuus* seed extracts from crude methanol, ethanol as well as n-hexane fraction of the methanol and ethanol. There was no mortality at 2000 mg/kgbody weight concentration of the extracts for all of the four extracts.

**Table 4.10: Subacute Toxicity studies of *Helianthus annuus* Seeds Extracts**

|  |  |  |  |
| --- | --- | --- | --- |
| **Groups** | **Extracts** | **Concentrations (mg/kg bw)** | **Number of deaths** |
| 1 | Distilled water | 10 | 1/4 |
| 2 | Methanol crude | 10 | 0/4 |
|  |  | 300 | 1/4 |
|  |  | 600` | 0/4 |
| 3 | Ethanol crude | 10 | 0/4 |
|  |  | 300 | 0/4 |
|  |  | 600 | 0/4 |
| 4 | Methanol | 10 | 0/4 |
|  | hexane | 300 | 0/4 |
|  |  | 600 | 0/4 |
| 5 | Ethanol hexane | 10 | 1/4 |
|  |  | 300 | 2/4 |
|  |  | 600 | 0/4 |

# Subacute Toxicity Studies

The results in Table 4.10 show the sub-acute toxicity studies of *Helianthus annuus* seeds extracts the test organism. At 10 mg/kg bw, one out of four (1/4) mortality was recorded for distilled water and ethanol hexane administered groups. There was no mortality at the same concentration for crude methanol, crude ethanol and n-hexane methanol administered groups. At 300 mg/kgbw, there was one out of four (1/4) mortality for crude methanol, two out of four (2/4) for hexane ethanol while there was no mortality for crude ethanol and methanol hexane. At 600 mg/kgbw, there was no recorded mortality for any of the extracts.

**Table 4.11: *In vivo* Antibacterial Activityof *Helianthus annuus* Seeds Extracts against *Salmonella spp* and *Escherichia coli***

|  |  |  |  |
| --- | --- | --- | --- |
| **Groups** | **Subgroups** | **Treatment** | **No of**  **deaths** |
| 1 | 1a | Distilled water + *Salmonella spp* | 0/4 |
|  | 1b | Distilled water +*Escherichia coli* | 1/4 |
| 2 | 2a | Ciprofloxacin 20 mg/kg+ *Salmonella spp* | 1/4 |
|  | 2b | Ciprofloxacin + *Escherichia coli* | 2/4 |
| 3 | 3a | Methanol crude 100 mg/kg + *Salmonella*  *spp* | 0/4 |
|  | 3b | Methanol crude 100 mg/kg + *Escherichia*  *coli* | 0/4 |
| 4 | 4a | Methanol crude 200 mg/kg + *Salmonella*  *spp* | 0/4 |
|  | 4b | Methanol crude 200 mg/kg + *Escherichia*  *coli* | 1/4 |
| 5 | 5a | Methanol crude 400 mg/kg + *Salmonella*  *spp* | 1/4 |
|  | 5b | Methanol crude 400 mg/kg + *Escherichia*  *coli* | 0/4 |
| 6 | 6a | Methanol hexane 100 mg/kg + *Salmonella*  *spp* | 0/4 |
|  | 6b | Methanol hexane 100 mg/kg + *Escherichia*  *coli* | 0/4 |
| 7 | 7a | Methanol hexane 200 mg/kg + *Salmonella*  *spp* | 0/4 |
|  | 7b | Methanol hexane 200 mg/kg + *Escherichia*  *coli* | 0/4 |
| 8 | 8a | Methanol hexane 400 mg/kg + *Salmonella*  *spp* | 1/4 |
|  | 8b | Methanol hexane 400 mg/kg + *Escherichia*  *coli* | 0/4 |

The results in Table 4.11 show the *in vivo* antibacterial activityof *Helianthus annuus* seeds extracts against four (4) test organism. As distilled water gave no mortality for *Salmonella spp* but gave one mortality *E. coli*. At 20 mg/kg Ciprofloxacin gave one mortality for *Salmonella spp* and two for *E. coli* while there was no mortality for crude methanol for both organisms. At 100 mg/kg crude methanol gave no mortality for either

of the organisms, but at 200 mg/kg there was one mortality for *E. coli.* The 400 mg/kg crude methanol gave one mortality for *Salmonella spp* but there was no mortality for *E. coli* at the same concentration. The 100 mg/kg and 200 mg/kg methanol-hexane gave no mortality for the two organisms. However, at 400 mg/kg there was one (1) mortality for *Salmonella spp* while there was no mortality for *E. coli.*

# Table 4.12: Serum Enzymes Biochemical Parameters of Rats treated with *H. annuus* Seed Extracts for 28 Days as compared to the Control Group

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Dose (mg/kgbw)** |  |  | **Biochemical parameters** | |  |
|  | **TP(g/L)** | **ALB(g/L)** | **ALT(IU/L)** | **AST(IU/L)** | **ALP(IU/L)** |
| MC10 | 26.15±1.39b | 15.45±1.80bc | 35.44±2.48bcd | 16.60±1.18c | 120.29±2.05ab |
| MH10 | 30.27±1.38c | 19.04±1.46c | 31.94±1.51b | 21.48±1.14de | 129.29±2.10bc |
| EC10 | 21.01±1.51a | 11.09±0.76a | 36.05±2.02bcd | 23.01±0.97de | 128.32±4.43abc |
| EH10 | 23.04±0.88ab | 15.43±1.41bc | 39.54±1.10cde | 23.53±2.14de | 160.07±3.33g |
| MC300 | 22.43±1.03ab | 16.10±0.84bc | 35.62±1.13bcd | 11.36±0.68a | 134.85±1.75cd |
| MH300 | 32.98±1.56cd | 21.07±1.46de | 30.84±2.08ab | 19.18±0.72cd | 151.76±1.47fg |
| EC300 | 22.42±1.03ab | 15.95±0.80bc | 41.06±1.65d | 24.73±1.64e | 120.42±3.76ab |
| EH300 | 31.66±1.15c | 20.49±1.19de | 34.95±1.19bcd | 21.14±1.14de | 138.23±3.29cd |
| MC600 | 23.17±1.45ab | 15.95±2.09bc | 25.64±1.89a | 11.88±1.18ab | 135.27±2.52cd |
| MH600 | 37.53±0.46e | 23.37±0.70e | 32.90±1.60bc | 15.74±1.21bc | 118.87±2.45a |
| EC600 | 25.13±0.98b | 16.03±1.44bc | 37.28±1.70bcde | 23.91±0.85e | 118.37±3.32a |
| EH600 | 35.62±1.12de | 22.05±0.92de | 33.17±3.43bc | 16.30±2.22c | 140.37±3.20de |
| NC | 22.38±1.31ab | 12.58±0.72ab | 42.99±2.77e | 21.15±1.30de | 148.60±4.79ef |

Values are presentedx asx Mean ± Standard Error of Meanx (SEM) of three replicates. Valuesx with different superscripts in a columnx are significantly different at p< 0.05. While MC= Methanol crude extract, MH= n-hexane fraction of methanol crude extract, EC= Ethanol crude extract, EH= n-Hexane fractionx of ethanol crude extract, NC= normal control. TP=Total protein, ALB= Albumin, ALT=Alanine transaminase, AST=Aspartate transaminase and ALP=Alanine phosphatase

The resultsx in Table 4.12 show the biochemical parameters of the rats treated withx the extract of *Helianthus annuus* seed. The biochemical parameters showedx significant difference among the extracts from the difference solvents. Crude methanol extracts showed decrease in the total protein with increase in concentrationx and was significantly (p <x 0.05) higher at 10 mg/kg body weight. At 10, 300 andx 600 mg/kg body weight, total proteinx concentration was significant (p<0.05) higher n-hexane fraction of the crude methanol extracts. The total protein alsox increased with increase in concentration for the same n-hexane fractionx of the crude methanol extracts andx the fraction performs better than the negative control. Similar resultx wasx obtained for crude ethanol whichx showedx significantly (p<0.05) higher total proteinx concentration at 600 mg/kgx body weight. The n-hexane fraction of the crude ethanol extractx showedx the same trend. When all the extracts were compared, the highest total proteinx concentration were obtained at 600 mg/kg body weight in n-hexane fraction of methanol crude extract (MHXN) and n- Hexane fraction of ethanol crude extract (EHXN) while the least were obtainedx at 10 mg/kg body weight crude ethanol extract, negative control, 300 mg/kg body weight crude ethanol extract and 300 mg/kg body weight crude methanol extract.

Albumin concentration atx 10 mg/kg body weight was significantly (p<0.05) highest in the n-hexane fraction of methanol crude extract (MHXN) compared tox other fractions at the same concentration. The albumin concentration increases withx increase inx concentrationx for the same fraction of the n-hexane fraction of crude methanol extract. The leastx albumin concentration was obtained in crude ethanol extract, but the albumin increased with increase in concentration. The alanine transferase (ALT) activity was significantly (p<0.05) higher for the n-Hexane fraction of crude ethanol extractx at 10 mg/kgx body weight but significantly (p<0.05)x leastx in n-Hexane fraction of crude methanol extract. At 300 mg/kg body weight, alanine transferase (ALT) activity wasx significantly (p<0.05)

higher for crude ethanol extract and significantly lowest (p<x 0.05) inx crude methanol extract. At 600 mg/kgx body weight, alanine transferase (ALT) activity was significantly (p<x 0.05)x higher for crude ethanol extract (ECR) and significantly (p< 0.05) lowest in n- hexane fraction of crude methanol extract (MHXN). The overall comparison showed that negative control had the highest alanine transferase activity which was significantly (p <x 0.05)x higher.

Aspartate transaminase activity decreasedx with increase inx concentration while alanine phosphatase increased with increase in concentration. Although there were significant (p<0.05) differences among the treated groups and the control in some cases, they were still within the normal range.

# Tablex 4.13: Serum Lipid Biochemical Parameters of Rats treated with *H. annuus* Seeds Extracts for 28 Days as compared to the Control Group

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Dose (mg/kgbw)** |  | **Biochemical parameters (mmol/L)** | |  |
|  | **TC** | **TRIG** | **HDL-C** | **LDL-C** |
| MC10 | 142.99±2.25c | 131.90±1.16ab | 100.23±1.22c | 109.58±2.46cde |
| MH10 | 146.33±3.24cd | 141.58±2.45cd | 119.42±2.59de | 104.10±2.62bcd |
| EC10 | 154.53±1.39e | 126.16±1.29a | 82.31±3.48a | 109.23±3.76cde |
| EH10 | 164.56±2.51f | 131.84±3.15ab | 111.36±4.28d | 113.43±3.53e |
| MC300 | 146.47±1.94cd | 143.71±2.46de | 115.93±2.68de | 111.97±2.72de |
| MH300 | 127.85±1.69b | 144.55±2.19def | 141.02±2.77fg | 82.54±1.66a |
| EC300 | 144.57±3.67cd | 126.75±3.57a | 88.89±0.97ab | 101.72±1.77bc |
| EH300 | 151.58±1.93de | 135.83±3.17bc | 124.67±2.50e | 95.99±2.10b |
| MC600 | 131.91±1.74b | 151.72±1.06f | 138.53±2.54f | 103.69±2.86bcd |
| MH600 | 116.57±3.78a | 149.00±1.80ef | 147.25±3.86g | 77.30±3.10a |
| EC600 | 144.68±2.63cd | 150.04±1.84ef | 93.44±2.34bc | 98.50±1.81b |
| EH600 | 147.61±0.99cde | 151.29±1.16f | 122.96±3.24e | 84.20±2.10a |
| NC | 174.83±2.58g | 146.65±2.15def | 95.44±1.61bc | 114.21±2.24e |

Values arex presentedx asx Mean ± Standard Error of Meanx (SEM) of threex replicates. Valuesx with different superscripts in a columnx are significantly different at p< 0.05. While MC= Methanol crude extract, MH= n-hexane fractionx of methanol crude extract, EC= Ethanol crude extract, EH= n-Hexanex fraction of ethanol crude extract, NC= normal control. TC=Total cholesterol, TRIG=Triglycerides, HDL-C= high density lipoprotein cholesterol and LDL-C=Low density lipoprotein cholesterol

The results in Table 4.13 shows thex lipidx component of the Biochemical Parameters of rats treated with *H. annuus* seeds extracts for 28 days compared to the control groups. The total cholesterol ranges from 116.57 mg/g found in the group administered 600 mg/kg body weightx of n-hexane fraction of crude methanol extract to 174.83 mg/gx found inx the group administered the control. Total cholesterol was significantly (p<0.05) higher in the negative control groupx as compared to the treated groups. Among the treated groups, the group treated with 10 mg/kgx body weight had the highest cholesterol level while the group treated with 600x mg/kg body weight of n-hexane fraction of crude methanol extract had significantly (p<0.05) lower value of total cholesterol (116.57±3.78 mg/g).

Triglyceride ranges from 126.16 mg/g found in 10 mg/kg body weight crude ethanol to

151.72 mg/g found in the group administered 600 mg/kg body weight. The triglyceride components in the groups administered 600 mg/kg body weight of crude methanol, crude ethanol, n-hexane fraction of crude methanol and n-Hexane fraction of crude ethanol extracts were significantly higher (p<0.05) than the control groupx while the control group was significantly (p<0.05) higher than the other groups treated with extracts concentrations lower than 300 mg/kgx body weight.

The high-density lipoproteins cholesterol (HDL-C)x ranged from 82.31 mg/g found inx the group of rat administered 10 mg/kg body weight to 147.25 mg/g obtained for the group administeredx 600 mg/kg body weight of n-hexane fraction of crude methanol extract. The 300 and 600 mg/kgx body weightx groupsx of n-Hexane fraction of crude ethanol extract of

*H. annuus* seeds were significantly higher (p<0.05) compared to the control group. The groups treated with 10 mg/kgx body weight crude ethanol andx 300 mg/kg body weight crude ethanol hadx significantly lower (p<0.05)x HDL-cholesterol level comparedx to the control group.

The resultsx of the low-density lipoprotein cholesterol (LDL-C) showed the LDL- cholesterol ranges from 82.54 mg/gx for group administeredx 300 mg/kgx body weight n- hexane fraction of crude methanol extract tox 114.21 mg/g for group administered the negative control. The normal control group hadx the highest valuex which wasx significantly (p<0.05) higher comparedx to all the other group exceptx the group administeredx 10 mg/kgx body weight n-Hexane fraction of crude ethanol extract.

# Tablex 4.14: Protein Components of the Serum Biochemical Parameters of Rats treated with *H. annuus* Seedsx Extracts for 28 Days as compared to the Control Group

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Dosex (mg/kg bw)** |  | **Biochemical parameters (mg/dL)** | | |  |
|  | **Creatinine** | **Total bilirubin** | **Indirect bilirubin** | **Uric acid** | **Urea** |
| MC10 | 7.74±0.68bc | 0.83±0.04ab | 0.33±0.04a | 7.74±0.68abc | 72.43±2.60a |
| MH10 | 8.89±0.71bcd | 1.04±0.05cd | 0.69±0.05de | 11.37±1.30efg | 72.85±2.01a |
| EC10 | 12.17±1.38de | 0.77±0.07a | 0.51±0.10bc | 8.74±0.63abcde | 75.31±2.08a |
| EH10 | 14.00±1.35e | 1.31±0.04e | 0.90±0.02f | 12.20±0.95fg | 77.00±1.26a |
| MC300 | 6.33±0.38ab | 1.12±0.08d | 0.56±0.02bcd | 8.42±0.34abcd | 69.83±2.35a |
| MH300 | 11.11±0.71cde | 1.00±0.05bcd | 0.63±0.05cd | 9.63±0.41cdef | 74.18±2.73a |
| EC300 | 10.96±0.97cde | 0.86±0.07abc | 0.55±0.07bcd | 7.31±0.97abc | 71.47±2.11a |
| EH300 | 21.08±1.54g | 0.99±0.08bcd | 0.50±0.02bc | 9.07±0.47bcde | 77.50±1.95a |
| MC600 | 4.30±0.47a | 1.45±0.06e | 0.49±0.01bc | 6.36±0.44ab | 73.64±1.90a |
| MH600 | 10.63±0.98cde | 1.08±0.06d | 0.78±0.02ef | 12.49±1.65fg | 78.03±3.41a |
| EC600 | 17.11±0.83f | 0.74±0.03a | 0.50±0.02bc | 5.86±0.54a | 73.46±3.72a |
| EH600 | 13.03±1.49e | 0.87±0.03abc | 0.61±0.01bcd | 10.98±1.07defg | 71.81±2.21a |
| NC | 17.79±1.40f | 0.86±0.07abc | 0.48±0.02b | 12.69±1.13g | 76.60±1.76a |

Values arex presentedx asx Mean ± Standard Error of Meanx (SEM) of threex replicates. Valuesx with different superscripts in a columnx are significantly different at p< 0.05. While MC= Methanol crude extract, MH= n-hexane fraction of methanol crudex extract, EC= Ethanol crudex extract, EH= n-Hexane fractionx of ethanol crude extract, NC= normal control

The resultsx in Table 4.14 show protein components of the serum biochemical parameters of rats treated withx *H. annuus* seeds extractsx for 28 days asx comparedx tox the control group. The creatinine component ranges from 4.30 mg/g to 21.08 mg/g among thex rat groups treated with the various extract of *H. annuus* seed. The rat group administered 300 mg/kg body weightx n-hexane fraction of crude ethanol hadx significantly (p<0.05) higher serum creatinine level compared to the control group. However, the creatinine level in the control group was significantly (p<0.05) higher than the restx of treated groups exceptx the group administered 600 mg/kg body weight of crude ethanol which had the same level of significant (p<x 0.05) with the control group.

Total bilirubin ranges from 0.77 mg/g in the group administered 10 mg/kgx body weight crude ethanol to 1.45 mg/g for the groupx administered 600 mg/kg body weight crude methanol. There was significant (p<0.05) difference among the various groups with some groups sharingx the same level of significantx (p< 0.05). The group administeredx 10x mg/kgx body weight n-hexane fraction of the crude methanol, 10 mg/kg body weight n-hexane fraction of crude ethanol, 300 mg/kg body weight of crudex methanol, 300 mg/kgx body weight of n-hexane fraction of crude methanol, 600 mg/kg body weight of crude methanol and the group administeredx 600 mg/kg body weight had significantly (p<0.05) higher over the control. The control group and the group treated with 300 mg/kgx weight crude ethanol andx 600 mg/kg body weight of n-hexane fraction of crude ethanol had the same level of total bilirubin. The remainingx groups had significantly (p< 0.05) lower total bilirubin when compared to control groups.

The indirectx bilirubin concentration ranges from 0.33 mg/L in group administeredx 10 mg/kg body weight crude methanol to 0.90x mg/L inx group administered n-hexane fraction of the crude ethanol extract. The indirect bilirubinx level was significantly (p<0.05) higher in all the other groups except the group administeredx 10 mg/kgx body weight crude

methanol extract. Uric acid concentration ranges from 5.86 mg/Lx in the group administeredx 600 mg/kg body weight crude ethanol to 12.69 mg/Lx in the control groups. There was significant difference (p< 0.05) among the various groups, and the control group had significantly (p<0.05) higher uric acid concentration comparedx the rest of the group. Urea ranges from 69.83 mg/Lx inx the group administered 300x mg/kg body weight of crude methanol to 78.03 mg/Lx in the group administeredx 600 mg/kg body weight of n- hexane fraction of crude ethanol extract. There wasx nox significant (p< 0.05) difference in the urea concentration for all the groups including the control group.

# Tablex 4.15: Serum Biochemical Mineral Components of Rats treated with *H. annuus* Seeds Extracts for 28 Days as compared to thex Control Group

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Dosex (mg/kg bw)** |  | **Biochemical parametersx (mEq/L)** | |  |
|  | **Sodium** | **Potassium** | **Chloride** | **Bicarbonate** |
| MC10 | 172.00±3.97de | 8.00±0.07a | 88.23±2.66abc | 36.89±1.34ab |
| MH10 | 163.77±2.05bc | 10.99±0.96bc | 92.04±2.77bcd | 35.13±1.73ab |
| EC10 | 179.01±2.72ef | 11.45±1.15c | 93.83±2.14cde | 41.63±2.43bc |
| EH10 | 174.86±2.54de | 12.09±1.01cd | 88.45±1.09abc | 49.46±1.55e |
| MC300 | 181.98±2.18f | 12.55±1.16cd | 80.43±2.25a | 40.36±1.83abc |
| MH300 | 157.70±1.70b | 13.12±0.87cd | 105.00±4.30f | 33.44±2.24a |
| EC300 | 178.98±0.96ef | 11.47±0.78c | 86.66±1.99abc | 35.17±2.21ab |
| EH300 | 157.51±1.64b | 13.22±0.64cd | 82.96±2.55ab | 42.05±2.03bcd |
| MC600 | 158.65±2.19b | 14.74±0.63d | 85.79±2.63abc | 44.15±1.89cde |
| MH600 | 146.99±2.17a | 10.63±0.98abc | 90.03±2.48bc | 36.27±1.64ab |
| EC600 | 168.12±3.34cd | 10.92±1.00bc | 101.94±2.15ef | 38.84±2.73abc |
| EH600 | 162.92±2.64bc | 14.58±1.02d | 99.57±3.56def | 48.77±2.94de |
| NC | 178.33±2.55ef | 8.49±0.62ab | 90.39±4.72bc | 51.02±3.26e |

Values arex presentedx asx Mean ± Standard Error of Meanx (SEM) of threex replicates. Valuesx with different superscripts in a columnx are significantly different at p< 0.05. While MC= Methanol crude extract, MH= n-hexane fraction of methanol crude extract, EC= Ethanol crudex extract, EH= n-Hexane fraction of ethanol crude extract, NC= normal control

The results in Table 4.15 show the serum mineral component of the biochemical parameters of rats treated with *H. annuus* seedx extracts. The sodium level in the serum ranges from 146.99 mg/Lx in the group administered 600 mg/kg body weight of n-hexane fraction of crude methanol tox 181.98 mg/Lx in groupx administered 300 mg/kg methanol crude (MCR). The group administeredx 300 mg/kgx body weight crude methanol hadx significantly (p<0.05) higher sodium concentrationx compared to the control group. The control group, the group administered 300 mg/kgx body weight of crude ethanol and 10 mg/kg body weight shared the same significant (p< 0.05) level and were significantly higher over the other groupx except the group administered 300 mg/kgx body weight of crude methanol. Potassiumx concentration in the rats administered the various extract of *H. annuus* seedx ranges from 8.00 tox 14.74 mg/L. The potassium concentrationx was significantly lower in the group administered 10 mg/kg body weightx of crude methanol compared to the restx of the groups. In the same manner, the potassium concentrationx was significantly lower in the control group whenx compared to the rest of the groupx other than the group administered 10 mg/kg body weight which was significantly lower.

The chlorine concentration ranges from 80.43 mg/L to 105.00 mg/L. The control group and the group administered 600 mg/kgx body weight of n-hexane fraction of the crude methanol shared the same level of significant. They were significantly higher compared to the group administeredx 10 mg/kgx body weightx crude methanol, 300 mg/kg body weight of crude methanol and the group of rats administered 300 mg/kg body weight of n-hexane fraction of the crude ethanol. However, they were significantly lower when compared tox the rest of the groups. The bicarbonate was lower among the treated groups compared to the control group. It ranges from 33.44 mg/L to 51.02 mg/L found in the control group. The bicarbonate concentration was significantly higher in the control over all the other groups except the group administeredx 10 mg/kgx body weight of n-hexane fraction of

crude ethanol which share the same significant level as the control group.

# Table 4.16: Haematological values of Rats treated with *Helianthus annuus* Seed Extracts for 28 Days compared to the Control Group

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Haematological parameters** | | | | |
| **Dosage**  **(mg/kg bw)** | **Hb(g/dL)** | **PCV(%)** | **MCV(fL)** | **MCH(pg)** |
| MC10 | 16.40±1.31ab | 40.00±1.15ab | 47.00±2.31bc | 20.50±1.44abcd |
| MC300 | 20.11±1.92abcd | 41.50±3.75ab | 47.50±1.44bcd | 17.50±0.87a |
| MC600 | 22.40±1.30cd | 34.50±2.60a | 56.50±1.44f | 20.50±2.02abcd |
| MH10 | 23.47±1.24d | 40.00±0.58ab | 48.50±2.02cd | 22.50±1.44abcde |
| MH300 | 20.37±0.84abcd | 39.50±2.02ab | 54.50±2.02ef | 25.00±1.15cde |
| MH600 | 21.10±1.34bcd | 45.50±2.02bc | 53.00±1.73def | 19.00±1.15ab |
| EC10 | 22.11±1.93cd | 39.50±1.44ab | 48.50±2.02cd | 19.50±2.02abc |
| EC300 | 24.59±1.56d | 36.88±1.09a | 46.00±1.91bc | 26.25±1.11ef |
| EC600 | 22.53±1.14cd | 41.00±2.31ab | 50.50±2.02cde | 22.50±2.60abcde |
| EH10 | 21.53±1.91cd | 40.50±3.75ab | 42.50±2.02ab | 19.00±2.31ab |
| EH300 | 18.32±1.20abc | 51.50±4.50c | 37.75±1.75a | 27.00±1.00e |
| EH600 | 23.50±1.25d | 40.00±1.73ab | 39.50±0.87a | 21.50±2.02abcde |
| NC | 15.95±0.97a | 37.50±1.44a | 37.50±0.87a | 23.50±2.02bcde |

Values are presented as Mean ± Standard Error of Mean (SEM) of three replicates. Values with different superscripts in a column are significantly different at p<0.05. Key: MC= Methanol crude extract, MH= n- hexane fraction of methanol crude extract, EC= Ethanol crude extract, EH= n-Hexane fraction of ethanol crude extract, NC= normal control. Hb=haemoglobin, PCV=Packed cell volume, MCV=Mean cell volume and Mean cell Haemoglobin

The results inx Table 4.16 show the hematological parameters of the rats treated withx *Helianthus annuus* seed extracts. The hemoglobin value was significantly (p<0.05) lower in the control group compared to the treatment groups. The same trend was observed in the value of the packedx cell volume (PCV). The control group had significantly (p<0.05) lower packedx cell volume (PCV), exceptx that the group sharedx the same significant (p<x 0.05)x level with the rat group administered 600 mg/kg body weight of crude methanol andx 300 mg/kg body weight of crude ethanol.

The MCV values ranges from 37.50 mg/L to 56.50 mg/L. The least value of MCV was obtained for the control group whichx significantly (p<0.05) differs from the treatment groups except for the groups treated with 300 andx 600 mg/kgx body weightx of n-Hexane fraction of crude ethanol extract. Thex control group shared the same level of significance (p< 0.05) with the 300 and 600 mg/kgx body weight of n-Hexane fractionx of crude ethanol extract and they were significantly (p< 0.05) lower than the rest of the groups. MCH values ranges from 17.50mg/L in the group administered 300 mg/kgx body weight of crude methanol to 27.00 mg/Lx in the group treated with 300 mg/kg body weight of n- Hexane fraction of crude ethanol extract. The MCH of the control group was significantly (p<0.05) higher in some cases and significantly (p<0.05) lower in others while sharing the same level of significant (p<x 0.05) with the other treatmentx groups.

# Table 4.16: Haematological values of Rats treated with *Helianthus annuus* Seed Extracts for 28 Days compared to the Control Group

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Haematological parameters** | | | | |
| **Dosage**  **(mg/kg bw)** | **MCHC(g/dL)** | **RBC(1012/L)** | **PLC(106/L)** | **TWBC(1012/L)** |
| MC10 | 42.00±3.46b | 7.70±0.69abc | 149.00±3.46ab | 7.15±0.43bc |
| MC300 | 38.50±3.75ab | 9.95±0.26def | 148.50±0.87ab | 9.40±0.52de |
| MC600 | 41.50±2.60b | 8.70±0.52bcde | 153.00±2.31b | 10.95±0.78ef |
| MH10 | 42.00±1.15b | 7.00±0.81ab | 153.00±1.73b | 7.25±0.95bcd |
| MH300 | 43.50±3.18b | 8.50±0.35abcd | 154.50±2.02b | 7.30±0.81bcd |
| MH600 | 40.50±2.60b | 7.75±0.61abc | 148.50±6.06ab | 11.55±0.49f |
| EC10 | 39.50±1.44ab | 6.60±0.58a | 142.00±1.73a | 7.90±0.17bcd |
| EC300 | 42.38±2.13b | 8.11±0.64abcd | 147.13±3.56ab | 6.06±0.83ab |
| EC600 | 32.00±1.73a | 10.85±0.95f | 155.50±2.02b | 4.80±0.17a |
| EH10 | 36.50±1.44ab | 8.30±0.52abcd | 149.00±2.31ab | 8.55±0.43cd |
| EH300 | 37.50±3.50ab | 8.08±0.58abcd | 147.50±2.50ab | 6.93±0.53bc |
| EH600 | 40.50±1.44b | 10.55±0.49ef | 150.50±3.75ab | 7.05±0.95bc |
| NC | 42.50±1.44b | 9.55±0.55cdef | 146.00±1.73ab | 9.45±0.61de |

Values are presented as Mean ± Standard Error of Mean (SEM) of three replicates. Values with different superscripts in a column are significantly different at p<0.05. Key: MC= Methanol crude extract, MH= n- hexane fraction of methanol crude extract, EC= Ethanol crude extract, EH= n-Hexane fraction of ethanol crude extract, NC= normal control. MCHC=Mean cell haemoglobin concentration, RBC=Red blood cells, PLC=Platelet count and TWBC= Total white blood cells

The results inx Table 4.16 show the hematological parameters of the rats treated withx *Helianthus annuus* seed extracts. MCHC values ranged from 32.00 % in the group treated with 600 mg/kg body weight of crude ethanol to 43.50x % in the group treated with 300 mg/kg body weight of n-hexane fraction of crude methanol. The MCHC of the control group was notx significantly (p<0.05) different from most of the treatedx groups exceptx inx the groups treatedx with 300mg/kg body weight of crude methanol, 300 andx 600 mg/kg body weightx of crude ethanol as well as 10 andx 300 mg/kg body weight of n-Hexane fraction of crude ethanol extract where it was significantly (p<0.05) higher.

The redx blood cell (RBC) of the rats ranges from 6.60 in the group treated with 10 mg/kgx body weight of crude ethanol extracts tox 10.85 in the group treated with 600 mg/kgx body weight of crude ethanol extract. The RBC value of the control group was significantly (p<0.05) higher thanx mostx of the treatment groups except inx the groups treated with 300 mg/kg body weight of crude ethanol extract. It however shared the same level of significance (p< 0.05) with the group treated with 600 mg/kg body weightx of n-Hexane fraction of crude ethanol extract.

The plateletx count (PLC) value ranges fromx 142.00 mg/L in group treated withx 10 mg/kg body weightx of crude ethanol to 155.50 mg/L in the group treated with 600 mg/kg body weight of crude ethanol. Only the platelet of the group treated with 10 mg/kgx body weight of crude ethanol was significantly (p<0.05) lower thanx the control group. The remaining groups either shared the same significant level (p<0.05) with or had significantly (p<0.05) higher PLC than the control group. The groups with platelet values significantly (p<0.05) higher than the control includes the groups treatedx 600 mg/kg body weightx of crude methanol, 10 and 300 mg/kg body weightx of n-hexane fractionx of crude ethanol extracts and 600 mg/kgx body weight of crude ethanol extract.

The total white blood cells (TWBC)x ranges from 4.80 mg/L in the groupx treated withx 600 mg/kg body weight of crude ethanol to 11.55 mg/kg body weightx of n-hexane fraction of crude ethanol. The TWBC was significantly (p<0.05)x higher in the group treated with the control comparedx to mostx of the other groups treatedx withx the various extract except in the group administered 300 and 600 mg/kgx body weight of crude methanol and 600 mg/kg body weight of n-hexane fraction of the crude ethanol. While the groupx treatedx with 300 mg/kg body weight of crudex methanol shared the same level of significance (p< 0.05) with the control group, the 600 mg/kgx body weight of crude methanol and 600 mg/kgx body weight of n-hexane fraction of the crude ethanol were significantly (p<0.05)x higher than the control groups

**Table 4.17: Relative Body Weight of Rats infected with *Escherichia coli* and treated with *H. annuus* Seed Extracts**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **Body weight (g)** |  |
| **Dose (mg/kg bw)** |  | ***E. coli*** |  |
|  | WEEK-0 | WEEK-1 | WEEK-2 |
| MC100 | 115.75±21.10a | 120.40±8.75ab | 140.16±8.76a |
| MC200 | 106.23±27.27a | 114.02±13.31a | 129.24±10.07a |
| MC400 | 120.21±26.71a | 123.94±20.17ab | 147.53±17.61a |
| MH100 | 116.01±30.09a | 115.32±20.71a | 131.10±17.30a |
| MH200 | 100.08±14.29a | 114.25±9.84a | 129.94±8.92a |
| MH400 | 104.01±17.81a | 112.72±17.00a | 130.58±16.84a |
| Cipro 20 | 106.23±23.37a | 129.37±12.35ab | 145.09±14.88a |
| Negative | 164.74±32.72a | 177.82±36.64b | 174.62±39.91a |

Values are presented as Mean ± Standard Error of Mean (SEM) of three replicates. Values with different superscripts in a column are significantly different at p<0.05. While MC= Methanol crude extract, MH= n- hexane fraction of methanol crude extract, negative = distilled water.

The results inx Tablex 4.17 show relative body weight of rats infected withx *Escherichia coli* and treated with *H. annuus* seeds extracts. There was nox significant difference (p< 0.05) in the rat treated with the variousx extracts, the negative and the positive control groupsx at the start of the administration at week 0 with the group infected with *E. coli* but there was significant (p<0.05) difference at week 1. The relative body weight of the group administeredx with the negative control was significantly higher (p<0.05) over the other groups. The relative body weight of the group administered 20 mg/kgx body weight ciprofloxacinx positive control and the groups administered 100 and 400 mg/kg body weight shows no significant difference (p< 0.05), butx were significantly difference (p<0.05) from the other groups and the negative control. At week 2, the significant

(p<0.05) difference observedx in thex relative body weight in weekx 1 was nox longer visible for rats infectedx withx *E. coli*.

**Table 4.18: Relative Body Weight of Rats infected with *Salmonella spp* and treated with *H. annuus* Seed Extracts**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **Body weight (g)** |  |
| **Dose (mg/kg bw)** |  | ***Salmonella sp.*** |  |
|  | WEEK-0 | WEEK-1 | WEEK-2 |
| MC100 | 116.70±21.58ab | 138.78±17.95ab | 155.70±15.16b |
| MC200 | 115.97±2.42ab | 138.60±3.80ab | 151.76±2.41b |
| MC400 | 90.07±12.82ab | 109.57±14.79ab | 129.11±11.64ab |
| MH100 | 132.27±15.74ab | 140.69±15.69ab | 144.83±17.11b |
| MH200 | 158.78±30.05b | 172.20±27.53b | 163.39±22.23b |
| MH400 | 160.82±23.74b | 164.98±18.41b | 157.35±12.86b |
| Cipro 20 | 82.69±13.29a | 91.02±10.45a | 116.53±7.66a |
| Negative | 145.63±25.34b | 143.93±26.61b | 152.61±19.95b |

Values are presented as Mean ± Standard Error of Mean (SEM) of three replicates. Values with different superscripts in a column are significantly different at p<0.05. While MC= Methanol crude extract, MH= n- hexane fraction of methanol crude extract, negative = distilled water.

The results in Table 4.18 show relative body weight of rats infected with *Salmonella spp* and treated with *H. annuus* seeds extracts There were significant (p<0.05) difference inx the relative body weight of rats infected withx *Salmonella spp* atx week 0 andx 1 butx the significant difference (p<0.05)x also was no longer visible at week 2*.* The relative body weight of the group administered 200 mg/kg body weight and 400 mg/kgx body weight of n-hexane fraction of crude methanol extracts were significantly (p<0.05) higher over the

rest of the groups including the control groups at week 0 for the groups infected with

*Salmonella spp.* Similar results were replicated at week 2 as earlier observed.

**Table 4.19: Total Bacteria Load (*Salmonella spp* and *Escherichia coli*) in Rats at 7 days before treatment with *H. annuus* Seed Extracts**

|  |  |
| --- | --- |
| Colony Forming Units (×106CFU/mL) | |
| *E.coli* | *Salmonella sp.* |
| 12.00±1.73a | 16.00±2.89a |
| 12.00±0.58a | 18.50±0.87a |
| 17.00±1.15b | 17.00±3.46a |
| 13.00±1.73ab | 17.00±2.31a |
| 13.50±0.87ab | 13.50±0.87a |
| 9.50±0.29a | 14.00±1.73a |
| 13.00±2.31ab | 14.00±2.31a |
| 13.50±0.29ab | 15.00±1.15a |

Values are presented as Mean ± Standard Error of Mean (SEM) of three replicates.Values with different superscripts in a column are significantly different at p<0.05.MC= Methanol crude extract, MH= n-hexane fraction of methanol crude extract, negative = distilled water.

The results in Table 4.19 show the colony forming unit of *E. coli* and *Salmonella sppx* before treatment with extractsx from *Helianthus annuus* seed using different solvents, ciprofloxacin and the negative control. The colony forming unitsx ranges from 9.50±0.29a to 17.00±1.15b (×106CFU/mL) for groups infected with *E. coli*. The group to be treated with Methanol crude had significantly (p<0.05) higher colony forming unit comparedx to other groups. While other groups share the same level of significance. There was no significant difference in the colony forming units of *Salmonella* as all the groups share equal level of significancex (p<0.05).

**Table 4.20: Totalx Bacteria Load (*Salmonella spp*x and *Escherichia coli*) inx Rats after treatment with *H. annuus* Seed Extracts**

|  |  |  |
| --- | --- | --- |
| Dose (mg/kgx bw) | Colony Forming Unit (×106CFU/mL) | |
|  | *E. coli* | *Salmonella sp.* |
| Negative | 13.50±2.02d | 10.50±2.02c |
| Ciprofloxacin | 0.00±0.00a | 0.00±0.00a |
| MC100 | 4.00±0.58c | 2.00±0.58b |
| MH100 | 1.50±0.29b | 0.00±0.00a |
| MC200 | 1.00±0.58b | 0.00±0.00a |
| MH200 | 0.00±0.00a | 0.00±0.00a |
| MC400 | 0.00±0.00a | 0.00±0.00a |
| MH400 | 0.00±0.00a | 0.00±0.00a |

Values are presented as Meanx ± Standard Error of Meanx (SEM) of three replicates. Values with different superscripts inx a column are significantly different at p< 0.05.While MC= Methanol crude extract, MH= n-hexane fraction of methanol crude extract, negative = distilledx water.

The results inx Table 4.20 show the colony forming unit of *E. coli* and*x Salmonella spp* treated with extracts from *Helianthus annuus* seed usingx different solvents, ciprofloxacin and the negative control. A dose dependent effectx of the plant extract against both bacteria was observed. The total bacteria load decreased with increasingx plant extract concentration. The negative control had significantly (p<0.05) higher colony forming unit for both *E. coli* andx *Salmonella spp* compared to the other extracts. The crude methanol extract had four (4) units for *E coli* and two (2) for *Salmonella sppx* while n-hexane fraction of crude methanol extract at 100 mg/kg body weight and crude methanol at 200 mg/kg body weight concentration had 1.5±0.29 and 1±0.58(×106CFU/mL) respectively. The other extracts atx different concentrationx had no colony forming unit (Significantly lower than the negative control butx comparable tox the standard drugx group).

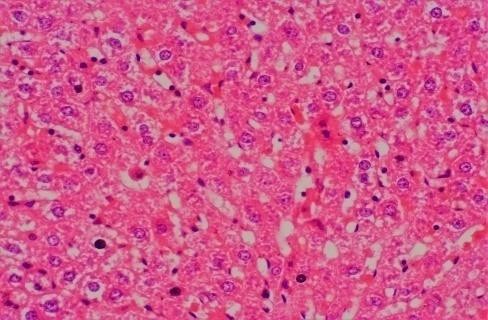
# Table 4.21: Relativex Body Weight of Rats treated with *H. annuus* Seed Extracts

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Dose (mg/kgbw)** |  |  | **Body weight (g)** | |  |
|  | **Week-0** | **Week-1** | **Week-2** | **Week-3** | **Week-4** |
| MC10 | 88.56±17.71a | 95.19±18.26a | 104.43±18.37a | 109.96±17.19a | 122.00±14.28a |
| MH10 | 92.79±7.73a | 102.27±7.02a | 110.66±6.81a | 118.76±6.33a | 127.63±5.34ab |
| EC10 | 89.49±10.93a | 100.80±11.45a | 111.95±12.53a | 122.81±12.63a | 134.41±13.23ab |
| EH10 | 104.65±5.81a | 112.02±4.51a | 124.25±4.52a | 131.96±3.80a | 141.08±4.06ab |
| MC300 | 98.73±4.13a | 105.56±5.31a | 116.12±6.03a | 125.29±7.31a | 134.19±7.25ab |
| MH300 | 91.66±7.09a | 101.79±6.55a | 111.79±6.03a | 121.99±5.36a | 128.15±4.55ab |
| EC300 | 102.38±16.46a | 115.46±17.46a | 126.52±19.90a | 140.77±19.36a | 154.09±18.39b |
| EH300 | 104.11±9.35a | 113.38±9.12a | 124.53±8.59a | 135.39±8.57a | 145.81±8.32ab |
| MC600 | 87.96±10.93a | 97.68±10.91a | 106.73±9.89a | 117.40±9.99a | 128.66±10.03ab |
| MH600 | 80.50±5.75a | 88.84±6.62a | 100.45±5.68a | 109.59±4.83a | 119.36±4.19a |
| EC600 | 98.05±8.17a | 106.15±7.98a | 116.32±5.93a | 123.44±5.86a | 131.91±5.90ab |
| EH600 | 89.80±6.15a | 98.95±5.48a | 111.14±5.05a | 120.18±4.12a | 128.67±3.90ab |
| CONTROL | 94.26±5.66a | 103.24±3.74a | 112.83±3.00a | 120.83±2.76a | 129.49±1.91ab |

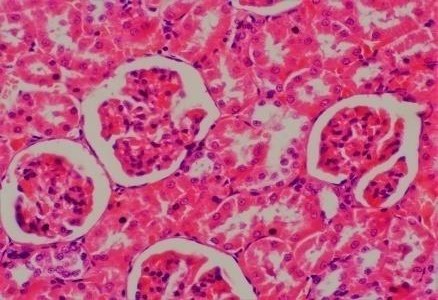
Values arex presented as Meanx ± Standardx Error of Meanx (SEM) of threex replicates. Values withx different superscripts in a columnx are significantly different at p< 0.05. While MC= Methanol crude extract, MH= n-hexane fraction of methanol crude extract, EC= Ethanol crude extract, EH= n-Hexane fraction of ethanol crude extract

The results in Table 4.21 show the relative body weightx of rats treated with the various extract of *H. annuus* seeds compared to control group. Although the groupsx administeredx 10x mg/kg body weight n-Hexane fraction of crude ethanol and 300 mg/kgx body weight n- Hexane fraction of crude ethanol extracts hadx higher relative body weight at the start of the administrationx compared tox the control and the other groups, there was nox significant difference (p< 0.05) in the relative body weight of the rats atx week 0 when the treatment started. The trend continued up tox the thirdx week. However, there was significant difference (p<0.05) amongx the various groups treated with difference extract of *H. annuus* seeds at week four (week 4) whichx marked the end of the treatment. The relative body weight of the group treated withx crude ethanol was significantly (p<0.05) higher over all the other extracts includingx the control group. The controlx shared the same properties with most of the extract except the 300 mg/kg body weight crude ethanol, 10 mg/kg body weight crude methanol andx 600 mg/kgx body weight n-hexane fraction of crude methanol extract. The relative body weight of the group administered 10 mg/kg body weight crude methanol andx 600 mg/kg body weight n-hexane fraction of crude methanol extracts were significantly (p<0.05) lower than the control group.

# Histopathology Results

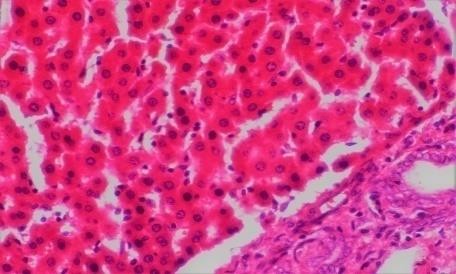


**Plate I: Histopathology of the Liver of Rat Treated with Negative Control (NC-L)** The result in Plate I shows the histopathology of the liver of rat treated with the negative control. The section shows hepatic tissue with preserved architecture composed ofx cords ofx normal hepatocytes. There are foci showingx lymphocytic infiltration of the portal tracts and the few are seen in the sinusoids. There are no features of acute or chronic toxicity or damage.



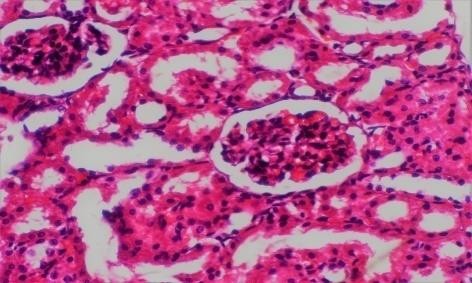
# Plate II: Histopathology of the Kidney of Rat Treated with Negative Control (NC-K)

The resultx inx Plate II shows the histopathology of the kidney of rat treated with Negative control. The section shows renal tissue withx preserved architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic toxicity or damage.



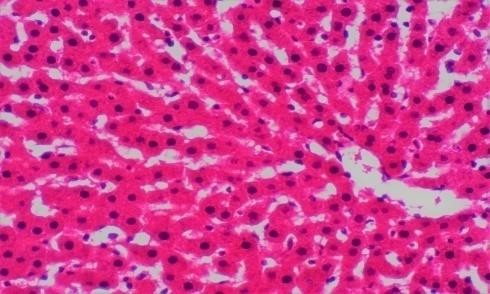
# Plate III: Histopathology of Liver of Rat Treated with n-hexane Fraction of Crude Methanol Extractat 10 mg/kg (MH10 L)

The result in Plate III shows the histopathology of the liver of rat treated with n- hexane fraction of crude methanol extract at 10 mg/kg (MH10L). The section shows hepatic tissue with preserved architecture composed ofx cords ofx normal hepatocytes. There are foci showing lymphocytic infiltration ofx the portal tracts and the few are seen in the sinusoids. There are no features ofx acute or chronic damage.



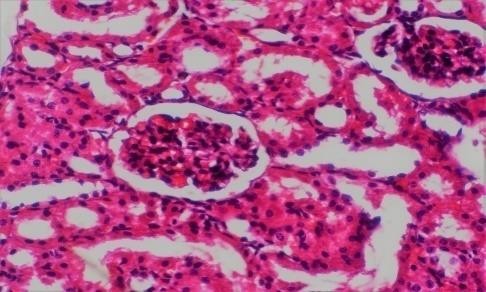
# Plate IV: Histopathology of Kidney of Rat Treated with n-hexane Fraction of Crude Methanol Extract at 10 mg/kg (MH10 K)

The result in Plate IV shows the histopathology of the kidney of rat treated with n- hexane fraction of crude methanol extract at 10 mg/kg (MH10 K). The section shows renal tissue with preserved architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic damage.



# Plate V: Histopathology of the Liver of Rat Treated with n-hexane Fraction of Crude Methanol Extract at 300 mg/kg (MH300K).

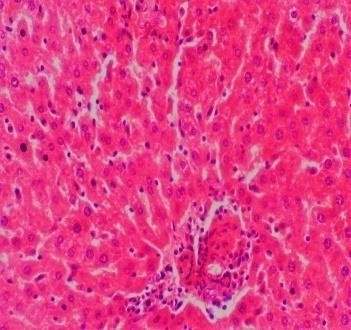
The result in Plate V shows the histopathology of the liver of rat treated with n-hexane fraction of crude methanol extractat 300 mg/kg (MH300 L). The section shows hepatic tissue with preserved architecture composed of cords of normal hepatocytes. There are foci showing lymphocytic infiltration of the portal tracts and the few are seen in the sinusoids. There are no features of acute or chronic damage.



# Plate VI: Histopathology of the Kidney of Rat Treated with n-hexane Fraction of Crude Methanol Extractat 300 mg/kg (MH300 K)

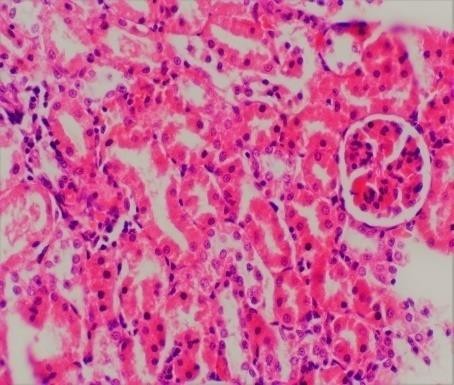
The result in Plate VI shows the histopathology of the kidney of rat treated with n- hexane fraction of crude methanol extractat 300 mg/kg (MH300 K). The section shows

renal tissue with preserved architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic damage.



# Plate VII: Histopathology of the Liver of Rat Treated with n-hexane Fraction of Crude Methanol Extract at 600 mg/kg (MH600L)

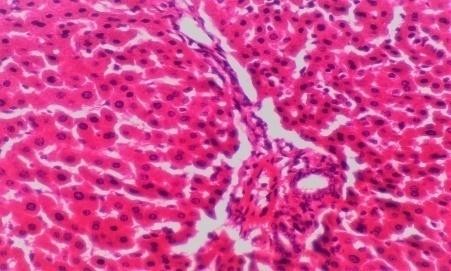
The result in Plate VII shows the liver histopathology of rat treated with n-hexane fraction of crude methanol extract at 600 mg/kg (MH600 L). The section shows hepatic tissue with preserved architecture composed of cords of normal hepatocytes. There are foci showing lymphocytic infiltration of the portal tracts and the few are seen in the sinusoids. There are no features of acute or chronic damage.



# Plate VIII: Histopathology of the Kidney of Rat Treated with n-hexane Fraction of Crude Methanol Extract at 600 mg/kg (MH600K)

The result in Plate VIII shows the histopathology of the kidney of rat treated with n- hexane fraction of crude methanol extractat 600 mg/kg (MH600 K). The section shows

renal tissue with preserved architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic damage.



# Plate IX: Histopathology of the Liver of Rat Treated with Crude Methanol Extractat 10 mg/kg (MC10L)

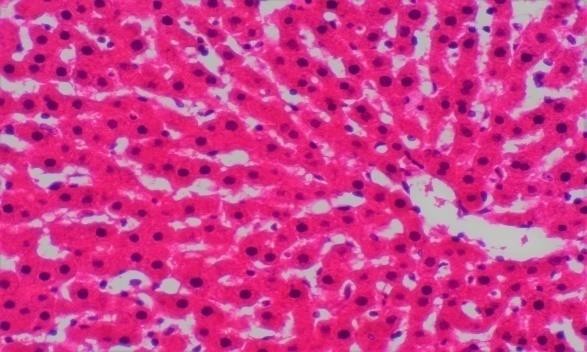
The result in Plate XIII shows the histopathology of the liver of rat treated with crude methanol at 10 mg/kg (MC10 L). The section shows hepatic tissue with preserved architecture composed of cords of normal hepatocytes. There are foci showing lymphocytic infiltration of the portal tracts and the few are seen in the sinusoids. There are no features of acute or chronic damage.



# Plate X: Histopathology of the Kidney of Rat Treated with Crude Methanol Extractat 10 mg/kg (MH10K)

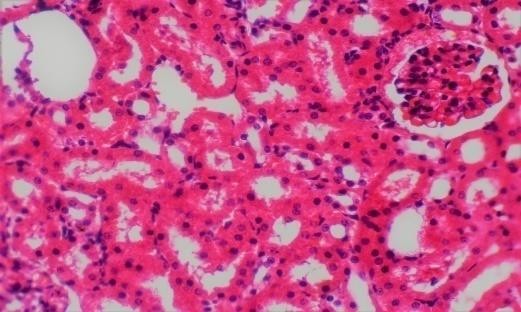
The result in Plate X shows the histopathology of the kidney of rat treated with crude methanol at 10 mg/kg (MC10 K). The section shows renal tissue with preserved

architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic damage.



# Plate XI: Histopathology of the Liver of Rat Treated with Crude Methanol Extract at 300 mg/kg (MH300L)

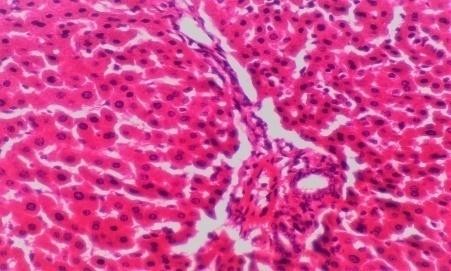
The result in Plate XI shows the histopathology of the liver of rat treated with crude methanol at 300 mg/kg (MC300 L). The section shows hepatic tissue with preserved architecture composed of cords of normal hepatocytes. There are foci showing lymphocytic infiltration of the portal tracts and the few are seen in the sinusoids. There are no features of acute or chronic damage.



# Plate XII: Histopathology of the Kidney of Rat Treated with Crude Methanol Extract at 300 mg/kg (MH300K)

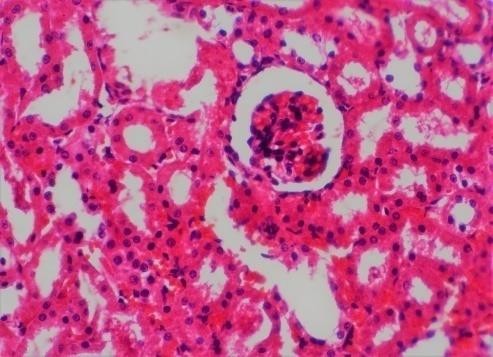
The result in Plate XII shows the histopathology of the kidney of rat treated with crude methanol at 300 mg/kg (MC300 K). The section shows renal tissue with preserved

architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic damage.



# Plate XIII: Histopathology of the Liver of Rat Treated with Crude Methanol Extractat 600 mg/kg (MH600L)

The result in Plate XIII shows the histopathology of the Liver of rat treated with crude methanol at 600 mg/kg (MC600 L). The section shows hepatic tissue with fairly preserved architecture and composed of centrilobular distribution of hepatocyte with cytoplasmic clearing and eosinophilic granular structures in the cytoplasm. The sinusoids appear dilated in areas.



# Plate XIV: Histopathology of the Kidney of Rat Treated with Crude Methanol Extractat 600 mg/kg (MH600K)

The result in Plate XVIII shows the histopathology of the Kidney of rat treated with crude methanol at 600 mg/kg (MC600 K). The section shows renal tissue with preserved

architecture composed of normal glomeruli, tubules and unremarkable interstitium. There are no features of acute or chronic damage.

# 4.2 Discussion

*Helianthus annuus* seed hadx been previously reportedx tox containx a goodx number of phytochemicals. Subashini and Rakshitha (2012) reported thatx methanolic extract of *Helianthus annuus* seeds contain carbohydrates, flavanoids, saponins, phytosterols, tannins, alkaloids andx steroids. The qualitative results in Table 4.1 confirmed some of the claims made by Subashini and Rakshitha (2012). The *Helianthus annuus* seed extracts contain alkaloids, saponins, steroids, tannins as well as othersx like flavonoids, phenols and terpennoids were present. Although, *Helianthus annuus* seed hadx been reported in some to containx anthraquinone andx cardiac glycosides, they were absentx from the finding of this study which is in line with the finding of Verma *et al*. (2017) where these two components were also absent. This variation may be attributed on the type of extractingx solvent employed, the method of extraction engaged or the plant partx used.

The significantly high phenol yield obtained from the quantitative phytochemical analysis of *Helianthus annuus* seed extracted with ethylacetate (META) and aqueous methanolic (MAQS) fractions showed thatx ethylacetate and aqueous methanol were better solvent to extract the phenol component of the seed. Crude methanol, crude ethanol andx aqueous fraction of ethanol were better in the extraction of flavonoids and tannins because they gave significantly higher yield. The n-hexane fraction of methanol and n- hexane fraction of ethanol were better solvent for the extraction of *Helianthus annuus* seed as they yield significantly higher quantitatively. Crude, ethylacetate andx aqueous fraction of methanol were better solvent for the extractionx of alkaloids. The biological activities that plantx extracts elicit arex basically a function of the array of phytochemicals

which act individually or synergistically to show ax broad spectrum of pharmacological activities.

The inhibitory activities of *Helianthus annuus* seed extracted from different solvents againstx *Salmonella spp* revealedx that the aqueous fraction of methanol (MAQS), ethyacetate fractionx of ethanol (META), aqueous fraction of ethanol (EAQS) andx ethylacetate fraction of ethanol (EETA) had no zonex of inhibition. This meansx thatx the above extracts are not effective against *Salmonella spp*. However, crude methanolic extract (MCR), n-hexane fraction of methanol (MHXN), crude ethanol extract (ECR)x n- hexane fraction of ethanol (EHXN) and ciprofloxacin had inhibitory activity againstx *Salmonella spp.*

Amongx the four extracts thatx have inhibitory activities, methanol crude extractx (MCR) and n-hexane fractionx of methanol (MHXN) have significantly higher inhibitory activities compared to crude ethanol extract (ECR) and n-hexane fraction of ethanol (EHXN). Their inhibitory activities were however, lower compared tox ciprofloxacinx usedx as standards or positive control. The larger zonex of inhibition around an antibiotic- containing disk, according to Kirby-Bauer Disk Susceptibility (2021) indicates thatx the bacteria or the test organisms are more sensitive to the antibiotic inx the disk. This means the ciprofloxacin control was more effective against *Salmonella spp* comparedx to the extracts. Inhibitory activities increased with increase in concentration for all the extracts. This means increasing concentrationx of the various extracts of *Helianthus annuus* seedx will increase effect against *Salmonella spp.*

The aqueous fraction of methanol (MAQS), ethylacetate fraction of ethanol (META), aqueous fraction of ethanol (EAQS), crude ethanol extract (ECR), n-hexane fractionx of ethanol (EHXN) and ethylacetate fractionx of ethanol (EETA) hadx no zone of inhibition

againstx *Escherichia coli.* This means these solvent extraction of the *Helianthus annuus* seed were not potent against *Escherichia coli.* Crude methanolic extractx (MCR), n-hexane fraction of methanol (MHXN) and ciprofloxacin which serves as positive control had inhibitory activities against *Escherichia coli.* This means extracting *Helianthus annuus* seed with crude methanol and n-hexane fraction of methanol will be effective against *Escherichia coli.*

Ciprofloxacin wasx more effective against *Escherichia coli* compared to methanol crude extract (MCR) and n-hexane fraction of methanol (MHXN) at 60 mg/ml because it had larger value of inhibitory zone (Kirby-Bauer Disk Susceptibility Test, 2021). At 120 mg/ml, MCR and MHXN were at the same level of significance with the control.The inhibitory zones for MHXN at 240 mg/ml and 480mg/ml was significantly higher than the control. The zone of inhibition also increased withx increase in concentrations of *Helianthus annuus* seed extractx which means *Helianthus annuus* seed extracts will be more potent against *Escherichia coli* at higher concentrations.

The minimum inhibitory concentration (MIC) value (which is the least concentrationx thatx have inhibitory effects) observedx in methanol crudex extractx against *E. coli* and ethanol crude extract against *Salmonella spp* which was comparable to the standardx drug (Ciprofloxacin) corresponds to that observed in the study of Subashini and Rakshitha, (2012) who evaluated the antioxidantx as well asx antimicrobial activity of methanolic extract of seeds from *Helianthus annuus* L. The authorsx reported thatx thex seed extractx showed high sensitivity to *Salmonella typhi.* Similarly, report from the study of Liu *et al*. (2016) showed that sunflower essential oil (SEO) had ax higher antibacterial effectx against

P. aeruginosa, *E. coli* and *S. aureus* thanx monomer mixtures andx each monomers. The work of Eze *et al*. (2015) on in vitrox antibacterial, antioxidant characteristics of *Helianthus annuus* leave extract onx some infectious causative bacteriax revealed an MIC

thatx ranged from 125-500 ug/ml *against E. coli, Staphylococcus aureus, Salmonella enterica* and *Shigella* spp.

The significant lower minimum bactericidal concentration (which represents the least concentration that can kill a testx organism) value of ethanol crude extract against *Salmonella spp.* andx methanol crude extract against *E. coli* were more significant compared to other extracts. In consonance to thex presentx study, Adetunji *et al*. (2010), reportedx similar MBC of ethanol extract against *Staphylococcus aureus, Aspergilus niger* and *Candida albicans*. Also in agreement with the present study, Aboki *et al*. (2012) who evaluatedx the physicochemical andx anti-microbial properties of sunflower (*Helianthus annuus* L.)x seed oil revealedx a low MBC value (thatx is, high bactericidal effect) of the extract against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis* and *Candida albicans*.

The inhibitory and bactericidal effect of *H. annuus* extracts as observed in the present study may be due to the presence of various phytoconstituent (active ingredients) available in the plant sample which act againstx the testx bacteria. It has beenx suggestedx thatx some of the chemical constituents of plants extracts (terpenoid, alkaloidx andx phenolic compounds) interactx withx some enzymes as well as proteins present on the cell membrane of the microbial cell. This then triggers disruption andx disperse a flux of protons towards cell exterior which induces cell death or may inhibit enzymes necessary for aminoacids biosynthesis (Burt, 2004; Gill andx Holley, 2006). Some other researchers have attributed the inhibitory effect of these plant extracts to the hydrophobicity characters of these plants extracts which enable them to react with proteinx of microbial cell membrane and mitochondriax distrupting their structures andx changingx their permeability ( Tiwari *et al*., 2013). Tannins and flavonoids have also been reported to possess as well as induce antibacterial activity against microbes as a result of their ability to inactivate microbial

adhesions, enzymes, cell envelope transport proteins (Cowan, 1999; Ashraf *et al*., 2018). Several alkaloidsx have beenx reportedx tox interfere with DNA, telomeres, telomerase, topoisomerase, the cytoskeleton or protein biosynthesis as a result inducing apoptosis (Wink, 2015; Eid *et al*., 2012; El-Readi *et al*., 2013; Noureini andx Wink, 2014; Wink andx Schimmer, 2018).

It was observed that ethanol extract of *Helianthus annuus* seed showed higher inhibitory and bactericidal effects on *Salmonella spp* while methanol crude extracts showed higher inhibitory and cidal effect on *E. coli* than other extracts. This may be attributed tox the differences in nature as well as concentration of the active ingredients presentx in them (Evans, 2009: Eze *et al*., 2015). Also, the methodx of extraction, part of the plant material being extracted as well as the type of extracting solvent may play vital roles in the varying degrees of antimicrobial activities elicited by the extracts.

The acute toxicity studies revealed the extracts of *Helianthus annuus* seed were non-toxic to the test organisms (rats) as mostx of the extracts did notx record any mortality. Hence both the aqueous andx organic extract are in category 5 of Global Harmonizationx System (>2000-5000 mg/kg b.w.t) (Onzago *et al*., 2014). This is inx agreement with the study of Onoja *et al*. (2018)x who reportedx anx LD50 of hydromethanol extractx of *Helianthus annuus* to be greater than 2000 mg/kg bw. Extracts of *H. annuus* have been reported to be usedx as ingredient in snackx bar, bread roll, chocolates, edible oil, creams among others (Adeleke andx Babalola, 2020). However, only mild reactions were recordedx among few individuals that consumed products containingx *Helianthus annuus* (cometic ingredient review, 2015). Sunflower seed/oil have been reported to have loads of health benefits which are attributed tox its nutritional composition which include highx concentration of monounsaturated and polyunsaturatedx fats, proteins, tocopherols, phytosterols, copper, zinc, folate, iron and vitamin B possessing antimicrobial, antidiabetic, anti‐inflammatory,

antihypertensive, andx antioxidants (Nandha *et al*., 2014; Adeleke and Babalola, 2020). The toxicity of certain plant materials or extracts may be due to the presence of an active constituents lethal to animalsx or substances which interact with others in the system to elicitx harmful effects.

The sub-acute toxicity test which lasted for twenty eight days revealed 50 % mortality for group administered 300 mg/kg body weight of n-hexane fractionx of the crude ethanol. There was also one-out-four mortality record for 300 mg/kgx body weight of crude methanol. Although, the acute toxicity test showed that the plant extracts were safe, a large possibility exists that the presence of the solventx used for extraction might have elicited complications that resulted to death over the period of administration. The death observed inx the group (negative control) administered 10 mg/kg body weight of distilledx water may be termed as a chance event. Generally, It is noteworthy, that the non-toxicity and antibacterial potential of thisx plant extractx may be takenx advantage of by incorporating them into food, skin care, oral health products.

The in vivo antibacterial activity of *Helianthus annuus* seedx extracts against *Salmonella spp* andx *Escherichia coli* is shown in the total bacterial load present in the treated groups. The significant similarity in bacterial loadx between the extracts of *H. annuus* (400 mg/kgx of methanol crude extract and n-hexane methanol fraction) and the standard drugx (Ciprofloxacin) as well as the significantx lower bacterial load shows thatx the extracts have high antibacterial features andx may be used as potential treatment against *E. coli* andx *Salmonella* infections.The zero bacterial load at higher concentrations indicates that the plantx extract has bactericidal effect as thex standard drug.

The standardx drugx (Ciprofloxacin) is a flouroquinolone whichx has been evaluated to be particularly effective againstx gram-negative bacteria at lower concentrations than the

other agents with similar antibacterial spectrax (Masadeh *et al*., 2015). It is used inx the treatment of several infections such as cystitis, urinary tract infections, sinustitis andx certain bacterial infections amongx others (Castro *et al*., 2013). All the extracts of *H. annuus* in the present study contained alkaloid whichx has quinolone. The major mechanism of action of quinolones is inhibition of nucleic acid synthesis. Here, DNA gyrase which is responsible for supercoilingx andx uncoilingx of DNA strands, involved in repair, replication as well as transcription is inhibited (Wink, 2015). It has been reportedx that quinolones induce changesx in the permeability of membranesx and hence disrupt the cell integrity, allows the efflux of the constituentsx of cytoplasm and the deathx of cells (Nam *et al*., 2015).

The histopathology results revealed that there was neither liver nor kidney damage tox the various groups administered differentx concentration of various extracts. This was indicatedx by the fact that both the structure of the liver and kidney as well as their minor organelles were well preserved. This agreedx with the earlier result that *Helianthus annuus* seed was non-toxic even at doses as high as 2000 mg/kgx body weight.

The lipid component of the biochemical parameters revealed that some of the extracts hadx significantly lower total cholesterol, triglycerides and low-density lipoproteins cholesterol (LDL-C). Hyperlipidemia isx one among the major factors that predisposes subjects to cardiovascular diseases (Piche *et al*., 2018). High levels of LDL-C and total cholesterol have tendency to promote atherosclerosis, hence, any intervention that lower the concentrations may be a potential route to prevent or manage cardiovascular related diseases. Evidence from the present study suggests *Helianthus annuus* seed extracts can lower total cholesterol, triglyceridesx and low-density lipoprotein cholesterol slightly. The presence of certain active compounds (like phenols) in the extracts of *H. annuus* may backup the evidence of *H. annuus* having hypolipidemic effects. Polyphenols have been

reportedx to possess cholesterol lowering effects. They bind to cholesterol andx bile acids form complexes as suchx enhancing their evacuation through feaces. This inx turnx reduce the formation of micelle while lipid uptake from intestine to the blood is reduced (Sommella *et al*., 2019). Flavonoids have been reported to have many health benefits as an antioxidantx (Guo *et al*., 2017), and can serve as inhibitor for low-density lipoprotein (LDL) oxidation, and as ax scavenger for DPPH radical activity (Lee *et al*., 2005; Grassi *et al.,* 2010).

Gou *et al*. (2017) reported that *Helianthus annuus* seed extracts have hepatoprotective and lowers the risk of diabetes, arteriosclerosis and hypertension. Among the four lipid components analysed, only high-density lipoprotein cholesterol (HDL-C) was significantly higher in the testx group as comparedx to the control group. Increase inx the high-density lipoprotein had been linked to prevention of cardiovascular diseases according to the report of Connelly *et al*. (2016).

The serum protein component of the biochemical parameters was all within the acceptable level even though there was significant difference among the various extracts at different concentrations. This implied that *Helianthus annuus* seed extracts hadx no overall negative impact on the treatedx experimental rats. Alagawany *et al*. (2015) statedx thatx *Helianthus annuus* seed extracts had no effect on proteinx biochemical parameters. The richness of sunflower in sulfur-rich proteins are ideal for different human metabiological needs such as insulinx production, muscular andx skeletal cell development, and antioxidants (Gou *et al*., 2017).

There was no marked significant difference inx mineral constituents of the biochemical parameters as the varied within the normal range. This supportedx the earlier finding thatx *Helianthus annuus* seed extracts are rich in nutrients. Gou *et al*. (2017) reported thatx

*Helianthus annuus* seedx extracts help to maintain serumx mineral level thereby helping to maintained healthx and boost thex immune system (Nandha, 2014). This benefitx may be linked to the presence of phytochemical like flavonoids. Flavonoids are essential metabolites foundx in the sunflower family. Non-nutrient phytochemicals withx antioxidantx potential in diet are dominatedx by flavonoidx and isoflavone consumption. Arai *et al*. (2000), demonstrated that consumption of high flavonoids and isoflavones lowers the incidence of coronary heart disease as HDL-C carries cholesterol away from the bloodx back tox the liver. Isoflavone isx a phytoestrogen that has been linked to a variety of health benefits, including antioxidation (Goux *et al*., 2017).

The significantly higher total proteins (EHXN andx MHXN treated groups) and the albumin observedx in the test groups showed that there was nox cells destruction andx muscle wasting. The activities of the liver enzymes were within normal range showingx there was nox oxidative stress or liver damage. This may be due to rich antioxidants in *Helianthus annuus* seeds. Antioxidants have long been known to protect cells from oxidative damage andx reduce the risk of chronic diseases (Goux *et al*., 2017). Certain enzymes (catalase, glutathione dehydrogenase amongst others), peptides (reduced glutathione), carotenoids, and phenolic compounds are all examples of natural antioxidants of tocopherols, flavonoids and phenolic acids foundx in *Helianthus annuus* seed. Natural antioxidants and antiglycatives are more efficientx inx treating andx preventingx diabetes (Xi *et al*., 2008) by preventing the formationx of reactive oxygen species (ROS), which trigger a variety of biochemical pathways linkedx tox diabetic complications.

The significant change in weight with time observedx in all the treatment groups for both *Salmonella spp* andx *E. coli* infected rats as well as in comparison with the control groups agrees withx the report of Onoja *et al*. (2015). Contrary tox the result obtained in thisx work, Leverrier *et al*. (2019) reportedx that different concentrations of *H. annuus* extracts

inducedx decrease inx weight of the subjects. They found that, itx decreasedx fat and brown adipose tissue, inx high-fat-diet-fed rats by activation of liver Adenosine monophosphate- activated protein kinase (AMPK)x pathway in a dose-dependent manner. However, there was no significant difference in the weight of treated rats in this study. Although, the presence of certainx phytochemicals have been reported to induce weight gain. Terpenes (Diterpene), a phytochemical present in *H. annuus* canx act directly on adenylate cyclase ( Tucci, 2010). Adenylate cyclase is an enzyme that activates cyclic adenosine monophosphate (cAMP). In turn cAMP promotes lipolysis which increases the body's basal metabolic rate, and increases utilisation of body fat (Litosch *et al*., 1982; Tucci, 2010).

The levels of enzymes (AST, ALT and ALP) in serum are markers used to ascertain functionx state asx well as injury inx some organs. ALP is ax marker enzyme for the plasma membrane andx endoplasmic reticulum (Adeyemi *et al*., 2015), hence an ectoenzyme of the plasma membrane. This enzyme is oftenx employed in assessing the integrity of the plasmax membrane, as its presence in either the tissue and/or serumx wouldx indicate probable damage to the plasma membrane of the cell. Whenever an increase in ALP level ensue, it may indicate possible damage of the membrane, this is being that ALP is a membrane boundx enzyme (Kumar and Gill, 2018). High levels of serum ALP activity is usually noticed in liver damage, cancer andx heart infections.

The transaminase (AST and ALT) enzyme are markers for heart andx liver integrity as well as functions (Adeniyi *et al*., 2010). These enzymes are majorly released from hepatocytes that have beenx injured or damaged (Biegus *et al*., 2016). Elevationx of these enzymes in the serum have been reported to indicate cellular damage, tissue necrosis, as well as a calculated riskx for cardiovascular diseases, withx higher risk of cardiovascular disease andx elevatedx myocardial infarction being attributed to elevation of ALT and AST

respectively. Tissue injury and damage arex majorly as a result of inflammation caused by certain reactions, interactions between the tissue in questionx and other substances.

The significant reduction in concentration of AST, ALT and ALP among rats administeredx extracts of *H. annuus* compared to the negative control, indicates that the plantx extracts have anti-hepatotoxic and tissue healing properties. Thisx may be due to the activities of certain active components present in the plantx extract. Certainx phenolic compounds have been described to possess anti-inflammatory potential. They inhibit NF- κB activation and reduce pathological inflammatory conditions. For example, resveratrol (Kowluru andx Kanwar, 2007) and kaempferol (Luo *et al*., 2015) have been reported to inhibitsx the activation of NF-κB by downregulating IKK phosphorylation, resulting in the suppression of inflammatory cytokines, such as TNF-α, IL-12, and IL-6.

The importance of hematological parameters as indices of physiological as well as pathological status for animals and humans can in no way be played down. Hence, it is employed as an investigative and diagnostic tool for disease. The significant increase observed in some haematological (Hb and MCV) indices of some of thex treatment groups compared tox the normal control group indicates is not in agreement with the report of Onoja *et al*. (2018) who investigated on the ameliorative effects of *Helianthus annuus* againstx nephrotoxic, cardiac and haematological disorders in albino rats. The authors reportedx that there was no significantx difference inx haemoglobinx (Hb) concentrationx andx MCV between the control and treatment groups. The significant increase in PCV level in groups administered 600 mg/kg bw of n-hexane fraction of methanol crude extract and n- hexane fraction of ethanol crude extractx comparedx to other treatment and control groups suggests that at ax high concentration, the plant may be used to ameliorate anaemia (anti- anaemic). Thisx feature may be attributed tox its enhancement of erythropoietinx synthesis by the kidney. Onoja *et al*. (2018) reported that *H. annuus* may possess kidney damage

reversal potential. In agreement tox thex present study, Adesina *et al*. (2017) reportedx non- significant difference inx MCH andx MCHC values of differentx groups fed different percentages of *H. annuus* inclusion into diet. Similarly, the work of Onoja *et al*. (2015) on the ameliorative effects of *H. annuus* on some diseased state in rats agree with the findings in this study as the haematological parameters (MCH and MCHC) were comparable to the control groups.

# CHAPTER FIVE

# CONCLUSION AND RECOMMENDATIONS

# Conclusion

From the findings of this study, it can be concluded that *Helianthus annuus* seed extracts contained alkaloids, saponins, steroids, tannins, flavonoids, phenols and terpennoids while anthraquinone and cardiac glycosides were absent.

The crude methanolic extract (MCR), n-hexane fraction of methanol (MHXN), crude ethanol extract (ECR) n-hexane fraction of ethanol (EHXN) had inhibitory activity (zone of inhibition) against *Salmonella spp* while crude methanolic extract (MCR), n-hexane fraction of methanol (MHXN) had inhibitory activities (zone of inhibition) against *Escherichia coli.*

The inhibitory activity in each case was lower compared to that of ciprofloxacin used as control. Also, the extract of *Helianthus annuus* seed have bactericidal effect against *Salmonella spp* and *Escherichia coli.* Ethanol crude extract of *H. annuus* had the lowest MIC and MBC (that is, most effective against the bacteria) which was closest to the standard drug.

The *in vivo* antibacterial study which was depicted in total bacteria load in infected rats showed that there was no bacteria load (CFU) in rats infected *Salmonella spp* treated with 100, 200 and 400 mg/kg bw which were comparable to the standard drug (ciprofloxacin) group. Meanwhile, only 100 mg/kg bw of Methanol crude extract group had (2) colonies of *Salmonella spp*. The total bacteria load decreased with increasing plant extract concentration in rats infected with *E.coli* and treated with methanol crude and methanol hexane fraction. There were no colonies found at higer concentrations, the

extracts of *H. annuus* may therefore be presumed to have more antibacterial potential against *Salmonella spp* than *Escherichia coli* in vivo.

Most biochemical parameters were within normal range even among the group of rats. Most of the plant extracts significantly reduced total cholesterol, LDL-C, triglycerides, ALT, AST, ALP but increased HDL-C. This may therefore presents the extracts of *H. annuus* to possess anti lipidemic as well as hepato protective properties. As a result, the extracts of *H. annuus* may be useful in cardiac related diseases. The usage of this plant extract will be without fear because it is safe, as the LD50 was recorded to be greater than 2000 mg/kg bodyweight. Moreso, it was observed that the plant extract may possess growth/weight inducing properties as there was progressive weight gain among all the treatment groups through time. The haematological study revealed that MPV, Hb and PCV Levels were increased in most *H. annuus* extracts treated groups. No damage was observed to either the kidney or liver.

# Recommendations

This study investigated the *in vivo* antibacterial effect of different extracts of *Helianthus annuus* seed and found inhibitory as well as bactericidal effect against *Salmonella spp* and *Escherichia coli* in some of the fractions. Hence, the following recommendations can be made based on observations from the study:

* + 1. Fractions of *Helianthus annuus* seed extracts that showed bactericidal activity should be further isolated and characterised to identify the exact active compound or compounds.
    2. Fractions of the *Helianthus annuus* seed extract that recorded mortality for either *Salmonella spp* or *Escherichia coli* should be systematically studied to establish whether those mortality or mortalities were chance event or actual potency of those fractions against the test organisms.
    3. The consumption of *Helianthus annuus* seeds is therefore encouraged due to the antibacterial activity inferred in this study.

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# APPENDIX A (MATERIALS)

1. **Apparatus**

Petri dishes, syringes, gloves, beakers, and conical flasks, test tubes, cotton wool, glass rod, masking tape, permanent marker, whatsmann filter paper and foil paper.

# Chemicals and Reagents

The methanol, ethanol, ethylacetate and hexane used were of analytical grade and were products of BDH Chemicals Ltd, Poole, England. Dimethylsulfoxide a product of Guangdong Guanghua Science Technology Co. Ltd, China. Crystal violet, iodine, alcohol, safranin and methyl-red reagent.

# APPENDIX B (ETHICAL APPROVAL)

