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الملخص

القسط الهندي C. speciosus نبات ينتمي إلى أنواع القسط المستخدمة كغذاء وظيفي كما هو موصوف في الطب النبوي ويستخدم على نطاق واسع في الأدوية التقليدية لعدة آلاف من السنين .استهدفت هذه الدراسة التعرف على تحديد المركبات الفينولية للمستخلص المائي والإيثانولي والميثانولي لنبات C. speciosus ، كذلك تحديد المركبات الفينولية بواسطة جهاز التحليل الكروماتورافي السائل عالي الاداء (HPLC) ، وتقييم التأثير المضاد للأكسدة ، التأثير المضادة للسرطان والتجلط وتقييم الدور الوقائي للمستخلص الا يثانولي ضد الإجهاد التأكسدي الناتج عن الزيرالينون (ZN) في الفئران .وكانت اهم النتائج المتحصل عليها هي إجمالي محتوى الفالفونويد والبوليفينول في المستخلص الإيثانولي كان الأعلى يليه المستخلص الميثانول ثم المستخلص المائي وزاد الناتج من هذه المركبات بزيادة وقت الاستخلاص . أظهرت نتائج تحليل HPLC وجود 13 مركبًا في كل مستخلص بتركيز مختلفة وكانت مركبات الكيرسيتين وحمض الكافيك وحمض الغاليك عالية في المستخلص المائي ، بينما كانت المستخلصات الكحولية غنية بحمض السيرنجيك وحمض الكلوروجينيك والنارينجينين. أوضحت النتائج ايضا أن جميع المستخلصات المختبرة لها تأثيرمضاد للأكسدة مع بعض الاختلافات الطفيفة باستثناء اختبار بيتا كاروتين ، reducing power ions, chelating assay حيث أظهرت المستخلصات الكحولية نشاطًا أعلى ب 50٪ مقارنة بالمستخلص المائي.أظهر المستخلص الميثانولي خاصية قويه كمضاد للسرطان ضد الخلايا السرطانيةHePG2 متبوعًا بالمستخلص الإيثانولي، ثم المستخلص المائي الذي أظهر خاصية ضعيفة. أظهرت نتائج الأنشطة المضادة للتجلط مخبريا او في الفئران أن المستخلصات تؤدي الى زيادة زمن التجلط حيث بشكل ملحوظ زمن الثرومبوبلاستين الجزئي المنشط (aPTT) ووقت البروثرومبين (TT) ووقت الثرومبين (PT) مقارنة بالمجموعات المقارنة .أظهر الإجهاد التأكسدي الناجم عن ZN في الفئران إلى زيادة معنوية في نشاطية الأنزيمات ALT ، AST، ALP، ومستويات الكرياتينين ، حمض اليوريك ، اليوريا ، الكوليسترول ، الدهون الثلاثية ، LDL، نشاط عامل الوخز الورمي ، ألفا فيتو بروتين، TNFα، interleukin-6 والمالونالدهايد مصحوبًا بانخفاض كبير في البروتين الكلي ، الألبومين والاحماض الدهنيى منخفضة الكثافة (LD L) ومحتوى الكبد والكلية من الانزيمات لمضادة للاكسده (TAC, GPx, CAT) حيث أستطاع المستخلص الايثانولي احداث تحسنا في جميع القياسات محل الدراسة في اتجاه القيم الطبيعية للحيوانات المقارنة. أدت المعامله بـ ZN في حدوث تغيرات نسيجية كبيرة في الكبد والكلى أثبتت النتائج أن المعالجة بالمستخلص الايثانولي بمفرده لم يكن لها تاثيرات علي التركيب النسيجي للكبد والكلى . كما أشارت النتائج ان المعالجة بجرعة عالية منعت التغيرات النسيجية في الكبد والكلى الناتجة عن الزيرالينون.نستخلص من هذه الدراسة أن مستخلصات جذور القسط أظهرت إمكانات عالية للأنشطة البيولوجية وخصائص وقائية لكبد ولكلى تجاه الكرب التاكسدي المحدث بواسطة الزيرالينون والتي ارتبطت بمحتواها العالي من البوليفينول والفلافونويد.

الكلمات المفتاحية : القسط الهندي, البوليفينول ,HPLC,مضاد للأكسدة ,مضاد لسرطان ,مضاد لتجلط, الزيرالينون, الإجهاد التأكسدي , حماية الكبد والكلية.

Abstract

Costus specious is a plant that belongs to *Costus species*, used as functional food as described in the Prophetic medicine and used widely in traditional medicines for many thousand years. This study aimed to identify the chemical profile of the phenolic compounds of the aqueous, ethanolic and methanolic extracts of C. *speciosus* under different extraction conditions, to determine the phenolic by HPLC, and evaluate, *in vitro* and *in vivo,* their antioxidant, anticoagulant and anticancer activities. The protective role of the ethanolic extract against zearalenone (ZN) as an inductor of oxidative stress in the liver and kidney in rats was also established. The obtained results indicated that : The content of total falvonoids and polyphenol in the ethanolic extract was the highest followed by the methanolic then the aqueous extracts and is was increased by increasing or extraction time. The HPLC analysis identified a total of 13 compounds in each extract with different concentrations. Quercetin, caffeic acid, and gallic acid were high in the aqueous extract, while the alcoholic extracts were rich in syringic acid, chlorogenic acid and naringenin. All the tested extracts have an appreciable antioxidant inhibiting effect with some slight variations except for beta-carotene test, reducing power ions, chelating assay where the extracts showed an activity higher than 50% compared to the aqueous extract. The methanolic extract showed a potential anticancer property against HePG2 cancer cell lines followed by the ethanol extract, then the water extract which showed a weak anticancer property. The *in vitro* and *in vivo* anticoagulant activities tests showed that the extracts prolonged significantly the activated partial thromboplastin time (aPTT), prothrombine time (TT) and thrombin time (PT) compared to the negative control. The oxidative stress induced by ZN in the rat showed a significant increase in serum ALT, AST and ALP activites ,level of creatinine, uric acid, urea, cholesterol, triglycerides, LDL, carcinoembryonic antigen, alpha-fetoprotein, TNFα, interleukin-6, malondialdehyde and a significant decrease in the content of serum TP, albumin, HDL, hepatic and renal TAC, CAT and GPx. Treatment of the stressed rats with the ethanolic extract resulted in improvement of all biochemical markers by restoring their values to normal. Histologically, the induced stress also caused significant tissue changes in the liver and kidneys. However, most of them were counteracted by ethanolic extract administration and the high dose was more effective than the low dose. In conclusion, the extracts of *c. speciosus* have a high potential biological activities and preventive and protective properties towards the liver and the kidneys these activities were linked to their high contents of polyphenols and flavonoids.

Keywords: *Costus speciusus* Phenolic Compounds, HPLC, Antioxidant, Anticoagulant, Anticancer, Zearalenone, Oxidative stress, Hepato-nephroprotectve.

Resumé

Costus speciosus est une plante qui appartient au genre *Costus*, c'est un aliment fonctionnel qui a été décrit en médecine prophétique et qui est largement utilisé dans les médicaments traditionnelle depuis plusieurs milliers d'années. Cette étude vise a établir le profil chimique des composés phénoliques des extraits aqueux, éthanoliques et méthanoliques de *C. speciosus* sous différents conditions d'extraction, à déterminer les composés phénoliques par HPLC, en évaluant, *in vitro* et *in vivo*, leurs activités antioxydantes, anticoagulantes et anticancéreuse. Le rôle protecteur de l'extrait méthanolique contre le stress oxydatif induit par la zéaralénone (ZN) dans le foie et les reins chez le rat a été aussi établi. Les résultats obtenus indiquent que: La teneur totale en polyphénols et flavonoïdes dans l'extrait éthanolique est plus élevée que dans les extraits méthanoliques et aqueux et augmente avec le temps d'extraction. L'analyse par HPLC a permis d'identifier 13 composés dans chaque extrait à des concentrations différentes. La quercétine, l'acide caféique et l'acide gallique sont les principaux composés de l'extrait aqueux, tandis que les extraits alcooliques sont riches en acide syringique, en acide chlorogénique et en naringénine. Tous les extraits testés ont un effet antioxydant significatif avec de légères variations sauf pour les tests de bêta-carotène, du pouvoir réducteur et chélateur où les extraits alcooliques ont montré une activité supérieure de 50% par rapport à l'extrait aqueux. Les extraits méthanoliques et éthanoliques ont montré une propriété anticancéreuse potentielle contre la ligné cellulaire HePG2, tandis que l'extrait aqueux a présenté une faible activité. Les tests des activités anticoagulantes, *in vitro et in vivo*, des extraits ont montré que les extraits prolongent de manière significative le temps de thromboplastine partielle activée (aPTT), le temps de prothrombine (TT) et le temps de thrombine (PT) par rapport au témoin négatif. Le stress oxydatif induit par la ZN chez le rat a montré une augmentation significative de l'activité del' ALT sérique, de l'AST et de l'ALP, de la teneur de la créatinine, de l'acide urique, de l'urée, du cholestérol, des triglycérides, du LDL, de l'antigène carcinoembryonnaire, de l'alpha-foetoprotéine, du TNFα, de l'interleukine-6, du malondialdéhyde et une diminution importante de la teneur en TP sérique, albumine, HDL, TAC hépatique et rénale, CAT et GPx. Le traitement des rats stressés avec l'extrait éthanolique a amélioré tous les marqueurs biochimiques en rétablissant leurs valeurs à la normal. Par ailleurs et sur le plan histologique, le stress induit a entrainé des modifications tissulaires importantes au niveau du foie et des reins. L'extrait éthanolique a exercé un effet préventif et protecteur du foie et des reins contre toute altération à la dose élevée.En conclusion, les extraits de *C. speciosus* possèdent un fort potentiel d'activités biologiques et des propriétés préventives et protectrices vis-à-vis du foie et des reins qui étaient liées à leurs teneurs élevées en polyphénols et des flavonoïdes.

Keywords: *Costus specious*, Polyphénols, Analyse HPLC, Antioxydantes, Anticoagulantes, Anticancéreuse, zéaralénone, stress oxydant, hepatonéphroprotecteur.

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ABTS	2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AFP	Alpha-fetoprotein Tumor Marker
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
AST	Aspartate Amino Transferase
APTT	Activated partial thromboplastin
BHT	Butylatedhydroxytoluene
BHA	Butylatedhydroxyanisole
CSE	Costus speciosus ethanolic
DF	Degrees of freedom
DPPH	2, 2-diphenyl-1-picryl-hydrazyl
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immuno-sorbent assay
FBS	Fetal bovine serum
GGT	Gamma Glutamyltransferase
GPX	Glutathion Peroxidse
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSH-PX	Glutathione Peroxidase
GSSG	Glutathione disulfide
GAE	Gallic acid equivalent
IL6	Interleukin6
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein cholesterol
HPLC	High Performance Liquid Chromatography
IC50%	Inhibitory concentration for 50% of activity
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MS	Mean square

MN	Micronucleus (MN)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MNPCE	micronucleated polychromatic erythrocytes
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NCE	normochromic erythrocytes
OTM	Olive tail moment
PT	Prothrombin time
PC	Protein C
PCE	polychromatic erythrocytes,
PPP	Platelet poor plasma
ROS	Rective oxygen species
RNS	Reactive nitrogen species
TAC	Total antioxidant capacity level
TG	Triglycerides
TAC	Total antioxidant capacity
TMB	Thiobarbituric acid
TPP	Tripolyphosphate
TPA	Plasminogen activator
TNFα	Tumor necrosis factor alpha
TM	Tail moment
TFPI	Tissue factor pathway inhibitor
VLDL	Very low-density lipoproteins
WHO	World Health Organization
ZN	Zearalenone

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Introduction

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer (Kinnula and Crapo, 2004), cardiovascular disease (Singh and Jialal, 2006), neural disorders (Sas *et al*., 2007), Alzheimer's disease (Smith *et al*., 2000), mild cognitive impairment (Guidi *et al*., 2006), Parkinson's disease (Bolton *et al*., 2000), alcohol induced liver disease (Arteel, 2003), ulcerative colitis (Ramakrishna *et al*., 1997), aging (Hyun *et al*., 2006) and atherosclerosis (Upston *et al*., 2003). Bioactive compounds, such as polyphenols and the secondary metabolites flavonoids and proanthocyanidins, derived from plants have been associated with various health benefits (Vuong *et al*., 2014; Dailey and Vuong, 2015).

Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Alothman *et al*., 2009 ; Bonoli *et al*., 2004) . Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Pérez *et al*.,2008). The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects (Loliger, 1991). Therefore, many attempts have been made to extract and isolate bioactive compounds from plant materials for utilization in the food and pharmaceutical industries. As bioactive compounds range from very polar to very non-polar compounds, the extraction solvent plays an important role in extraction efficiency of bioactive compounds from the plant materials (Pinelo *et al*., 2005; Ye *et al*., 2015).

In Arabic civilization or tradition, prophetic medicine corroborated by clinical and epidemiological researches and evidences constitute a platform for mankind to cure their ailments (Sheikh *et al*., 2017; Hussain and Hussain, 2016). In this context, Costus plant species which include *C speciosus* is one the functional food cited in Prophetic medicine used widely in traditional medicines for many thousand years (Choudhary *et al*., 2015). *Costus speciosus* (family Costaceae) is an herbaceous plant widely growing in South East Asian countries such as India, Malaysia, Srilanka and Indonesia (Anonymous, 2007; Rani, 2012). The rhizome is used in the traditional system to treat bronchitis, fevers, dyspepsia,

inflammations, anaemia, rheumatism, lumbago and hiccough (El-Far *et al*., 2016 ; El-Far *et al* 2018). The natives of North-east India use the rhizome in urinary troubles, fever, headaches and also to dissolve kidney stones (Kumar, 2012; Jha *et al*., 2010)

The aims of this study are:

- ✓ Studying the effects of different solvents and extractions time on the yield and of biological compounds of *C speciosus.*
- ✓ Determination of the bioactive compounds in the extracts using HPLC.
- ✓ Determination of total polyphénols and flavonoides contents in *C speciosus* extracts.
- ✓ Evaluation of the antioxidant activity of *C speciosus* extracts using different assay (DPPH, ABTS, chelation of iron, Cuprac, β-carotene and Phenanthroline).
- ✓ Evaluation *in vitro* of the anticancer activity of the extracts of *C speciosus.*
- ✓ Evaluation *in vitro* and *in vivo* of the antithrombotic activity of *C speciosus* extracts.
- ✓ Evaluation of the therapeutic potential protective effects of *C speciosus* ethanolic extract against zeralenone-induced oxidative stress, genotoxicity and histological changes in the liver and kidney of rats.

1. Medicinal plant

Throughout the ages, humans have relied on nature for their basic needs, for the production of food, shelter, clothing, transportation, fertilizers, flavours and fragrances, and medicines (Cragg and Newman, 2005). Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and probably thousands of years of use. The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2 600 BC (Heinrich and Teoh, 2004). Among the substances that were used are oils of *Cedrus species* (cedar) and *Cupressus sempervirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora species* (myrrh) and *Papaver somniferum* (poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation. (Beissert and Schwarz, 2002)

1.1 Traditional medicine

Plants have been utilized as medicines for thousands of years (Samuelsson, 2004). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Samuelsson, 2004). The specific plants to be used and the methods of application for particular ailments were passed down through oral tradition. Eventually information regarding medicinal plants was recorded in herbal phamacopoeias (Balunas, 2005)

1.2. African traditional medicine

African traditional medicine in its varied forms is holistic, involving both the body and the mind. The healer typically diagnoses and treats the psychological basis of an illness before prescribing medicines to treat the symptoms. Well known African medicinal plants include *Acacia senegal* (gum arabic), *Agathosma betulina* (buchu), *Aloe ferox* (Cape aloes), *Aloe vera* (north African origin), *Artemisia afra* (African wormwood), *Aspalanthus linearis* (rooibos tea), *Boswellia sacra* (frankincense), *Catha edulis* (khat), *Commiphora myrrha* (myrrh), *Harpagophytum procumbens* (devil's claw), *Hibiscus sabdariffa* (hibiscus, roselle), *Hypoxis hemerocallidea* (African potato), *Prunus africana* (African cherry) (Newman *et al*., 2000).

Madagascar has contributed *Catharanthus roseus* (rosy periwinkle) and has the potential of contributing more in view of the diversity of the flora and fauna (2000; Neuwinger, 2000).

1.3. Drug discovery from medicinal plant

Numerous methods used to acquire compounds for drug discovery include: isolation from plants and other natural sources synthetic chemistry combinatorial chemistry, and molecular modeling (Ley and Baxendale, 2002; Geysen *et al*., 2003; Lombardino and Lowe, 2004). Despite the recent interest in molecular modelling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, the natural products, and particularly that of medicinal plants, remain an important source of new drugs, drug leads and chemical entities (Newman *et al*., 2003; Butler, 2004).

1.4. plant secondary metabolite

In plants, as a result of metabolic processes, many different kinds and types of organic compounds or metabolites are produced. These metabolites are grouped into primary and secondary metabolites. The primary metabolites like chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids, play recognised roles in photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation. The secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or a taxonomically related group of species, whereas the basic primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 2006). During the past few decades, experimental and circumstantial evidence has made it clear that many secondary metabolites do indeed have functions that are vital for the fitness of a plant producing them. The main roles are:

- Defence against herbivores (insects, vertebrates)
- Defence against fungi and bacteria
- Defence against viruses
- Defence against other plants competing for light, water and nutrients
- Signal compounds to attract pollinating and seed dispersing animals
- Signals for communication between plants and symbiotic micro-organisms
- Protection against UV-light or other physical stress (Wink, 1999)

They have also provided an invaluable resource that has been used to find new drug molecules (Gurib-Fakim, 2006). Plant secondary metabolites can be grouped into three chemically distinct classes: terpenes, nitrogen containing and phenolics compounds.

1.4.1. Terpenes

Terpenes belong to the biggest class of secondary metabolites and basically consist of five carbon isoprene units which are assembled to each other (many isoprene units) by thousands of ways. Terpenes are simple hydrocarbons, while terpenoids are modified class of terpenes with different functional groups and oxidized methyl group moved or removed at various positions (Gonzalez-Burgos and Gómez-Serranillos, 2012). Terpenoids are divided into monoterpenes, sesquiterpenes, diterpenes, sesterpenes, and triterpenes depending on its carbon units. Most of the terpenoids with the variation in their structures are biologically active and are used worldwide for the treatment of many diseases. Many terpenoids inhibited different human cancer cells and are used as anticancer drugs such as Taxol and its derivatives. Many flavorings and nice fragrances are consisting on terpenes because of its nice aroma. Terpenes and its derivatives are used as antimalarial drugs such as artemisinin and related compounds. Meanwhile, terpenoids play a diverse role in the field of foods, drugs, cosmetics, hormones and vitamins (De Santana Souza *et al*.,2014; Maione *et al* ., 2013).

1.4.2. Nitrogen containing compounds

A large variety of plant secondary metabolites have nitrogen in their structures. Included in this category are such well-known antiherbivore compounds such as alkaloids and cyanogenic glycosides, which are of considerable interest because of their toxicity to humans and their medicinal properties. Most nitrogenous secondary metabolites are biosynthesised from common amino acids (Taiz and Zeiger, 2006)

- ✓ Alkaloids

The term ‘alkaloid’ has been defined as a cyclic organic compound containing nitrogen in a negative oxidation state, which has limited distribution in living organisms (Taiz and Zeiger, 2006). Based on their structures, alkaloids are divided into several subgroups: non-heterocyclic alkaloids and heterocyclic alkaloids, which are again divided into 12 major groups according to their basic ring structure. Mescaline is an example of a non-heterocyclic

or pseudo-alkaloid, tetrandrine is an example of a bisbenzylisoquinoline alkaloid while solasodine is a triterpene alkaloid (Gurib-Fakim, 2006)

- ✓ Cyanogenic glycosides

Perhaps the most obvious defence-related secondary metabolites are the cyanogenic glucosides (Vierheilig *et al*., 2000). They are not in themselves toxic but are readily broken down to give off volatile poisons when the plant is crushed. Cyanogenic glycosides release the well-known respiratory poisonous gas, hydrogen cyanide (Taiz and Zeiger, 2006)

1.4.3. Polyphenols

Polyphenols are natural compounds synthesized exclusively by plants, with chemical features related to phenolic substances and strong antioxidant properties. These molecules or classes of substances are mainly present in fruits, vegetables, green tea, and whole grains (Singla *et al*., 2019). Phenolic compounds are one of the most numerous and widely distributed group of aromatic compounds in the plant kingdom, with over 8000 phenolic structures currently known, of which more than 6000 are the flavonoids (Garcia-Salas *et al*., 2010; Tsao *et al*., 2010; Vladimir-Kneţević *et al*., 2012). From the chemical point of view, polyphenols are natural compounds with aromatic structures containing one or more aromatic rings with or without the vicinity of a heterocycle and which are grafted with hydroxyl, carboxyl, methoxyl and carbonyl functional groups.

- ✓ **Classes of polyphenols**

There are three main classes of polyphenols: phenolic acids, flavonoids, and other phenolics.

- **Phenolic acids**

Phenolic acids are a subclass of the larger phenolics category, occurring in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyfatty acids, sterols, and glucosides. Structurally, phenolic acids are phenols that possess one carboxylic acid moiety that can be directly attached either to the aromatic ring (benzoic acid derivatives) or attached to an alkyl residue (hydroxycinnamic acid derivatives). Although the basic skeleton remains the same, phenolic acids differ in the number and

position of the hydroxyl groups on the aromatic ring (Losada-Barreiro and Bravo-Díaz, 2017). The majority of phenolic acids are linked through ester, ether, or acetal bonds either to structural components of the plant (cellulose, proteins, lignin) or to larger polyphenols (flavonoids), or smaller organic molecules (glucose, quinic, maleic, or tartaric acids) or other natural products such as terpenes (Goleniowski *et al.*, 2013).

- **Flavonoids**

Flavonoids are the most studied class of polyphenols. Although they are low molecular weight secondary metabolites, their chemical diversity, size, three-dimensional shape, and physical and biochemical properties allow them to interact with multiple targets to influence biological activity in plants, animals, humans and microbes. Consequently, many therapeutic properties have been assigned to flavonoids (Francisco *et al.*, 2014).

Flavonoids comprise the most abundant class of plant polyphenols with more than 9000 of identified chemical structures (Wang *et al.*, 2018). They share a carbon skeleton of diphenyl propanes, two benzene rings (A and B) joined by a linear three carbon chain. This central chain usually forms a closed pyran ring (C) with one of the benzene rings. According to the degree of oxidation of the C ring, the hydroxylation pattern of the nucleus, and the substituent at carbon 3, the flavonoids can thus be classified into different subclasses: flavones, isoflavones, flavanols (catechins), flavonols, flavanones, anthocyanins and proanthocyanidins (Losada-Barreiro and Bravo-Díaz, 2017).

- **Other phenolics**

There are at least two major classes of tannins: **(*i*):** hydrolyzable and nonhydrolyzable (also known as condensed) tannins and **(*ii*)**: proanthocyanidins and procyanidins. Structurally, hydrolyzable and nonhydrolyzable tannins are richly hydroxlyated oligomers or polymers of hydroxybenzoic acids such as gallic acid or flavan-3-ols such as catechin, respectively. High-molecular-weight condensed tannins may contain 50 or more flavan-3-ols subunits attached by carbon-carbon bonds (Selma *et al.*, 2009). They are highly astringent and noticeable in unripe fruits.

Stilbenes are well-known class of naturally occurring phytochemicals. They bear classical C6-C2-C6 structures with two hydroxyl groups on the A ring and one on the B ring. Stilbenes are characterized by a double bond connecting the phenolic rings. These compounds

are stress metabolites produced in response to fungal infection. Though known as plant defense compounds, stilbens have an enormous diversity of beneficial human health effects. One of the most relevant and extensively studied stilbene is resveratrol found largely in grapes (Martinez *et al.*, 2014; Losada-Barreiro and Bravo-Díaz, 2017).

Lignans are found in all plants and show enormous structural diversity, with their molecular backbone consisting of two phenylpropane (C3-C6) units. The most lignans that are of special interest owing to their many powerful health benefits are tetrahydrofurofuran and sesamin (Martinez *et al.*, 2014).

2. Costus speciosus

Costus speciosus is a succulent, vertical, everlasting, herbaceous, ornamental, tuberous stem, sub-woody at the base, stout crawling rhizomes growing up to 2.0 - 2.7 m tallness with long lanceolate leaves and essential white flowers (Dubey *et al*., 2010; Karthikeyan *et al*., 2012; Najma *et al*., 2012). The herb blossom by July and August, whereas the aerial parts lose during the cold weather (Rajesh *et al*., 2009; Nehete *et al*., 2010). Flowers of *C. speciosus* resemble crepe paper. *C. speciosus* occurs in the moist and wet evergreen areas of the Indo-Malayan region and Sri Lanka (EL-far *et al*., 2013).The herb has a red color fruit, whereas the seeds are black (Fig. 01) (Pawar and Pawar. 2014). Its classification is as follows:

Kingdon: Plantae

Subkingdom: Tracheobinota

Super Division: Spermatophyta

Division: Mangoliophyta

Class: Liliopsida

Sub Class: Zingiberidae

Order: Zingiberales

Family: Costaceae

Genus: Costus

Species: Speciosus (Srivastava *et al*., 2011)

Figure 01. *Costus speciosus*

2.1.Traditional use of the Root

Ibn AI-Qayim stated in his book Al-Teb Al- Nabawi that *costus* contains different therapeutic advantages. It expels the phlegm and checks its excessive production and effective in common cold. It is good tonic for stomach and liver, increases the cutaneous circulation and removes the stains on the face if applied with honey. It is a good remedy for freckles if applied on face with vinegar and effectively used in cases of tetanus and intestinal parasites. Imam Zahbi said that it is useful in paralysis, an antidote for snake poison and its oil is effective in back pain. According to the authentic books of Al-Hadith, it is described accompanied with the description of cupping. Anas Bin Malik رضي الله عنه narrated that the Prophet صلى الله عليه وسلم stated "Out of those things which are being used by you for treatment, the cupping and Qust Bahri are the best treatment". (Sahih Bukhari 5371). This description might be interpreted to mean that if somebody fails to use to cupping, he may use the *Saussurea lappa* instead of cupping (Khan *et al*., 2001).

Hadith - Mustadrak-al-Hakim, Narrated Jabir Bin Abdullah رضي الله عنه narrates that Prophet صلى الله عليه وسلم stated " If someone's child gets Azra (upper respiratory tract infection) or headache then she should take Qust and after grinding it in water, apply it to the child" (Mustadrak-Al-Hakim). Azra is an ancient term, might be interpreted as tonsillitis, in which tonsils become swollen and painful with or without pus formation. Hence, this drug is especially effective against any type of tonsillitis and might be used in all types of phlegmatic diseases. It is effective in general weakness after diarrhea and cholera. The water extract of the root was used to wash the females' internal organs after the menstruation.

2.2. Phytochemistry of *Costus speciosus*

Phytochemical screening of *C. speciosus* detected the presence of alkaloids, glycosides, steroids, phenolic, (Rates, 2001; Singh *et al*., 2014) flavonoids, polyphenols, tannins, and β-carotene (Kotebagilu et al., 2014) Diosgenin, β-sitosterol, furostanol saponins-costusosides, β-D-glucoside, prosapogenins, dioscin, gracillin, dihydrophytylplastoquinone, and α-tocopherolquinone were isolated from *C. speciosus* and have a wide variety of biological activities, (Duraipandiyan *et al* ., 2012; Lijuan *et al* ., 2011). Moreover, β-amyrin, camphene, costunolide, diosgenin, α-Humulene, lupeol, and zerumbone for anticancer activity were recognized (Santos *et al*., 2012; Zhang *et al*., 2012). The chemical structures of some actives principals isolated from different parts of *C. speciosus* are presented in figure (02).

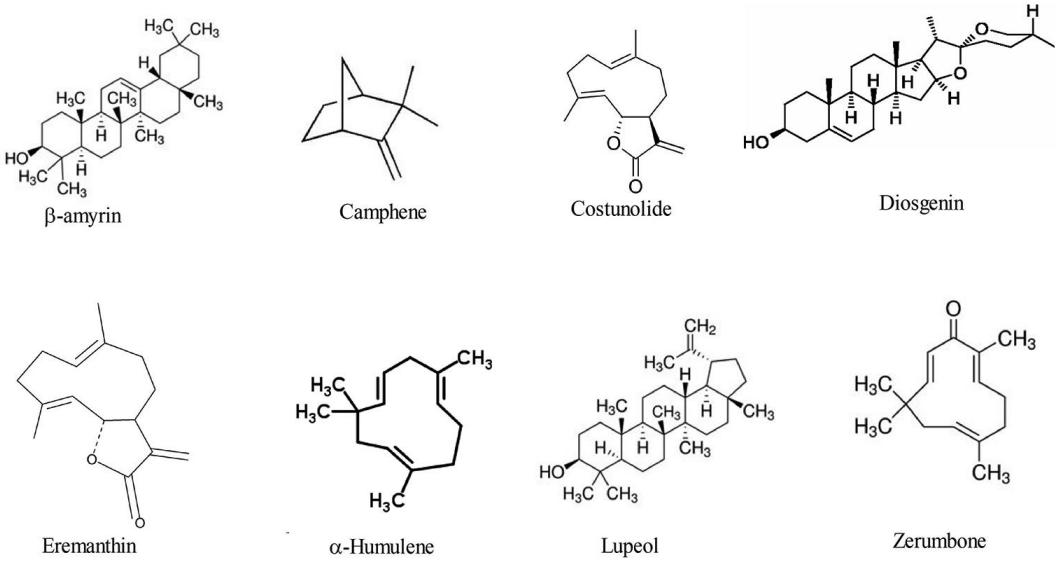

Figure 02. Chemical structures of some *C. speciosus* active ingredients (El-Far *et al*., 2018)

3. Oxidative stress

Oxidative stress resulted from an imbalance between the formation of Reactive oxygen species (ROS)/ Reactive nitrogen species (RNS) and the impaired ability of an organism to detoxify these reactive intermediates or to repair the damage that they cause (Poprac *et al.*, 2017).

3.1. Forms of ROS and RNS

All living aerobic multicellular organisms require molecular oxygen (O_2) to survive rather than oxygen, which is susceptible to radical formation due to its electronic structure.

Reactive oxygen species (ROS) are small molecules derived from oxygen molecules including free oxygen radicals, such as superoxide ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$), peroxyl ($RO_2^{\cdot}$), and alkoxyl (RO^{-}) as well as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), which are non-radicals. These non-radicals are either oxidizing agents or easily converted into radicals. Nitrogen-containing oxidants, such as nitric oxide ($NO^{\cdot}$) peroxynitrite ($ONOO^{\cdot}$), nitrogen dioxide (NO_2) are called reactive nitrogen species (RNS) (Bedard and Krause. 2007; Pisoschi and Pop, 2015).

3. 2. Sources of ROS and RNS

Both endogenous and exogenous sources contribute to intracellular ROS/RNS levels.

- **Endogenous sources**

✓ **Mitochondria**

Most of the intracellular ROS are derived from mitochondria. The superoxide radicals are produced at two major sites in the electron transport chain, namely complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase). The transfer of electrons from complex I or II to coenzyme Q or ubiquinone (Q) results in the formation of reduced form of coenzyme Q (QH_2). The reduced form QH_2 regenerates coenzyme Q via an unstable intermediate semiquinone anion (Q^{-})in the Q-cycle. The formed $^{\cdot}Q^{-}$ immediately transfers electrons to molecular oxygen leading to the formation of superoxide radical. The generation of superoxide is non-enzymatic and therefore higher the metabolic rate, the greater is the production of the ROS (Finkel and Holbrook, 2000) as shown in figure (03).

The superoxide anion is converted to hydrogen peroxide by the action of mitochondrial superoxide dismutase (MnSOD). However, H_2O_2 can be detoxified by the Catalase (CAT) and glutathione peroxidase (GPx). The other mitochondrial components which contribute to the formation of ROS include monoamino oxidase, aketoglutarae dehydrogenase, glycerol phosphate dehydrogenase and p66shc (Starkov *et al.*, 2008).

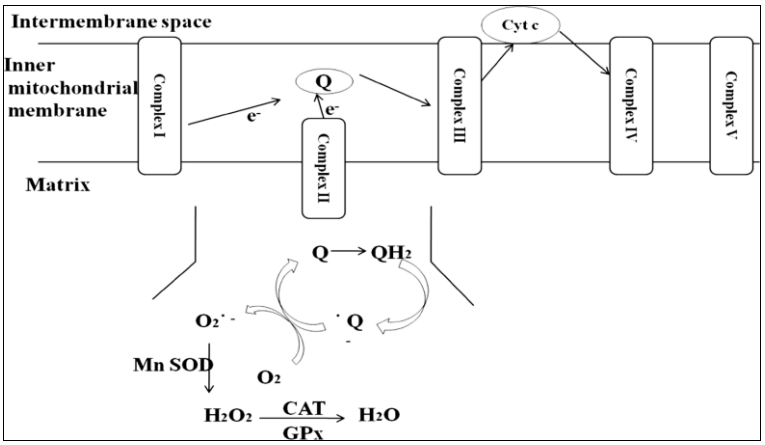

Figure 03 . Mitochondrial ROS production (Alugoju *et al*., 2015)

✓ **Peroxisomes**

In peroxisomes the respiratory pathway involves the transfer of electrons from various metabolites to the oxygen leads to H_2O_2 formation, but is not coupled to oxidative phosphorylation to produce ATP instead free energy is released in the form of heat. The other free radicals produced in peroxisomes include H_2O_2, $O2^{\bullet-}$ $OH^{\bullet}$ and $NO^{\bullet}$. The β-oxidation of fatty acids is the major metabolic process producing H_2O_2 in the peroxisomes. Additionally, the different peroxisomal enzymes such as acyl CoA oxidases, D-amino acid oxidase, L-α-hydroxy oxidase, urate oxidase, xanthine oxidase, D-aspartate oxidase have been shown to produce different ROS (Fahimi and Schrader, 2006).

✓ **Endoplasmic Reticulum**

The enzymes of endoplasmic reticulum such as cytochrome p-450, b5 enzymes and diamine oxidase contribute to the formation of ROS (Gross *et al*., 2006). Another important thiol oxidase enzyme, Erop1p catalyses the transfer of electrons from dithiols to molecular oxygen results in the formation of H_2O_2 (Droge, 2002).

- **Exogenous sources**

There are multiple external triggers that induce oxidative stress and have direct or indirect effects on responses. Air pollutants, tobacco smoke, ionizing and nonionizing radiations, foods and drugs, as well as xenobiotics can all contribute to oxidative stress. Chemical agents like quinones (Bolton *et al*., 2000), heavy metals such as lead, arsenic,

mercury, chromium, and cadmium; organic solvents; and pesticides are common exogenous sources of ROS (Yildirim *et al*., 2000).

- ✓ **Radiation and chemotherapy**

Ionizing radiation, such as x-rays, neutrons, as well as α, β, and γ rays, can all cause oxidative stress. α Particles have weak penetrative power, but the rest are very penetrating through the human body. Ionizing radiation can produce $HO^{\cdot}$ by radiolysis of water or ROS via secondary reactions (Roessner *et al*., 2008).

Cancer chemotherapy is often accompanied by toxic side effects, and ROS generation by chemotherapeutic agents is the primary event leading to induced toxicity. This is evident by increased lipid peroxidation, and reduced antioxidant and tissue GSH levels during chemotherapy. Both radiation and chemotherapy induce systemic oxidative stress and reduce levels of vitamin E and beta-carotene in patients (Conklin, 2004).

- ✓ **Cigarette smoke**

Cigarette smoke is another significant generator of ROS. It is comprised of more than 7,000 chemical compounds and oxidative agents, and tobacco smoke contains 1014-1016 free radicals per puff. The active chemicals include aldehydes, quinones, benzo(a)pyrene, epoxides, and peroxides . Cigarette smoke has a gas phase which contains $^{\cdot}NO$, peroxyl radicals, and carbon-centred radicals as well as a tar phase containing relatively stable polycyclic aromatic hydrocarbons and nitrosamines. In the presence of iron, tar semiquinone can generate $HO\cdot$ and H_2O_2 (Witschi.2005; Yang *et al*., 2008).

- ✓ **Foods and alcohol.**

Ingested food can generate $O_2^{\cdot-}$ and H_2O_2 in the gastrointestinal tract. Humans ingest macronutrients (carbohydrates, proteins, and fats), micronutrients (minerals and vitamins), food preservatives, as well as microorganisms. Dietary iron and also copper generate ROS by the Fenton reaction. Increased intake of Fe^{2+} generates ROS and RNS, lipid peroxidation, and oxidative stress, and its accumulation in tissues increases the risk of cancer and inflammation. Trans fatty acids in processed foods also generate ROS. Lipids from vegetable and animal origin, when heated in microwave ovens, generate free radicals (Fraga et al., 2010 ; Zapolska-Downar *et al*., 2005).

- ✓ **Drugs and xenobiotics**

Many drugs and xenobiotics contribute to the formation of free radicals in the body. Anticancer drugs such as anthracyclines and analogs, mitoxantrone and other quinones, actinomycin D, enediynes such as bleomycin, chartreusins, elasmin A and related compounds can cause oxidative stress (Deavall *et al*., 2012).

3.3. Molecular targets of free radicals

Since these free radicals are highly reactive, they can damage all the three important classes of biological molecules including nucleic acids, proteins, and lipids (Droge, 2002).

- ✓ **Deoxyribonucleic Acid (DNA)**

Both ROS/RNS can oxidatively damage the nucleic acids. The mitochondrial DNA ismore vulnerable to the ROS attack than the nuclear DNA, because it is located in close proximity to the ROS generated place. ROS, most importantly, the $OH^{\bullet}$ radical directly reacts with all components of DNA such as purine and pyrimidine bases, deoxyribose sugar backbone (Dizdaroglu *et al*., 2002) and causes a number of alternations including single and double stranded breaks in DNA. The $OH^{\bullet}$ radical abstracts hydrogen atoms to produce a number of modified puine as well as pyrimidine base by-products and DNA- protein cross links.

The RNS, most importantly, $OONO^{-}$ interacts with guanine to produce nitrative and oxidative DNA lesions such as 8-nitroguanine and 8-oxodeoxyguanosine respectively (Hiraku *et al*., 2010). 8-nitroguanine formed is unstable and can be spontaneously removed, resulting in the formation of an apurininc site .Conversely adenine can be paired with 8-nitroguanine during DNA synthesis resulting in a G-T transversions (Hofer et al., 2005). Accordingly, 8-nitroguanine is a mutagenic DNA lesion involved in carcinogenesis.

- ✓ **Ribonucleic acid (RNA)**

ROS can attack different RNAs produced in the body. The RNA is more prone to oxidative damage than DNA, due to its single stranded nature, lack of an active repair mechanism for oxidized RNA, less protection by proteins than DNA and moreover these cytoplasmic RNAs are located in close proximity to the mitochondria where loads of ROS are produced. Indeed, RNA is subjected to more oxidative damage than DNA in humans (Hofer

et al, 2005). 7, 8-dihydro-8-oxoguanosine (8-oxoG) is the most extensively studied RNA damage product and its levels are raised in various pathological conditions like Alzheimer's disease, Parkinson's disease, atherosclerosis, hemochromastosis and myopathies (Abe *et al*.,2002 ; Kikuchi *et al*., 2002 ; Martinet *et al*., 2004 ; Broedbaek *et al*., 2009).

- ✓ **Lipids**

The membrane lipids, especially the polyunsaturated fatty acid residues of phospholipids are more susceptible to oxidation by free radicals (Valko *et al*, 2007). The lipid peroxidation is very important *in vivo* because of its involvement in various pathological conditions. The lipid peroxidation results in the loss of membrane functioning, for example, decreased fluidity, inactivation of membrane bound enzymes and receptors (Tateyama, 2003).

- ✓ **Proteins**

The protein oxidation can be induced by radical species such as $O2^{\bullet-}$, $OH^{\bullet}$, peroxyl, alkoxyl, hydroperoxyl as well as by the non radical species such as H_2O_2, O3, HOCl, singlet oxygen, $OONO^-$ (Chevion *et al*., 2000). ROS oxidize different amino acids present in the proteins, causing the formation of protein- protein cross linkages, results in the denaturing and loss of functioning of proteins, loss of enzyme activity, loss of function of receptors and transport proteins (Haklar *et al*., 2001).

3.4. **Relationship between Oxidative stress and diseases**

Free radicals, especially ROS are eliminated by antioxidant molecules in normal cells. When this balance shifts and oxidative stress occurs, it results in damage of biopolymer molecules (i.e. lipids, protein and DNA) in the cell. Inflammatory pathways activated by oxidative stress could be the reason of cancer, diabetes, cardiovascular, neurological and pulmonary diseases (Bhole and Bhavsar, 2017).NO one of the most important molecules in the inflammatory pathways has different actions: vasodilation in acute inflammation, mediation to proinflammatory cytokines, such as IL-1 (interleukin-1) and cytotoxicity against microorganisms. When pathogens or cytokines stimulates iNOS (inducible nitric oxide synthase), it synthesizes NO and high concentrations and activates nuclear factors, like NF-kB (Aktan, 2004). Because of anti-inflammatory, cytotoxic and anti-aging properties, antioxidant molecules are very popular nowadays. There is a great deal of attention on natural products, due to carcinogenic effects of synthetic antioxidants used in food industry, such as BHT

(butylated hydroxytoluene), BHA (butylated hydroxy anisole) and BHQ (tertiary butylhydroquinone) (Barlow and Schlatter, 2010). Natural antioxidants, phenols, flavonoids or non-nutrient compounds of fruits and vegetables could have potential antioxidant, antimutagenic and/or anticarcinogenic and anti-inflammatory effects (Singh *et al*., 2008).

3.5. Defense system against oxidative stress

After ROS and RNS exposure from different sources, living organisms have a series of defence mechanisms against the oxidative stress including: preventative mechanisms, repair mechanisms, physical defences and antioxidant defences. The antioxidant defence mechanisms can be divided into enzymatic and non-enzymatic defences.

- **Enzymatic Antioxidants**

✓ **Superoxide dismutases (SOD)**

Three isoforms of SOD exist in humans: cytosolic copper and zinc-containing enzyme (Cu-Zn-SOD), manganese-requiring mitochondrial enzyme (Mn-SOD), and an extracellular Cu-Zn containing SOD (EC-SOD). Iron-containing SOD (Fe- SOD) is present in bacteria and plants but not in vertebrates and yeast, while nickel-containing SOD (Ni-SOD) is present only in prokaryotes (Nozik-Grayck *et al*., 2005).

✓ **Glutathione peroxidase (GP_X)**

Glutathione peroxidase (GP_X) converts glutathione (GSH), a tripeptide consisting of glutamate, cysteine, and glycine, into oxidized glutathione (also called glutathione disulfide, GSSG) and, during this process, reduces H_2O_2 to H_2O and lipid hydroperoxides (ROOH) to corresponding stable alcohols. The GP_X reaction is coupled to glutathione reductase (GSSG-R), which maintains reduced GSH levels. Neurons are most vulnerable to free radical damage as they have very low levels of GSH. GP_X serves an important role in protecting cells from the harmful effects of peroxide decomposition.(Chu *et al*., 2004; Dayer *et la*., 2008)

✓ **Catalase**

Catalase dismutates H_2O_2 to H_2O and O_2 and is found mainly in peroxisomes (Schrader and Fahimi, 2006). CAT are heme enzymes, but a manganese catalase is found in

prokaryotes. In humans, catalase is found largely in liver, kidney, and erythrocytes, although all organs express this enzyme. Catalase-expressing pathogens such as Campylobacter jejuni (Atack and Kelly, 2009), H. pylori, Helicobacter hepaticus, and enterobacteriaceae family bacteria including *Escherichia coli*, *Shigella*, and *Salmonella* synthesize. CAT to deactivate H_2O_2 to evade host response and survive within the host. Less catalase activity is noted in colorectal cancer (Chang *et al*., 2012), gastric adenocarcinoma and *H. pylori*-infected stomach (Iborra *et al*., 2011). Scientists even claim that catalase function is not to detoxify H_2O_2, but to protect cells from apoptosis (LeBlanc *et al*., 2011; De Moreno *et al*., 2015)

- **Non-enzymatic Antioxidants**

✓ **Glutathione**

Glutathione is found in all eukaryotic cells and is one of the key non-enzyme antioxidants in the body. It is generally present in its reduced form, GSH. This is ubiquitously expressed, and together with three enzymes, glutathione reductase (GR), and glutathione *S*-transferases (GST), form the glutathione system. In the gut mucosa, the GSH system serves as an antioxidative barrier. High intake of fruits and vegetables stimulate GSH-dependent enzymes which may account for at least some of the reported antioxidative benefit of these food groups (Hoensch *et al*., 2002)

✓ **Vitamin C**

Vitamin C or ascorbic acid is the primary antioxidant in plasma and cells. It is synthesized from glucose in the liver of most mammalian species, but not by humans and therefore must be ingested to avoid scurvy, a potentially lethal condition. Vitamin C can be obtained from fresh fruits and vegetables. Vitamin C donates electrons to other compounds and prevents their oxidation. The many relevant species reduced by vitamin C include various ROS, RNS, sulfur radicals, O_3, nitrosating compounds, and HOCl. Vitamin C reduces heavy metal ions (Fe, Cu) that can generate free radicals via the Fenton reaction, and thus it can have pro-oxidant activity although its main function is as an antioxidant (Padayatty *et al*., 2003).

✓ **Vitamin E**

Vitamin E (the most biologically active form is α-tocopherol) is an important and abundant antioxidant that protects cell membranes from lipid peroxidation. α-Tocopherol

terminates the activity of lipid peroxidation by scavenging lipid peroxyl radicals ($LOO^{\cdot}$) but itself is converted into a reactive radical during this reaction (Traber and Atkinson, 2007). α-Tocopherol can also reduce Fe or Cu, as a pro-oxidant . The ability of α-tocopherol to act as a pro- or antioxidant depends on the amount of α-tocopherol available to scavenge ROS (Xu *et al*., 2009).

- ✓ **Carotenoids including vitamin A**

Vitamin A, which is found in food, is referred to as carotenoids or provitamin A. Yellow and orange fruits as well as green leafy vegetables provide most of the carotenoids in our diet. Alpha- and beta-carotene, lycopene, and cryptoxanthin are the main carotenoids in food as well as in the body. Beta-carotene and other carotenoids exhibit antioxidant properties depending on the *in vitro* experimental system used. Antioxidant properties of biological carotenoids depend on retinol-binding proteins and other endogenous antioxidants *in vivo* (Kaneko *et al*., 2008; Rao and Rao, 2007). Beta-carotene has been shown to suppress lipid peroxidation in mouse models. Antioxidant properties can be reversed to pro-oxidant behavior depending on O_2 tension or carotenoid concentration (Zhang *et al*., 2000).

- ✓ **Minerals**

Zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), and selenium (Se) are key components of enzymes with antioxidant functions and are designated as antioxidant micronutrients. Zn, Mn, and Cu are cofactors of Cu/Zn-SOD (Ighodaro *et al* ., 2018). Fe is a component of catalase. Se is a major antioxidant in the form of selenoproteins that mitigates the cytotoxic effects of ROS. Cereals contain selenomethionine, a naturally occurring amino acid that is the most important nutritional source of Se. When Se-GPx is inhibited under physiological conditions, such as during Se deficiency, it leads to toxicity through increased $O2^{\cdot-}$, NO, and lipid peroxidation Thus again, proper nutrition and absorption of these micronutrients is essential to maintain redox homeostasis (Bhattacharyya *et al*., 2014).

- ✓ **Polyphenols including flavonoids**

Plant polyphenols are important antioxidants, and dietary intake of these compounds can be up to 50-800 mg/day (Pietta, 2000). Polyphenols comprise flavonoids, phenols, phenolic acids, lignins, and tannins. Flavonoid sources include fruits, vegetables, nuts, red wine, beer, tea, seeds, grains, spices, and medicinal plants. Flavonoids prevent superoxide

anion production by inhibiting XO. In addition, they inhibit COX, LOX, GST, microsomal monooxygenases, and NADH oxidase. Many flavonoids chelate free Fe and Cu that could otherwise increase ROS generation, and also reduce ROS such as $O_2^{\cdot -}$, and HO· (Rodrigo and Gil-Becerra, 2014).

4. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most frequent and fatal type of liver cancer - a cancer that arises when a cell in the liver becomes genetically mutated allowing it to grow relentlessly (Singal *et al*., 2014). The Cancer Statistics Report has recounted a higher incidence and mortality rate chiefly due to cancer of the liver (Siegel *et al*., 2016). With approximately five new cases being diagnosed every year, HCC has become the fifth most common cause of cancer worldwide. More often, patients diagnosed with HCC have very low survival rate, with merely 17.2 % of them surviving for 5 years or more after diagnosis (Cabibbo *et al*., 2010). Common treatment strategies for HCC include hepatectomy, liver transplantation, radiofrequency ablation, radiotherapy and chemotherapy (Pardee and Butterfield, 2012). Standard chemotherapy treatment involves administration of Sorafenib or Nexavar, a MAP Kinase inhibitor. Patients on Nexavar generally encounter adverse drug effects such as diarrhea, hand-foot skin disease, fatigue, anorexia and alopecia (Keating *et al*., 2009). Medicinal plants and natural products, which have considerable chemotherapeutic value and comparatively fewer side effects, are increasingly being studied for treating cancer (Hatcher *et al*., 2008; liu *et al*., 2007; Ding, 2006)

5. Blood Coagulation

Haemostasis is the normal physiological process of blood clotting which involves dissolving and lysing of clotted blood. Haemostasis can be divided into two major systems as follows; primary and secondary haemostasis (Abdel-Wahab *et al.,* 2006). These two processes happen concurrently and interrelated. The primary haemostasis is a short lived which involves platelets and vascular response to vessel injury which lead to the formation of initial ‘platelet plug’. The vascular wall is lined with endothelial cells which show antithrombotic properties due to coagulation inhibitors, neutral phospholipids and fibrinolysis activators (Anshu *et al.,* 2014).

On the other hand, the sub endothelial layer contains collagen and laminin which is highly thrombogenic. As a result, vasoconstriction slows the blood flow at the damaged blood vessel to limit the blood loss which usually last up to 30 minutes. These process is mediated by two controls; local and systemic controls. The local controls are the vasoconstrictors such as thromboxane (produced at injured site) whereas systemic control is the epinephrine produced by adrenal glands to stimulate vasoconstriction (Cohen *et al*., 2014). In addition, vasoconstriction enhances the platelet adhesion and activation which in turn lead to platelet accumulation and prevent blood loss.

Meanwhile, the secondary haemostasis is the formation of insoluble fibrin which is produced via proteolytic coagulation cascade. Blood coagulation is a complex process that forms blood clots to block/heal a lesion which involves cellular (platelet) and protein (coagulation factors). Mostly the coagulation factors are precursors of proteolytic enzymes, zymogens which circulate in an inactive form and a suffixing letter 'a' will be given to the activated zymogen (Anshu *et al.,* 2014). Clotting factors are classified into three groups as follows; fibrinogen family, Vitamin K dependent proteins and contact family (Table 01). In addition, coagulation process is control by inhibitor (plasminogen & protein C) which disrupt the clot formation as well as development of thrombus.

Table (01). Classification of coagulation factors (Palta *et al*., 2014)

Fibrinogen family	Vitamin K dependent	Contact family
fibrinogen	Factor II	Factor XI
Factor V	Factor VII	Factor XII
Factor VIII	Factor IX	HMWK
Factor XIIII	Factor X	Prekallikerin

HMWK- High molecular weight kininogen

Blood coagulation system can be divided into three pathways; extrinsic, intrinsic and common pathways which are enzymatic cascade that is activated upon tissue damage (Singh *et al.,* 2001).

5.1. Extrinsic Pathway

Based on Fig. (4), extrinsic pathway involves vascular and blood components which are activated upon the release of tissue factor/tissue factor pathway (Factor III) at the site of

injury and takes up to 10-15 seconds to complete the entire process (Funk *et al.,* 2012). Tissue factor is a glycoprotein which is found on surface of subendothelial tissue and exposed during vascular injury. Similar to activation of factor IXa in intrinsic pathway, tissue factor act as a cofactor for Factor VIIa which contain serine protease that cleaves factor X to Factor Xa in the presence of calcium ions. The association between factor VIIa and tissue factor is the principal step in clotting cascade. Next, factor Xa and factor Va convert prothrombin/factor II to thrombin which then cleaves fibrinogen to fibrin. Furthermore, inhibition of extrinsic pathway by Tissue Factor Pathway Inhibitor (TFPI) occurs at factor VIIa-Xa complex. TFPI also known as lipoprotein-associated coagulation inhibitor (LACI) consist of 3 protease inhibitor domains as follows, domain 1 will bind to factor Xa whereas domain 2 binds will to factor VIIa with the presence of factor Xa (Anshu *et al.,* 2014).

5.2. Intrinsic Pathway

Meanwhile, the intrinsic/contact pathway is activated when the blood contact with the negatively charged substances such as endothelial surface, prekallikrein, collagen, phospholipids or high-molecular-weight kninogen (HMWK) (Fig. 04). Factor I, II, V, VIII, IX and XII plays an important role in these pathway (Anshu *et al.,* 2014). Upon conversion of prekallikrein to kallikrein, it activates factor XII to XIIa which then activates factor XI to XIa. Factor XIa then activates factor IX (vitamin K dependent) to IXa in the presence of calcium ions. Factor VIIa and Factor IXa forms complexes with phospholipid which then activate factor Xa. Similar to extrinsic pathway, activation of Factor Xa convert prothrombin to thrombin which then cleaves fibrinogen to fibrin. Furthermore, the production of thrombin can be controlled via negative feedback with the activation of Protein C (Cramer and Gale, 2011). Protein C binds to thrombomodulin and promotes the degradation of factor Va and VIIIa which then inhibits the activation of prothrombin.

5.3. Common Pathway

The common pathway is the meeting point of extrinsic and intrinsic pathway in which factors I, II, V and X takes part. Factor Xa convert prothrombin to thrombin which then cleaves fibrinogen to fibrin. The generation of Factor V, VIII, Thrombin, antithrombin and Protein C will either enhance or inhibit thrombin production via positive or negative feedback. In positive feedback regulation, factor Va and VIIIa will enhance thrombin

production by activating prothrombin or factor Xa (Funk *et al.*, 2012). Meanwhile, in negative feedback, Protein C will bind to thrombomodulin and promotes the degradation of factor Va and VIIIa which then inhibits the activation of prothrombin (Cramer and Gale, 2011). Thus, these regulations prevent excessive blood loss which could lead to stroke or heart attack.

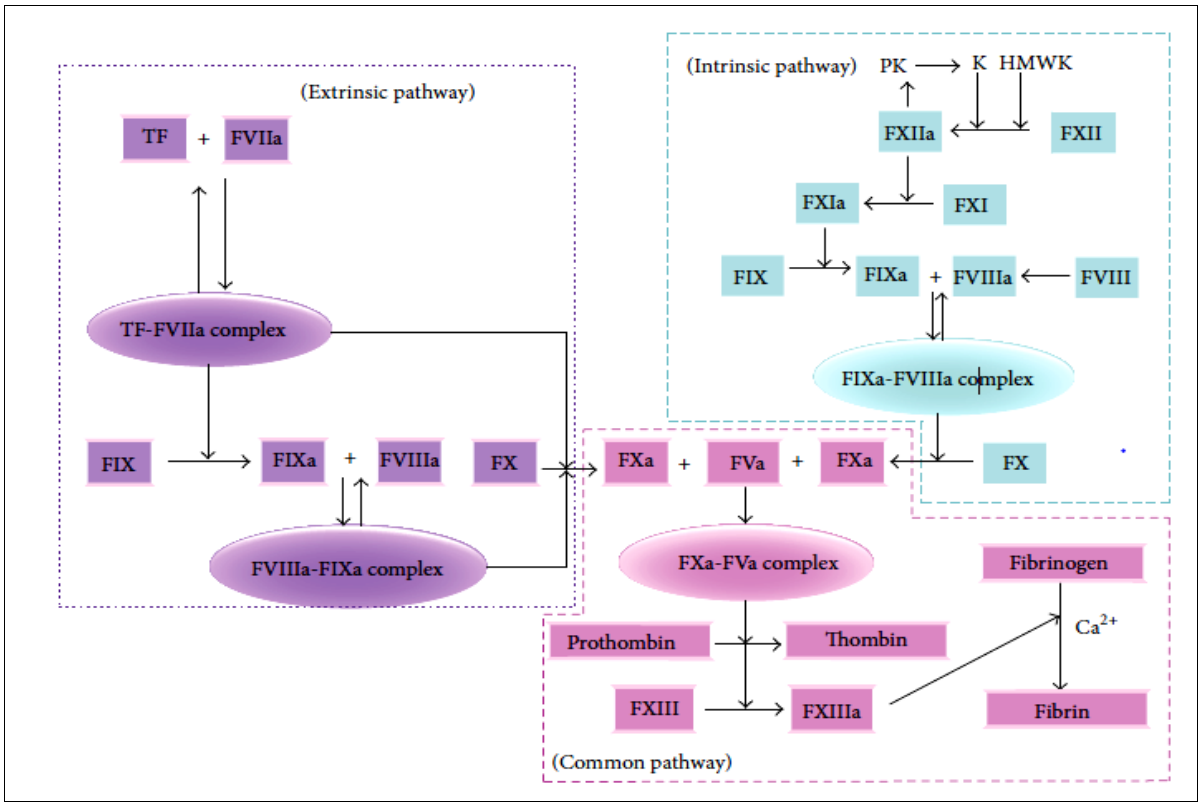

Figure 04. Extrinsic, intrinsic, and common pathways of blood coagulation during hemostasis and thrombosis. (PK: prekallikrein; K: kallikrein; HMWK: high molecular weight kininogen) (Chen *et al.*, 2015)

5.4. Fibrinolytic system

The final step in haemostasis involves the fibrinolytic system which is parallel to the activation of coagulation cascade. Fibrinolytic system involves primarily three serine proteases enzymes which dissolves fibrin clot into fibrin degradation products (FDPs) (Raber, 1990). Plasmin is produced from inactive plasminogen in liver by proteases tissue-type plasminogen activator (TPA) as well as urokinase-type plasminogen activator (uPA). Plasmin activity is inhibited by α-2 antiplasmin and α-2Macroglobulin which prevent fibrinolysis (Funk *et al.*, 2012). Meanwhile, plasminogen activator inhibitor main inhibitor of fibrinolysis which act by inhibiting t-PA and u-PA (Anshu *et al.*, 2014). In addition, thrombin activatable

fibrinolysis inhibitor (TAFI) which is a proenzyme produced by liver and is activated by thrombin act by reducing the affinity of plasminogen to fibrin (Mosnier and Bouma, 2006).

5.5. Haemostasis Screening Assay

Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) are used to detect the blood coagulation disorder. PT assay measure the deficiencies in the extrinsic and common pathway but does not measure Factor XIII activity or intrinsic pathway. Meanwhile, the APTT measures the intrinsic and common pathways but does not measure factors VII and XIII. The TT assay measures the rate of conversion of fibrinogen to fibrin (Gunendren *et al*., 2017).

5.6. Regulation of Coagulation

Regulation of coagulation cascade is vital to prevent inappropriate, excessive or miss localized clotting of blood and to prevent cardiovascular diseases such as deep vein thrombosis (Pike *et al*., 2005) happens at each level. Each phase associated with inhibitory factors, either by enzymatic inhibition or modulation of cofactor activity (Adams and Bird, 2009). Direct inhibitors of coagulation cascade include tissue factor pathway inhibitor (TFPI), antithrombin, protein C and protein S. They are essential to down regulation of thrombin generation and the control of thrombus formation. A deficiency in any of these can trigger a hypercoagulable state and expose the patient to thromboembolic disease (especially venous) (Assareh *et al*., 2014).

However, tissue factor is regulated by a serine protease, tissue factor pathway inhibitor (TFPI), which neutralizes the catalytic activity of factor Xa and inhibits the TF: FVIIa complex. It is predominantly produced by endothelium and bound to heparin sulfate, as well as other cell types, including platelets, and is found in circulating form bound to LDL. The endothelial form is released into circulation when administration of intravenous heparin or subcutaneous low molecular weight heparin (LMWH) (Adams and Bird, 2009).

The most important inhibitor of coagulation system is antithrombin, a serine-protease inhibitor (Adams and Bird, 2009). It inhibits a large number of serine proteases including thrombin (factor IIa) and factors IXa, Xa, XIa and XIIa. It acts preferentially on free enzymes, with factors involved with the tenase or prothrombinase complexes less accessible to inactivation. This means that its main function is to limit the coagulation process to the

injured site, without pathological extension of the clot. However, the action of antithrombin is greatly enhanced by the administration of heparin or heparin- like molecules expressed on endothelium. Also heparin cofactor II inactivates thrombinin the presence of heparin or heparin-like molecules (Pike *et al*., 2005).

Other coagulation factors are inhibited by inhibitory proteins. For example, FV/FVa, FVIII/FVIIa is inactivated by protein C (PC) with protein S as a cofactor. PC is activated when thrombin binds to the endothelial cell membrane surface protein thrombomodulin. Also Protein Z, another vitamin K-dependent protein, inhibits FXa via protein Z-dependent protease inhibitor (ZPI) (Corrál *et al*., 2007) but factor VIIIa is an inherently unstable molecule, and inactivation also occurs spontaneously, through dissociation of the A2 domain (Adams and Bird, 2009).

In contrast, the thrombin formed activates thrombin-activatable fibrinolysis inhibitor (TAFI), which serves to protect the clot from plasmin mediated fibrinolysis. TAFI proteolytically excises lysine residues from the fibrin clot, thus removing the binding sites of plasminogen to fibrin, and decreasing the effectiveness of plasmin-mediated clot lysis (Mosnier and Bouma, 2006).

5.7. Blood Coagulation Disorders

Blood coagulation factors and platelet are the two crucial components to maintain haemostatis. Bleeding disorder can be inherited or acquired and occur due to deficiency to any of the components. Examples of inherited bleeding disorder are von Willebrand's disease and haemophilia A whereas example of acquired bleeding disorder is liver disease. These disease can be treated with antifibrinolytic or procoagulant to enhance coagulation cascade. In addition, the formation of blots/thrombus (thrombosis) can also lead to various diseases such as pulmonary embolism and cerebral vascular accident (CVA) (Pandey and Madhuri, 2010). However, anticoagulant therapy can be given to prevent thrombus formation. Anticoagulant can be obtained by two ways; conventional therapy (drug) or natural/herbal medicine.

5.8. Mechanisms of Cancer-Associated Thrombosis

The molecular mechanisms leading to the predisposition of cancer patients to thromboembolic events are poorly understood. However, several mechanisms that can promote a hypercoagulable state are seen in cancer patients, thereby increasing the risk of

thromboembolic events. This section will focus on both direct (Fig.05) and indirect (Fig.06) (Abdol *et al*., 2018).

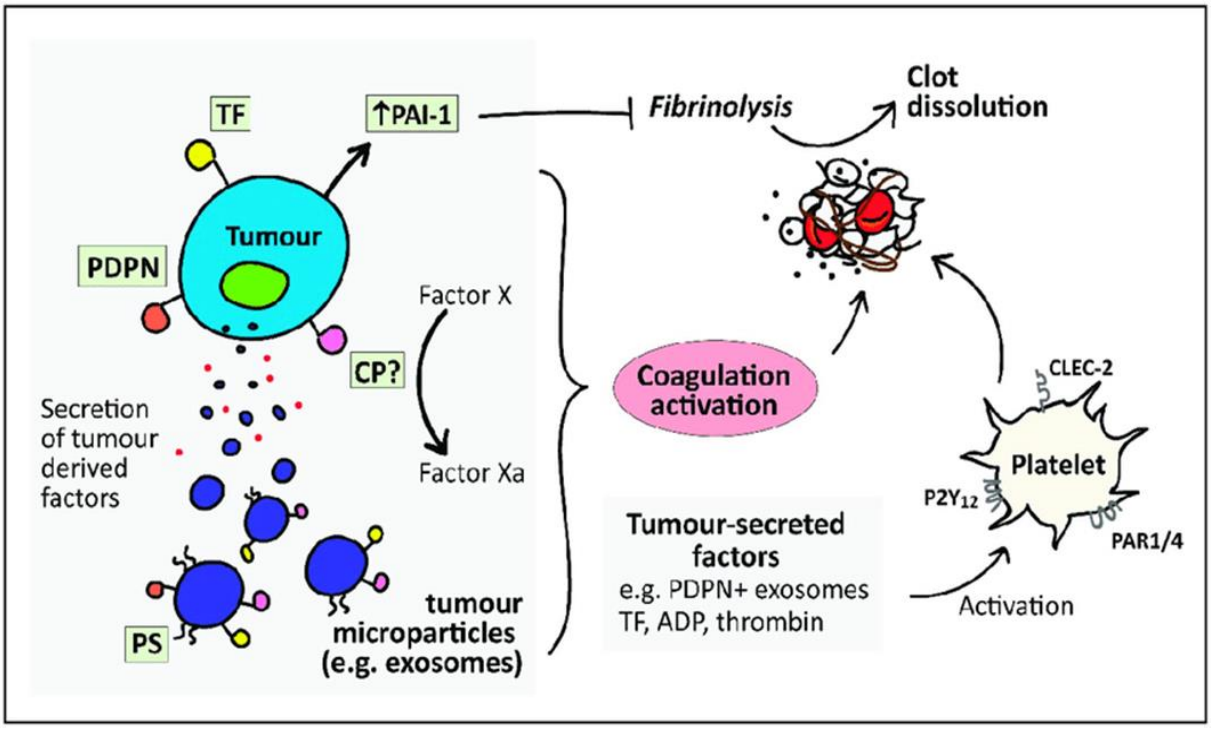

Figure 05. Direct mechanisms involved in cancer-associated thrombosis (Elyamany *et al*., 2014)

Direct activation of coagulation and platelets can occur through several factors expressed on or released from cancer cells. These include the expression of tissue factor (TF), the key initiator of the coagulation cascade, which can also be released by TF-positive microparticles. Podoplanin (PDPN) expression can directly cause platelet activation and aggregation via the C-type lectin-like receptor 2 (CLEC-2) receptor on platelets. Plasminogen activation inhibitor-1 (PAI-1), a key inhibitor of fibrinolysis, is highly expressed in cancer cells. Cancer cells also secrete platelet agonists such as ADP and thrombin, thus further promoting platelet activation through P2Y12 and protease-activated receptors 1 and 4 (PAR1/4), respectively. Phosphatidyl serine (PS) expressed on tumour microparticles may also promote coagulation as PS serves as a surface for formation of coagulation complexes. Cancer procoagulant (CP) has been shown to directly activate coagulation by activating Factor X (Brose *et al*., 2008; Khorana, 2009)

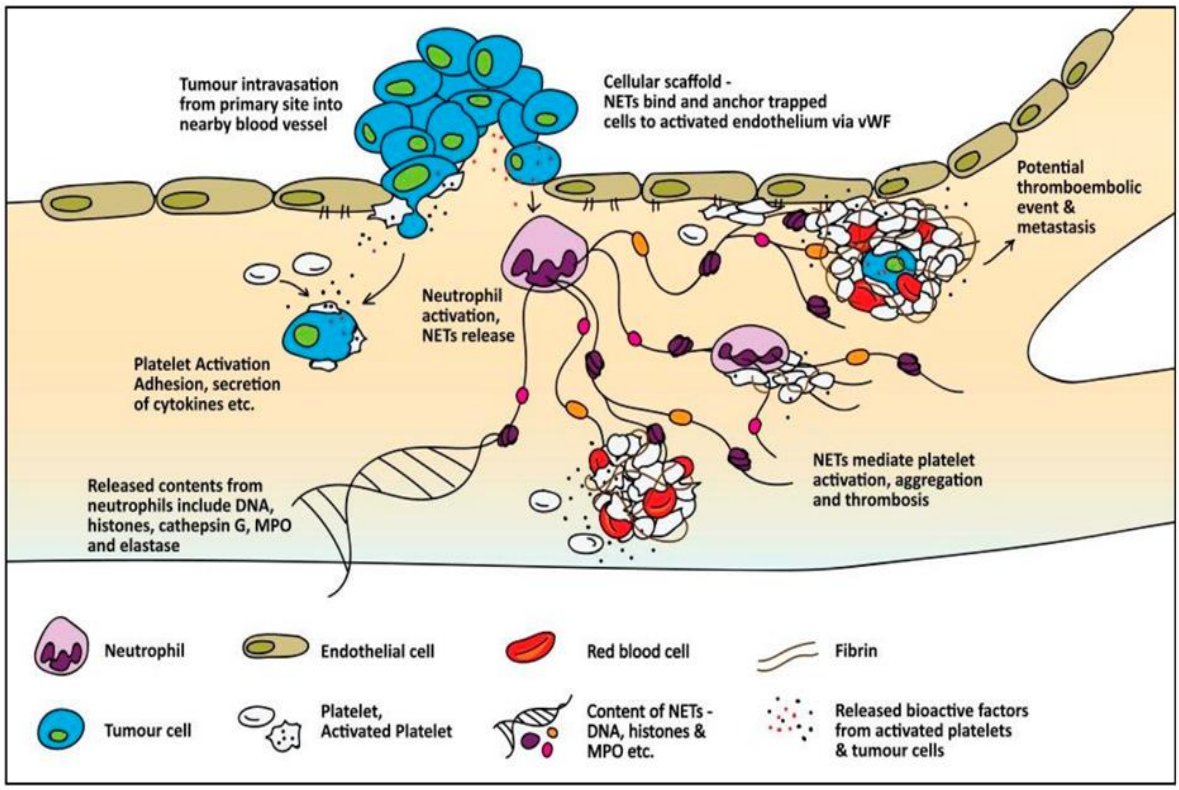

Figure 06. Indirect mechanisms promoting thrombosis in cancer (Elyamany *et al*., 2014).

Tumours can be highly metastatic, resulting in cancer cell dissemination and into nearby blood vessels. Inflammatory cytokine secretion from tumour cells of platelets and promote a procoagulant phenotype in endothelial cells. Cancer-derived factors also stimulate neutrophils to release neutrophil extracellular traps (NETs). NETs serve as a scaffold that can physically entrap platelets, or activate platelets through NET-associated histones, ultimately leading to profound platelet activation, fibrin deposition, and entrapment of red blood cells, exacerbating clot formation (Brose *et al*.., 2008; Mandala *et al.*, 2011; Elyamany *et al*., 2014).

6. Zeralenone

Zearalenone (ZN) is a nonsteroidal oestrogenic mycotoxine biosynthesized through a polyketide pathway by a variety of *Fusarium fungi*; including *Fusarium graminearum* (*Gibberella zeae*) (formerly named *F. roseum*) is the main producer of ZN. *F. culmorum, F. verticillioides, F. cerealis, F. Semitectum, F. crookwellense, F. pseudograminearum*, and *F.equiseti*. These fungi are common soil fungi in temperature and warm countries, also they are found to be regular contaminants of cereal crops worldwide. Zearalenone was known previously as F-2 toxin. (Bennet and klich, 2003). ZN is a resorcyclic acid lactone, chemically described as 6-[10-hydroxy-6- oxo-trans-1-undecenyl]-B-resorcyclic acid lactone, structure

resembles that of naturally occurring estrogens (Fig. 07). (Gromadzka *et al*., 2008), and in fact this mycotoxin binds to estrogenic receptors (Boyd and Wittliff, 1978; Greenman *et al*., 1979). Accordingly, low concentrations of ZN found to cause various estrogen-disrupting effects, including infertility, reduced serum testosterone concentrations and sperm counts, enlargement of ovaries and uterus, reduced incidence of pregnancy, and decreased progesterone levels in animals (Zinedine *et al*., 2007). So, the reproductive system has been regarded as a major target of ZN toxicity (Tiemann and Danicke, 2007; Minervini and Dell'Aquila, 2008).

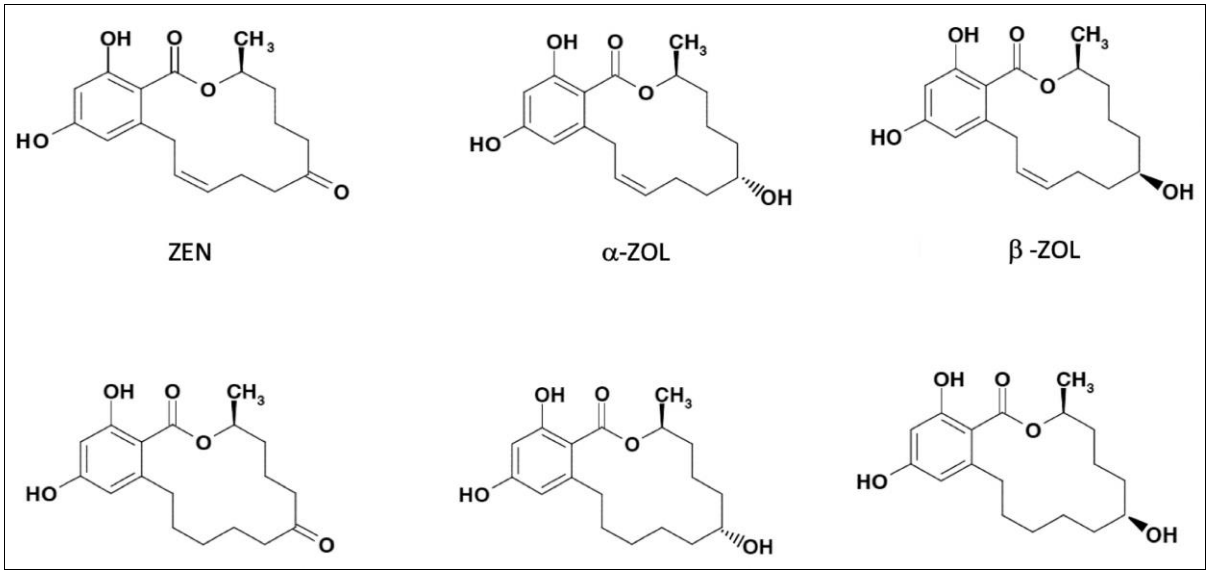

Figure 07. Chemical structures of zearalenone (ZN/ZEA/ZON) and its modified forms (α-zearalenol (α-ZEL/α-ZN/α-ZOL), β-zearalenol (β-ZEL/β-ZN/β-ZOL), zearalanone (ZAN) α-zearalanol(α -ZAL), and β-zearalanol (β-ZAL) (Urraca *et al*., 2004).

6.1. Occurrence of Zearalenone (ZN)

Fusarium species are able to grow in moist and cool conditions and invade crops both in preharvest, postharvest, and under poor storage conditions, but toxin production usually occurs at postharvest activities and storage (European Commission, 2000; Zinedine *et al*., 2007**)**. ZN can easily contaminate crops such as corn, maize, wheat, barley, rice, oats, millet, and sorghum (European Commission, 2000; JECFA, 2000; Zinedine *et al*., 2007). ZN has been reported in cereal products (for example malt, beer, soybeans, and flour), corn silage, corn by-products, and soya meal (Schollenberger *et al*., 2007; Zinedine *et al*., 2007). ZN has also been reported in eggs (Sypecka *et al*., 2004). Very low levels of ZN and its metabolites

(usually below the limit of quantification) might be found in milk (Seeling *et al*., 2005). ZN is a heat-stable mycotoxin, but under alkaline conditions, a temperature higher than 150 ◦C can degrade the toxin (European Commission, 2000). Children are more affected by ZN-contaminated foods due to high consumption rate of cereal-based food products, (Bhat *et al*., 2010).

6.2. Pharmacokinetics of Zearalenone (ZN)

ZN is rapidly absorbed, following oral administration, by body and high level of toxin can be measured in serum. It was reported that oral bioavailability of ZN can reach to 80% to 85% of the ingested dose. ZN is widely distributed and slowly eliminated from body tissues (Kuiper-Goodman *et al*., 1987; Fink-Gremmels and Malekinejad, 2007). ZN can be deposited in body tissues and carry over into milk. As reported by Prelusky *et al*., (1990), ZN and its major metabolites (α and β Zearalenols) were detected in plasma and milk of lactating cows. Metabolism of ZN is by reduction to its major metabolites (α and β Zearalenols) by 3-α and 3-β-hydroxysteroid dehydrogenase as catalysts (Olsen and Kiessling, 1983; Othmen *et al*., 2008). This biotransformation mostly occurs in liver, but gut microflora and intestinal mucosa were reported to be able to metabolize ZN (Biehl *et al*., 1993; Kollarczik *et al*., 1994; Danicke *et al*., 2001, 2002). In most animal species (except rabbits), ZN and its metabolites are excreted in the bile but in rabbits urine is the main route (Kuiper-Goodman *et al*., 1987). In the elimination process, the alcoholic metabolites (α and β Zearalenol) are excreted as free compounds and glucuronide conjugates through faeces and urine (Danicke *et al*., 2001; Othmen *et al*., 2008).

6.3. Adverse effects and toxicity of Zearalenone (ZN)

6.3.1 Endocrine toxicity

Due to the estrogenic activity of ZN, farm animals fed with ZN-contaminated feed show alterations in the reproductive tract, decrease in fertility, increase in number of fetal resorptions and implementation failure, and reduced litter size. The alterations in reproductive tract are permanent. ZN can cause infertility, abortion, reproduction problems (especially in swine), and is associated with cervical cancer (Bhatnagar *et al*., 2002 ; El-Nezami *et al*., 2002). Ingestion of contaminated feed results in interference with the exocrine and endocrine

systems. Like other environmental estrogens, ZN has the potential to disrupt sex steroid hormone functions (Bennett and Klich, 2009; Bhat *et al*., 2010). ZN and its metabolites bind to estrogen receptors and activate gene transcription. Besides, they interfere with the regular activity of the endocrine glands (Malekinejad *et al*., 2005; Fink-Gremmels and Malekinejad, 2007). Among animals, pigs are the most sensitive and poultry are the least affected by ZN (Bhat *et al*., 2010). In pigs, ZN poisoning is usually associated with feminizing syndromes or hyperestrogenic activity and causes urinary/genital problems (Danicke *et al*., 2005). According to the Joint FAO/WHO Expert Committee, the safety of ZN is evaluated on the basis of the dose that has no hormonal effect in pigs (JECFA. 2000).

6.3.2. Hematotoxicity

ZN was also shown to be haematotoxic. According to Maaroufi *et al*. (1996), dysfunction of the blood coagulation process in rats and some blood parameters changes (hematocrit, MCV, the number of platelets and WBC) as well as some biochemical markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum creatinine, bilirubin, were observed in vivo indicating liver toxicity. ZN was also found to be hepatotoxic. The effects of low (10 µg/kg b.w.) and high (100 µg/kg b.w.) doses of ZN on selected blood serum enzyme activities of AST, ALT, ALP, gammaglutamyl transferase (GGT), and total lactate dehydrogenase (LD) of rabbits were studied by ConKova´ *et al*.(2001). Results from this study reported a significant increase in ALP activity at 168 and 336 h during the experiment with the first group (10 µg/kg b.w.). In the second 100 µg ZN sera group, significant increases in activities of AST, ALT, ALP, GGT, and LD were observed at 168 and 336 h, indicating possible liver toxicity due to chronic effects of the toxin. ZN and derivatives were evaluated by IARC and are classified, in the group 3 (IARC, 1999).

6.3.3. Reproductive and developmental toxicity

ZN and its metabolites can bind competitively to estrogen receptors which subsequently activate estrogen response elements, resulting in the dimerization of the receptors and a variety of induced estrogenic effects (Kuiper *et al.*, 1987; Ahmad *et al*., 2018). In addition, the competitive binding products can also bind to the DNA template through the regulation of uterine target gene transcription and protein synthesis. This results in animal reproductive

hormone disorders, thus affecting the animal's reproductive development system (Li *et al*.,2015 ; Zhang *et al*., 2017). Studies have shown that exposure to ZN in the early pregnancy stage can affect the development of the placenta and embryo, and even lead to the deformity or death of the embryo (Zhang *et al*., 2014).

6.3.4. Immunotoxicity

ZN can also bind to ERs on the surface of the cells of the immune system and thus regulate a variety of metabolic pathways of the immune response. It has been confirmed that ZN not only activates immune response-related genes, but also interferes with the immune system of the spleen, changes the phenotypes of spleen lymphocytes, and even causes lymphocyte atrophy (Abbès *et al*., 2006; Hueza *et al*., 2014). In addition, ZN can in duce immunosuppression by reducing immunoglobulins in serum and cytokines in lymphoid organs (Pistol *et al*., 2015).

6.3.5. Cytotoxicity

ZN can bind to the estrogen receptors in cytoplasm and result in lipid peroxidation (lipid peroxide can cause serious damage to cell membranes, lipoproteins, and other organelles and cell components containing lipid structures) which then produces a series of cytotoxic effects (Hou *et al.*, 2013; Zheng *et al*.,2018). Early studies show that ZN-induced apoptosis is related to mitochondrial apoptosis or the endoplasmic reticulum stress pathway, which is characterized by the mass production of reactive oxygen species and aggravation of lipid peroxidation (Ben Salem *et al*., 2015; Ben Salem *et al*., 2017). In recent years, it has been confirmed that the apoptosis of kidney cells in mice due to the presence of ZN in their diet are caused by the activation of the endoplasmic reticulum stress pathway (Wang *et al*., 2018).

4.5. Protective effect of plants against zearalenone toxicity

Indeed, they have already demonstrated that almost all ZN toxic effects are significantly prevented *in vivo* and *in vitro* using Vitamin E (Abid-Essefi *et al*., 2003; Ouanes *et al*., 2003, 2005; Hassen *et al*., 2007). Furthermore, Zourgui *et al.* (2008) demonstrate a total protection obtained by cactus cladodes extract against ZN induced oxidative damage and DNA fragmentation, using Balb/c mouse as a sensitive laboratory animal model with ZN (40 mg/kg b.w). A study, carried out by Ben Salah-Abbes *et al*. (2009) has demonstrate the protective

role of the radish (*Raphanus sativus*) aqueous extract against ZN induced reproductive toxicity, oxidative stress and genotoxicity in Balb/c mice model with ZN (40 mg/kg b.w.).

1. Materials

1.1. Chemicals

The chemicals, reagents and kits used in the present study as well as their sources are displayed as follows:

Table 02. The chemicals, reagents and kits

Name of chemical or reagent	Source
2,2-diphenyl-1-picrylhydrazyl (DPPH) 2,2′- azinobis-3- ethylbenzothiazoline-6- sulfonic acid (ABTS) linoleic acid β-carotene 2,6-di-tert-butyl-4-hydroxytoluene (BHA), Tween 40 neocuproine Doxorubicin RPMI-1640 medium MTT DMSO Alanine aminotransferase reagent kit (ALT)	Sigma Aldrich (MO, USA)
Aspartate aminotransferase reagent kit (AST) Alkaline phosphatase kit (ALP) lipid peroxidase (MDA) glutathione peroxidase (GPx) catalase (CAT) activities cholesterol (CHO) triglycerides (TG) high density lipoprotein (HDL) low density lipoprotein (LDL) Interleukin-6 (IL-6), carcinoembryonic antigen (CEA)	Eagle Diagnostics (Dallas, TX, USA)
tumor necrosis factor-alpha (TNF-α) Createnine kit Thiobarbituric acid	Orgenium (Helsinki, Finland)
Trichlroacetic acid Total antioxidant capacity kit Urea kit Uric acid kit	Bio-diagnostic, Egypt

1.2. Plant materiel

The C. speciosus roots were purchased from local herb stores in Sétif, Algeria and were imported from India. The plant was authenticated by a plant taxonomist at the Department of Botany, Faculty of Natural and Life Science, University of Ferhat Abbas Setif 1, Algeria.

2. Methods

2.1. Plant extraction

The roots of the plant were washed and dried in the oven (40°C) for one week until their weight was constant. The dried roots were ground to reach a particle size of 200 to 500 µm and were extracted with : water, 70% ethanol or 70% methanol at the room temperature under agitation for 24 or 36 h. The extracts were evaporated under reduced pressure and the final residue was a dried to obtain powdered material which was then used to assess different assays. The percentage of the extract yield was calculated as follows:

$$R\ (\%) = (\text{Mass of extract} / \text{Mass of plant powder}) \times 100$$

2.2. Phytochemical Characterization

2.2.1. Determination of polyphenols of *C speciosus* extracts by HPLC

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.02% tri-floro-acetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0-5 min (80% A); 5-8 min (40% A); 8-12 min (50% A) and 12-16 min (80% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the sample solutions and the column temperature was maintained at 35°C**.**

2.2.2. Determination of total phenolic content of *C speciosus* extracts

Total phenolic content (TPC) was determined spectrophotometrically, using the Folin-Ciocalteau reagent according to the method described by Li et al. (2007). Folin-Ciocalteu reagent is a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungsten (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol

oxidation. This reaction occurring under alkaline conditions is carried out with sodium carbonate. Blue coloration is measured at 765 nm and reflects the quantity of polyphenols usually expressed as gallic acid equivalent (GAE).

A 200 µl of the tested extract was mixed with 1 ml of Folin-Ciolcalteu reagent (10%) then a volume of 800 µl of sodium carbonate solution (75 g/l) was added after 4 min. The mixture was incubated for 2 h in dark and at room temperature to react then the absorbance was read at 765 nm using UV-Vis spectrophotometer. To establish the calibration curve, gallic acid was used as reference. The concentration of polyphénols was calculated and the results were expressed in µg equivalents of gallic acid per mg extract (µg GA/mg) (Fig.08).

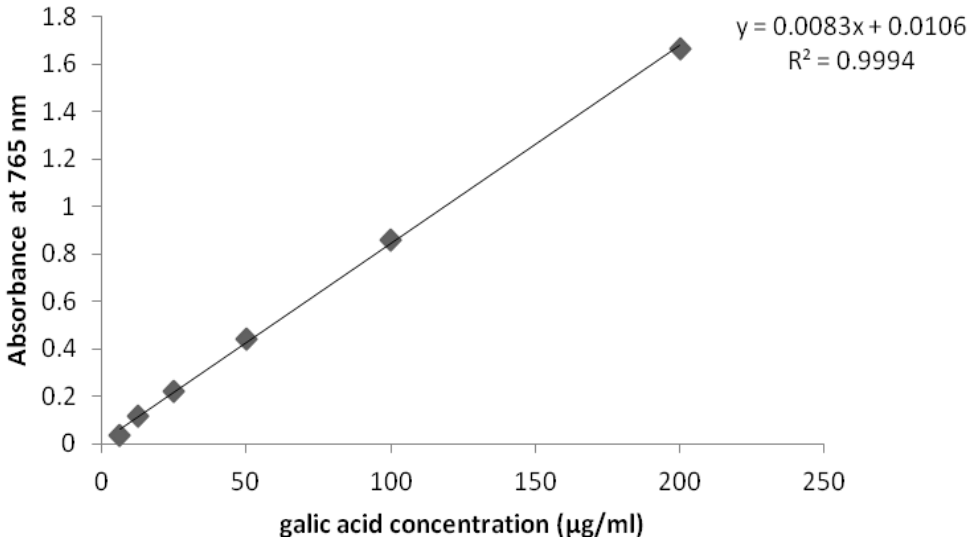

Figure 08. Standard curve of gallic acid used in the determination of total polyphenols in various plants extracts

2.2.3. Determination of total flavonoid content of *C speciosus* extracts

The basic principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoides. rutin is reported to be suitable for building the calibration curve. Therefore, standard rutin solutions of various concentrations were used to build up the calibration curve (Bhaigyabati et al., 2014) [2].

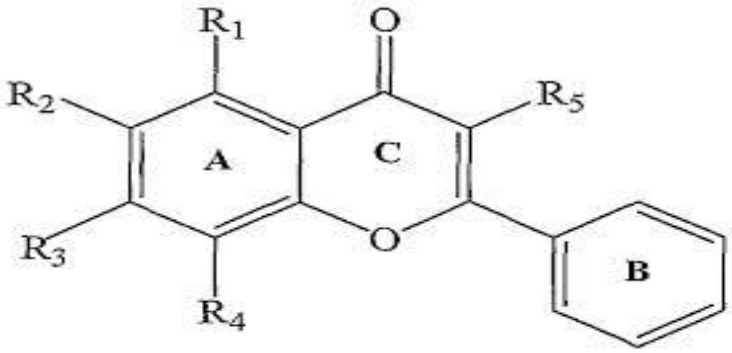

Figure 09 . Basic structure of flavonoids

The TFC of different extracts was determined using the Trichloro aluminum method described by Turkoglu *et al*. (2007). Briefly, 1 ml of $AlCl_3$ methanolic solution (2%) was added to 1 ml of each extract and the mixture was incubated for 30 min at room temperature, then the absorbance was read at 430 nm. To estimate the flavonoides concentration, rutin was used as a standard and the results were expressed as µg equivalent of rutin/ mg (µg ER/mg) (Fig.10).

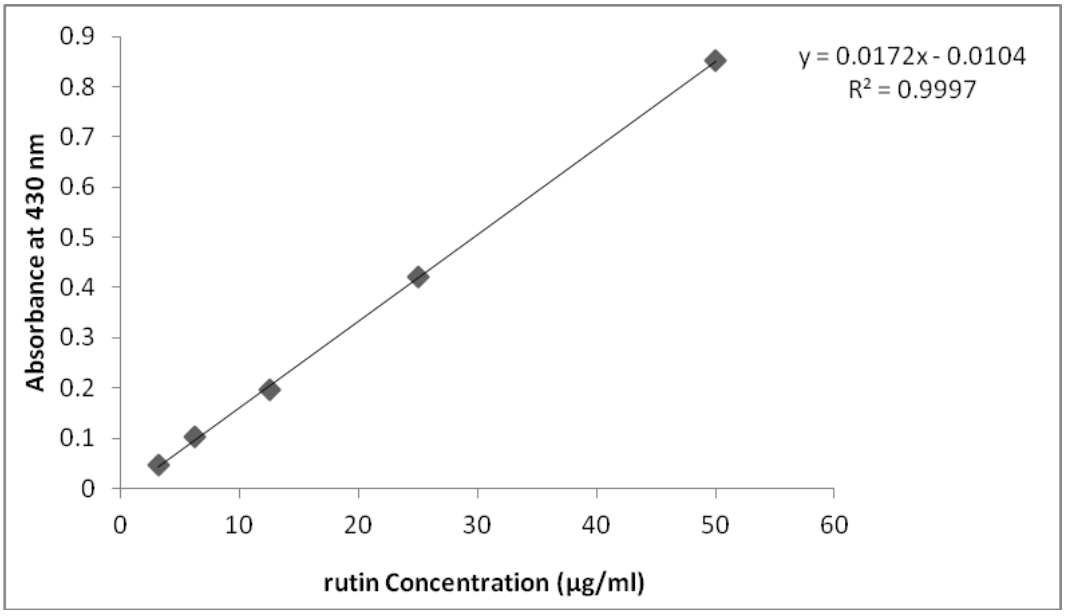

Figure10. Standard curve of rutin used in the determination of total flavonoids in various plant extracts.

2.3. Antioxidant activity of *C speciosus* extracts

The antioxidant activity of different plant extracts was evaluated using seven methods: total antioxidant activity**,** DPPH assay, the ABTS test, CUPRAC method, the β-carotene bleaching test, ferrous chelation and phenanthroline test.

2.3. 1. Total antioxidant capacity of *C speciosus* extracts

A spectrophotometric method has been developed for the quantitative determination of antioxidant capacity. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. The method has been optimized and characterized with respect to linearity interval, repetitivity and reproducibility, and molar absorption coefficients for the quantitation of several antioxidants (Prieto *et al*., 1999).

The total antioxidant capacity of each extract was carried out using the method described by Prieto *et al*. (1999). In brief, 0.1 ml aliquot of the plant extract was added to 1 ml of the reagent solution (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulfuric acid). The tubes were incubated at 95 °C for 90 min. The samples were then left to cool to room temperature, the absorbance of the mixture was measured at 695 nm and a standard curve was performed with the ascorbic acid solution. The antioxidant capacity of the samples was expressed as µg of ascorbic acid equivalents per mg of extract.

2.3.2. Free radical scavenging activity by DPPH assay

DPPH (Fig.11) is a stable radical owing to stabilization by delocalization on to aromatic rings. DPPH$^{\cdot}$ can trap other radicals easily but does not dimerize. Because a strong absorption band is centered at about 515 nm, the solution of DPPH radical form in deep violet in color and it becomes colorless to pale yellow when reduced upon reaction with hydrogen donor. The decrease in absorbance depends linearly on antioxidant concentration (Gupta, 2015)

DPPH scavenging activity was evaluated according to the method of Blois (1958). In brief, 160 µl of DPPH solution (0.04 mg/ml) were added to 40 µl of different dilutions of the extracts (aqueous, ethanolic and methanolic) or standards (BHT, BHA). The mixture was incubated for 30 min in obscurity and the absorbance of each solution was read at 517 nm using a microplate reader (Fig. 09). The percent of inhibition was calculated as follow:

$$I\,(\%) = (Ab - As/A\,b) \times 100$$

Where: *Ab* is the absorbance of the control reaction /*As* is the absorbance of the test compound

The IC50 was calculated from the graph plotting inhibition percentage against extract concentration.

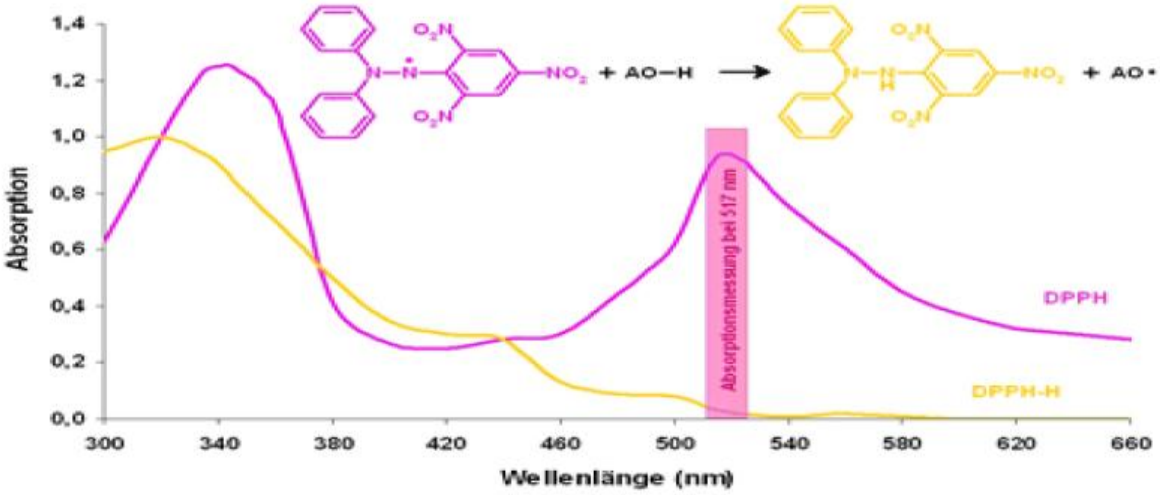

Figure11. Reaction mechanism of DPPH with antioxidant. (H = antioxidant radical scavenger)

2.3.3. ABTS decolorization assay of *C. speciosus* extracts

ABTS is test frequently used by the food industry and agricultural researchers to measure the antioxidant capacities. In this assay, ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The $ABTS^{\cdot+}$ radical cation is reactive towards most antioxidants including phenolics, thiols and Vitamin C. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form. The reaction may be monitored spectrophotometrically.

The ABTS decolorization assay of extracts was determined according to the method of Re *et al*. **(**1999**).** The $ABTS^{\bullet+}$ solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate using the water as solvent. The solution was left in the dark at room temperature for 18 h before used. The reaction mixture was contained 160 µl of $ABTS^{\bullet+}$ solution extract and 40 µl of different dilutions of extracts or standards (BHT, BHA). After incubation for 10 min at room temperature, the absorbance was measured at 734 nm using a 96-well microplate reader. The percentage of ABTS radical scavenging activity was calculated as follow:

ABTS radical scavenging activity (%) = [A control - A sample / A control] x 100

The IC50 was calculated from the graph plotting inhibition percentage against extract concentration.

2.3.4. CUPRAC assay of *C speciosus* extracts

The method involves the reduction of the copper-neocuproine complex $[Cu(Nc)_2]^{2+}$ by the antioxidant (AOX) in the presence of ammonium acetate to form copper-neocuproine complex $[Cu(Nc)_2]^{+}$, a yellow compound (Fig. 12), with a maximum absorption at $\lambda = 450$ nm (Özyürek *et al*., 2011; Trofin *et al*., 2019).

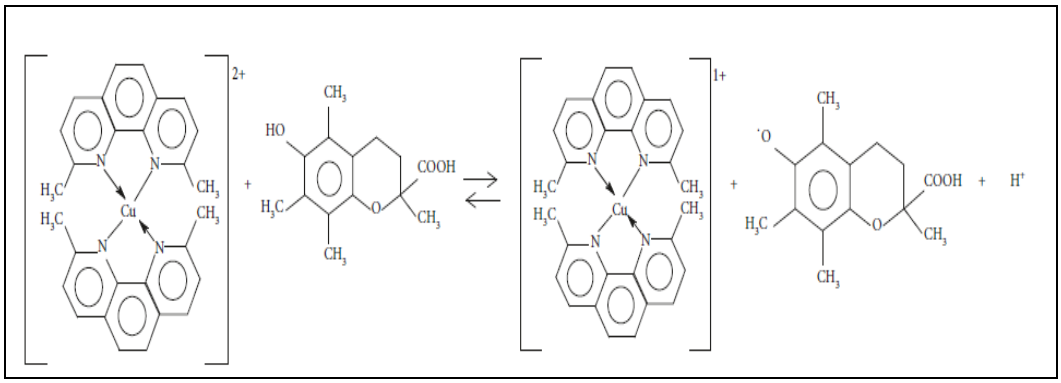

Figure 12. The redox reaction between copper-neocuproine complex and trolo(Özyürek *et al*., 2011).

The cupric reducing antioxidant capacity was evaluated according to the method described by Apak *et al*. **(**2004). In brief, 40 µl of different dilutions of extracts and 50 µl of $CuCl_2$ solution (10 mM) were added into a 96 well round-bottomed plate then 50 µl of neocuproine solution (7.5 mM) and 60 µl of NH_4Ac buffer (1 M, pH 7.0) solution were added to each well. After 60 min the absorbance was measured at 450 nm.

2.3. 5. β-carotene bleaching assay of *C speciosus* extracts

The method is based on the discoloration of yellowish color of a β-carotene solution due to the breaking of π-conjugation by addition reaction of lipid or lipid peroxyl radical (or) to a C=C double bond of β-carotene. The radical species is generated from the autoxidation of linoleic acid by heating under air atmosphere. When the appropriate antioxidant is added to the solution, the discoloration can be retarded by competing reaction between β-carotene and antioxidant with the subjected radicals (Djordjevic *et al*., 2004).

The β-carotene bleaching activity of different extracts and standards (BHA and BHT) was evaluated using the method described by Marco **(**1968). For the preparation of β-carotene

emulsion, 0.5 mg of β-carotene was mixed initially with 200 mg of Tween 40 and 25 µl of linoleic acid in 1 ml chloroform. After the evaporation of the chloroform under vacuum, 100 ml of distilled water saturated with oxygen were then added to the mixture under vigorous stirring. One hundred sixty µl of emulsion were added to 40 µl of samples (extracts or standards) at different concentrations. The mixtures were incubated at 50°C for 2 h, the absorbance was measured at 470 nm and the percentage of the antioxidant activity (AA) was calculated using the following formula:

AA% = (Abs of β-carotene content after 2 h/Abs of initial β-carotene content) × 100

2.3. 6. Ferrous ions chelating assay of *C speciosus* extracts

In the assay, ferrous iron (Fe^{2+}) reacts with Ferene S to produce a stable colored complex with absorbance at 593 nm. Ferric iron (Fe^{3+}) can be reduced to form Fe^{2+} enabling the measurement of total iron (Fe^{2+} and Fe^{3+}). The level of ferric iron (Fe^{3+}) is calculated by subtracting ferrous iron from total iron (Sudan *et al*., 2014).

The ferrous ion chelating potential of the extracts was measured according to the method of Decker and Welch **(**1990) with slight modifications. Forty µl of $FeCl_2$ (0.2 mM) was mixed with each extract at different concentrations. The reaction was initiated by the addition of ferene (0.5 mM). The mixture was shaken well then incubated for 10 min at room temperature and the absorbance was determined at 593 nm. EDTA was used as antioxidant standard and percent chelation was calculated using the following equation.

AA (%) = [Abs control - Abs sample / Abs control] x 100

2.3.7. Phenanthroline method

1,10-phenanthroline ($C_{12}H_8N_2$, ortho-phenanthroline or o-phen) is a tricyclic nitrogen heterocyclic compound that reacts with radical or metals such as iron, nickel, ruthenium, and silver to form strongly colored complexes. This property provides an excellent and sensitive method for determining these metal ions in aqueous solution the wavelength of maximum absorbance intensity, 510 nm (Skoog *et al*., 2005).

The Phenanthroline content in *C. speciosus* and standards (BHA and BHT) was evaluated using the method described by Szydlowska-Czerniaka (2008) with some modifications. In brief, 10 µl of different dilutions of the sample solutions were added to 50 µl

of the mixture of $FeCl_3$ (0.2%), 30 µl of 1, 10-phenanthroline (0.5%) and 110 µl of methanol. The mixture was left at room temperature in a dark for 20 min then the absorbance of was read at 510 nm against a reagent blank.

2.4. Cytotoxicity assay of C speciosus extracts

Hepatocellular carcinoma (HePG-2) cell lines were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. Doxorubicin was used as a standard anticancer drug for comparison. The cell lines were used to determine the inhibitory effects of the aqueous, methanolic and ethanolic extracts on cell growth using the MTT assay (Denizot and Lang, 1986). The cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 µg/ml streptomycin. The cell lines were seeded in a 96-well plate at a density of 1.0 $x10^4$ cells/well at 37 °C for 48 h under 5% CO_2 and were incubated for 24 h then the cells were treated with different concentrations of the tested extracts and incubated for another 24 h. After the incubation period, 20 µl of MTT solution (5 mg/ml) was added and incubated for 4 h. DMSO (100 µl) was added into each well to dissolve the purple formazan formed. The colorimetric assay was measured and recorded at absorbance of 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X 100 (Mosmann, 1983).

2.5. Anticoagulant activity of *C speciosus* extracts

2.5.1. *In vitro* evaluation of *C speciosus*

To determinate the influence of different concentrations of aqueous, ethanolic and methanolic extracts of C speciosus on the coagulation activity was evaluated using prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) by using a hemostasis analyzer (Stago-STA Compact Max®). The samples were dissolved in physiological serum. All analyses were carried out in triplicate.

2.5.1.1. Collection of blood and separation of plasma

About 10 ml of blood was collected through vein puncture from healthy volunteers of both sexes (ages 18-35 years old) with no medication history for at least one week before blood sample collection. One µl of 3.8% trisodium citrate solution was added to 9 µl blood to avoid natural coagulation process. The blood samples were immediately centrifugation for 15

min at 3000 rpm using cooling centrifuge and platelet poor plasma (PPP) were separated and stored at -4 °C until use.

2.5.1.2. Prothrombin Time (PT) test

The Prothrombin Time is a one-stage test based upon the time required for a fibrin clot to form after the addition of Tissue Factor, phospholipids and calcium to decalcified, platelet poor plasma. The prothrombin test specifically evaluates the presence of factors VII, V, and X, prothrombin, and fibrinogen. The normal reference range for PT assay is between 12.2 -14.2 sec. If there is deficiency in coagulation factor or presence of coagulation inhibitor, PT will be prolonged (Hood *et al*., 2008).

The action in extrinsic pathway was evaluated by PT test, The test was carried out using commercial reagent kits. Plasma (90 µl) was mixed with 10 µl of samples and incubated at 37°C for 5 min at 37°C. Then, 200 µL of PT assay reagent (rabbit brain extract and calcium chloride) pre-warmed at 37°C for 10 min was added and the clotting time was recorded by a digital coagulometer . Plasma alone (only with vehicle) was used as control (absence of anticoagulant activity).

2.5.1.3. Activated partial thromboplastin time (aPPT) test

APTT is also known as Kaolin Cephalin Clotting time (KCCT) or Partial Thromboplastin Time with kaolin (PTK). The aPTT test is used to measure and evaluate all the clotting factors of the intrinsic and common pathways of the clotting cascade by measuring the time (in seconds) it takes a clot to form after adding calcium and phospholipid emulsion to a plasma sample (Cramer and Gale, 2011).A prolonged APTT will be seen if any of these factors, prothrombin, fibrinogen, factors V, VIII, IX, X, XI and XII are abnormal (Cohen, 2014). The normal range for APTT is 31.7 - 44 sec. A prolonged APTT can be seen either in the presence of inhibitor for intrinsic pathway or reduced activity of the factors (<30%).

The action in intrinsic and common pathways was evaluated by aPTT test .The test was carried out using commercial reagent kits. Plasma (90 µL) was mixed with 10 µL of samples and incubated at 37°C for 5 min at 37°C, before the addition of prewarmed aPTT reagent and incubation at 37°C for 2 min. Pre-warmed (37°C) 25 mM calcium chloride was then added

and the clotting time recorded by a digital coagulometer. Plasma alone (only with vehicle) was used as control (absence of anticoagulant activity).

2.5.1.4. Thrombin time (TT)

TT assay measures the time taken for the conversion of fibrinogen to fibrin in the presence of thrombin. Usually TT assay tests for the acquired deficiency of fibrinogen which is due to coagulopathy or severe liver disease (Raber, 1990). The time taken for the formation of fibrin filaments is measured. The normal range for TT assay is 15-20 sec. Abnormalities of TT assay can be due to three reasons. First, it can be due to deficiency in volume of fibrinogen (<100 mg/dl) (Choi *et al*., 2003). In addition, it also can be due to the presence of inhibitor (heparin or fibrin degradation products, FDP) or abnormal fibrinogen. Similar to APTT and PT, mixing study can be performed in 1:1 ratio to distinguish between deficiency of fibrinogen or decrease in fibrinogen.

This assay measures the prolongation of thrombin generation. When human plasma is incubated with a compound which inhibits blood coagulation, the time taken for clot formation will be prolonged compared to the control (test devoid of inhibitor). In this assay, 130 µl of human plasma (pre-incubated at 37 °C for 5 min before use) was incubated with different concentrations of the 20 µl extract for 5 min at 37°C; normal plasma served as the controls. Concentrations (1 IU/ml) of heparin were used as the reference anticoagulant. A fixed concentration (150 µl) of bovine thrombin (2.5 U/ml, Sigma) was added to each sample to initiate reaction. The time for clot formation was recorded accordingly. Results were expressed as a prolongation time relative to controls. The Thrombin time value is expressed in seconds.

2.5.2. *In vivo* evoluation of *C speciosus* extracts (CSE)

2.5.2.1. Animals

The male Sprague Dawley rats weighing 150-160 g were purchased from Animal House Colony, National Research Centre, Dokki, Giza, Egypt. Animals were maintained on standard laboratory diet (metabolizable energy 12.08 MJ; protein: 160.4; fat: 36.3; fiber: 41 g/kg purchased from Meladco Feed Co., Aubor City, Cairo, Egypt). The animals were housed in filter-top polycarbonate cages in a room free from any source of chemical contamination,

artificially illuminated (12h dark/light cycle) and thermally controlled (25 ± 1°C) and humidity (50 ± 5%) at the Animal House Lab., National Research Centre, Dokki, Giza, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre and the National Institutes of Health (NIH publication 86-23 revised 1985)

2.5.2.2. Experimental design for in vivo anti-coagulate assay

Thirty rats were allocated randomly to three groups of ten animals each and treated for 15 day as follow:

Group A: Control group; rats were orally administered dose of physiological saline and served as negative controls.

Group B: Low dose CSE treated group; rats were orally administered with low dose of CSE (200 mg/kg).

Group C: High dose CSE treated group; rats were orally administered with high dose of CSE (400 mg/kg).

2.5.2.3. Anticoagulant assay

Blood samples were collected via the retro-orbital venous plexus of rats and mixed immediately with 3.2% trisodium citrate in volume ratio of 9:1. Then, the mixture was centrifuged at 3000 x g for 20 min to obtain citrated platelet poor plasma. Each citrated platelet poor plasma (PPP) obtained was transferred directly into a plastic tube to record the clotting time same methods use *in vitro*.

2.6. Evaluation of the protective role of ethanolic extract against Zearalenone-induced hepato-nephro toxicity

2.6.1. Experimental design:

Other sixty rats were divided into six groups (10 rats /group) and were maintained on their respective diet for 2 weeks as follows:

Group (1): normal control animals which fed on basal diet and water without any treatment.

Group (2): low dose CSE group; Rats were orally administered with low dose CSE (200 mg/kg b.w).

Group (3): high dose CSE group; Rats were orally administered with high dose CSE (400 mg/kg b.w).

Group (4): zearalenone (ZN) group; Rats were orally administered with ZEN at a dose 40 µg/kg b.w

Group (5): zearalenone plus low dose CSE group; Rats were orally administrated with ZN and the low dose of CSE

Group (6): zearalenone plus high dose CSE group; Rats were orally administrated with ZN and the high dose of CSE

2.6.2. Sample collection

The animals were observed daily for signs of toxicity during the experimental period. At the end of the treatment period (i.e. day 15), all animals were fasted for 12 hr, then blood samples were collected via the retro-orbital venous plexus under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at -20°C until analysis. All animals were sacrificed by cervical dislocation and samples of the liver, kidney and bone marrow were collected. Two samples of liver and kidney tissues for each animal within different groups were collected the first sample of each organ for each rat was homogenized in phosphate buffer saline. This homogenate was centrifuged at 1700 rpm and 4°C for 10 min; the supernatant was stored at -70 °C until analysis. This supernatant (20%) was used for the determination of MDA and it was further diluted with phosphate buffer solution to give 2% and 0.5% dilutions for the determination of hepatic GPx (2%), CAT (0.5%) activities and TAC level.

2.6.3. Determination of serum biochemical parameters

Serum levels of the ALT, AST, ALP, total protein and albumin as markers of hepatic damage and function were determined. The kidney damage was assessed by the determination of the levels of creatinine (CREA), uric acid (UA) and urea. Plasma levels of Cholesterol, triglyceride, HDL and LDL were measured according to standard procedure using pictus 200 Random Access Clinical Analyzer (Diatron). IL-6 , carcinoembryonic antigen (CEA) and TNF-α were determined according to the manufacturer instructions.

2.6.3.1. Determination of serum ALT

The amino group is enzymatically transferred by ALT present in the sample from alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate.

$$\text{Alanine + 2-Oxoglutarat} \xrightarrow{\text{ALT}} \text{Pyruvate + L-Glutamate}$$

Pyruvate is reduced to Lactate by lactate dehydrogenase (LDH) present in the reagent with the simultaneous oxidation of NADH to NAD^+. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH.

$$\text{Pyruvate + NADH + } H^+ \xrightarrow{\text{LDH}} \text{L-Lactate + } NAD^+$$

Endogenous sample pyruvate is rapidly and completely reduced by LDH during the initial incubation period so that it does not interfere with the assay (Huang et al.,2006)

Determination of serum ALT activity was carried out using a test reagent kit according to the method described by Reitman and Frankel (1957). An aliquot of 0.5 ml of reagent I [Buffer: phosphate buffer (100 mmol/l, PH 7.4), L-alanine (200 mmol/l) , α-oxoglutarate (2 mmol/l) was incubated for 30 min at 37°C after mixing with 0.1 ml serum. An aliquot of 0.5 ml color reagent II [2,4-dinitrophenylhydrazine (2 mmol/)] was then added, mixed and allowed to stand at room temperature for 20 min before the addition of 5 ml of NaOH .Five minutes later, the developed colour was measured at 546 nm against distilled water blank .

2.6.3.2. Determination of serum AST

The amino group is enzymatically transferred by AST present in the sample from L-Aspartate to the carbon atom of 2-oxoglutarate yielding oxaloacetate and L-glutamate.

$$\text{L-Aspartate + 2-Oxoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate + L- glutamate}$$

Oxaloacetate in the presence ofn NADH and malate dehydrogenase (MDH) is reduced to L-malate. In this reaction NADH is oxidized to NAD^+. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to oxidation of NADH to NAD^+.

$$\text{Oxaloacetate + NADH + } H^+ \xrightarrow{\text{MDH}} \text{L -malate + } NAD^+$$

Addition of LDH to the reagent is necessary to achieve rapid and complete reduction of endogenous pyruvate so that it does not interfere with the assay (Wang *et al* .,2016).

Determination of serum AST activity was carried out using a test reagent kit according to the method described by Reitman and Frankel (1957). An aliquot of 0.5 ml of reagent I [Buffer: phosphate buffer (100 mmol/l, PH 7.4), L-aspartate (100 mmol/l), α-oxoglutarate (2

mmol/l)] was incubated for 30 min at 37°C after mixing with 0.1 ml serum. An aliquot of 0.5 ml color reagent II [2,4-dinitrophenylhydrazine (2 mmol/)] was then added, mixed and allowed to stand at room temperature for 20 min before the addition of 5 ml of NaOH . Five minutes later, the developed colour was measured at 546 nm against distilled water blank.

2.6.3. 3. Determination of serum ALP

Alkaline phosphatase is an enzyme present in almost organisms. It catalyses the hydrolysis of Phenyl phosphate at pH 10.4 liberating phenol and phosphate is measured colorimetrically, in the presence of 4-aminophenazone and potassium ferricyanide (Niu et al.,2019)

$$\text{Phenyl phosphate} \xrightarrow{\text{ALP}} \text{phenol + phosphate}$$

Alkaline phosphatase was determined in serum according to Blfield and Goldberg (1971). The reagent was prepared by mixing of serum and buffer substrate [buffer PH: 10 (50 mmol) + phenyl phosphate (5mmol)] the components were incubated for 20 min. Exactly at 37°C, and then R3 [enzyme inhibitor : EDTA 100 mmol/l + 4-aminophenazone 50 mmol/l] was added The components were mixed well then R4 was added. The components were mixed; let stand for 5 min at room temperature in the dark and the absorbance of sample (A_{sample}) and standard ($A_{standard}$) was read against reagent blank. The enzyme activity was calculated as follows:

$$\text{Enzyme activity (U/L)} = \frac{A_{sample}}{A_{standard}} \times 75$$

2.6.4. Total Protein

Total protein level can be measure in serum. Proteins have ability to form stable complex with Cu^{2+} ions at alkaline pH and photometrically (Bertholf,2014).

In order to determine total protein level, 1 ml of reagent solution [NaOH (0.47 M), Potassium iodide (23.3 mM), Copper (II) sulphate (6.5 mM) and Sodium-Potassium Tartrate (22.1 mM)] was pipetted with 0.02 ml of sample or standard [Aqueous solution of Proteins equivalent to 50 g/l] and mixed with left to standing for 10 min at room temperature. The absorbance was determined at 540 nm in accompanied with blank. The total protein was calculated as follows: SA O.D/ST O.D × 5 = g of protrin /dl SAO.D: (Sample absorbance ST O.D: Standard absorbance).

2.6.5. Albumin

Albumin has ability to combine with bromcresol green to produce a coloured complex, which is photometrically measured (Doumas *et al*., 1971). The level of total protein was measured at wave length 630 nm. In order to measure total protein, add 0.01ml of either sample or standard to 2.5 ml of reagent [Succinate buffer pH (4.2 50 mM) and Bromcresol green (0.75 g/L)]. All are mixed, reading absorbance of sample and standard [Aqueous solution of Proteins equivalent to 50 g/L] minutes. Subtract reagent blank value from that of the sample and standard. The albumin was calculated as follows: SA O.D / ST O.D. X 5 = g albumin /dl. (SAO.D: Sample absorbance ST O.D: Standard absorbance).

2.6.6. Determination of Creatinine levels

Creatinine is the product of the degradation of creatine. Creatinine reacts with picric acid in an alkaline solution to form a reddish colored complex (Toora *et al*., 2002).

Creatinine was determined in serum according to **(**Bartles *et al.,* 1972). In this assay the working reagent contains always two references [R1: pinic acid (25 mmo/L) and R2: sodium hydroxide (0.4mmol/L)]. Equal volumes of reagents 1and 2 were mixed before assay. The components were mixed and after 20 seconds at 20-25°C, the absorbance A of the standard (R3: Standard: 2 mg/dl (177µmol/L) and sample were read at 495 (490-510 nm) against blank. The creatinine was calculated as follows:

$$A_{\text{sample}} - A_{\text{standard}} = \Delta A_{\text{sample}} \text{ or } \Delta A_{\text{standard.}}$$

$$\text{Creatinine in serum (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 2$$

2.6.7. Determination of Urea

Urea is the final degradation product of protein and amino acid metabolism. In protein catabolism the proteins are broken down to amino acids and deaminated. The ammonia formed in this process is metabolized to urea in the liver. This is the most important catabolic pathway for eliminating excess nitrogen in the body. Enzymatic determination of urea was carried out according to the following reaction:

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{Urease}} 2NH^+_4 + CO_2$$

The ammonium ions formed react with salicylate and hypochloride to give a green dye (2.2 dicarboxylindophenol).

The determination of serum urea level was carried out using a test reagent kit according to the modified Berthelot method described by Fawcett and Scott (1960)**.** An aliquot of 1000 µl of reagent I [Phosphate buffer (PH 6.7; 60 mmol), EDTA (1.5 mmol) , sodium salicylate (60 mmol/l and Sodium nitroprusside (5.2 mmol/l)] was mixed with 10 µl serum. An aliquot of 50 µl of reagent II (Urease > 5000 U/l) was then added, mixed and allowed to stand at 37°C for 3 min before the addition of reagent III [Sodium hypochlorite (18 mmol/l) and Sodium hydroxide (450 mmol/l)] and incubate for 5 min at 37°C. The developed green color was measured at 600 nm using spectrophotometer against reagent blank using 1 cm light path-cuvette. The concentration of urea was calculated as follows:

$$\text{urea conc. (mg/dl)} = \text{Standard conc} \times \frac{\Delta A_{sample}}{\Delta A_{standard}}$$

(ΔA_{sample}: absorbance of sample. $\Delta A_{standard}$: absorbance of standard)

2.6.8. Determination of uric acid

Uric acid is oxidized by uricase to allantoine and hydrogen peroxide ($2H_2O_2$) which under the influence of POD, 4-aminophenazone (4-AP) and 2-4 Dichlorophenol sulfonates (DCPS) forms a red quinoneimine compound (Galbán *et al* .,2001):

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{Urease}} \text{Allantoine} + CO_2 + 2H_2O_2$$

$$2H_2O_2 + 4\text{-AP} + \text{DCPS} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

Working reagent is prepared by the mixing the two reagents 1(Chromogen-buffer (25 ml),Tris buffer (50 mmol) and 3,5, Dichloro 2-hydroxybenzensulphonate (5.0 mmol/L) and reagents 2 (Surface-active agent, Peroxidase > 2000U/L Uricase > 500IU/L, 4-Aminoantipyrine 0.20 mmol/L) before the assay. An aliquot of 1 ml of reagent working was mixed with 0.05 ml sample or Standard (6 mg/dl) incubated for 10 min at 37°C. the absorbance was measured at 520 nm of the sample (A_{sample}) and the standard ($A_{standard}$) were read against blank . The concentration of urea acid was calculated as follows

$$\text{Uric acid in serum} = \frac{A_{sample}}{A_{standard}} \times \text{standard conc.}$$

2.6.9. Evaluation of serum lipid profile

2.6.9.1. Determination of CHL

Total Cholesterol is measured enzymatically in serum or plasma in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reactions by products, H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a color. Absorbance is measured at 500 nm. The color intensity is proportional to cholesterol concentration (Li *et al*., 2019).

Cholesterol standards, samples, and blanks were dissolved in isopropanol:NP40 (9:1, v:v) and treated similarly, using 1 ml to redissolve each well of cells from a 6-well dish. In a black 96-well plate, 10 µl of a 100 U/ml catalase solution was distributed in each well and 40 µL of each sample was mixed followed by 15 min incubation at 37°C in order to eliminate any peroxides present in reagents or samples. Then 150 µl of reagent A (0.1 M potassium phosphate buffer, pH 7.4, 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, 0.3 U/ml cholesterol oxidase, 1.3 U/ml HRP, and 0.4 mM ADHP) was added and mixed in each well. The plate was incubated at 37°C for an additional 15 min and fluorescence was read at an excitation wavelength of 530 nm and an emission wavelength of 580 nm. The cholesterol mass of the 40 µl aliquot of the unknown samples was determined by linear regression using the fluorescence emission of the blanks and the 40 µl cholesterol standards (20 to 800 ng range)(Robinet *et al*., 2010).

2.6.9.2. Determination of TG

Triglycerides are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase, and H_2O_2, one of the reaction products, is measured as described above for cholesterol. The reaction sequence is as follows:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerolkinase}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol-3p-oxidase}} \text{Dihydroace tonephosphate} + H_2O_2$$

$$2H_2O_2 + 4\text{–amin antipyrine} + \text{P-Chlorophenol} \xrightarrow{\text{poxidase}} \text{Quinoneimine} + 4H_2O + HCL$$

The method (Bucolo and David 1973) is based on hydrolysis of TG in the samples using lipoprotein lipase to form glycerol which in turn is converted to glycerol-3-phosphate under the influence of glycerol kinase. The Concentration of serum triglycerides was calculated as follows: $\frac{A_T}{A_S} X\ C_S$ = (mg/dl)

(Where A_T = absorbance the test sample, A_S = absorbance the standard sample and C_S = concentration of standard.)

2.6.9.3. Determination of HDL

In this assay, very low-density lipoproteins (VLDL) and LDL in the sample were precipitated with phosphotungstate and magnesium ions (Lopes-Virella et al., 1977) .The supernatant contains the HDL, which was then spectrophotometrically measured by means of the coupled reactions described below:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterd Oxidase}} \text{Cholesterol+ fatty acids}$$

$$\text{Cholesterol}+{}^1/_2\ O_2+H_2O \xrightarrow{\text{Peroxidase}} \text{Cholestenone} + H_2O_2$$

$$2H_2O_2+\text{4-aminoantipyrine+phenol} \xrightarrow{\text{Cholesterd esterase}} \text{Quinoneimine} + 4H_2O$$

A mixture of 200 µl sample and 500 µl reagent A [Phosphotungstate (0.4 mmol/L) and Magnesium chloride (20 mmol/L)] was left to stand for 10 min, at room temperature and then centrifuged at 4000 rpm for 10 min. A volume of 100 µl of the clear supernatant was pipetted into a spectrophotometer cuvette, followed by the addition of 1 ml of reagent B (color reagent) and incubation at 37 ^oC for 10 min. The absorbance was read at 500 nm within 30 min. against a blank containing 100 µl distilled water and l ml of reagentB. The sampling procedure was repeated using 100µl HDL standard (HDL-C) instead of the sample.

$$\text{Concentration of serum HDL-cholesterol (mg/dl)} = \frac{A_T}{A_S} X\ C_S X \text{ dilution factor}$$

(A_T = absorbance the test sample. A_S = absorbance of the standard sample. C_S =concentration of standard)

2.6.9.4. Determination of LDL

LDL (mg/dl) was calculated according to the following friedewald equation (Friedewald *et al*., 1972) using the measured TG, TC and HDL concentrations: LDL = TC - HDL-(TG/5)

2.6.10. TNF-α level

Tumor Necrosis Factor-alpha (TNF-α) is a non-glycosylated 17.5 kDa, 157 amino acid proteins that is a potent lymphoid factor, which exerts cytotoxic effects on a wide range of tumor cells and other target cells. TNF-α plays a pro-inflammatory role and has been detected in synovial fluid of patients with rheumatoid arthritis. TNF-α (tumor necrosis factor-α) is secreted by macrophages, monocytes, neutrophils, T-cells, NK-cells following their stimulation by bacterial lipopolysaccharides. Various pathological conditions are associated with the production of high levels of TNF-α such as septic shock syndrome, cachexia (e.g. HIV, tuberculosis, cancer), autoimmune diseases, hepatitis, leukemia, myocardialischaemia, organ transplantation rejection, multiple sclerosis, rheumatoid arthritis, and meningococcal septicemia (Waage *et al.,* 1987; Intiso *et al.,* 2004)**.**

TNF-α investigation depends on enzyme-linked immunosorbent assay in serum, plasma, cell culture supernatants and urine. This assay will be done through an antibody specific for human TNF-α coated on a 96-well plate. Standards, samples and biotinylated anti-human TNF-α are pipetted into the wells and TNF-α present in a sample is captured by antibody immobilized to the wells and biotinylated TNF-α specific detection antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm using ELISA reader.

2.6.11. Determination of IL-6

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal

antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured.

Samples and standards as instructed. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature or over night at 4ºC. Add 100 µl prepared biotin antibody to each well. Incubate 1 hour at room temperature. Add 100 µl prepared Streptavidin solution. Incubate 45 minutes at room temperature. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature. Add 50 µl Stop Solution to each well. Read at 450 nm immediately

2.6.12. CEA test

CEA stands for carcinoembryonic antigen. It is a protein found in the tissues of a developing baby. CEA levels normally become very low or disappear after birth. Healthy adults should have very little or no CEA in their body. This test measures the amount of CEA in the blood, and sometimes in other body fluids. CEA is a type of tumor marker. Tumor markers are substances made by cancer cells or by normal cells in response to cancer in the body.

All reagents and samples were brought to room temperature (18 - 25°C) before use and all standards and samples be run at least in duplicate. One hundred µl of each standard and sample were added into appropriate wells, the wells were covered and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking. The solution was discarded and washed 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel. The liquid was completely removed at each step and after the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. Then 100 µl of 1x prepared biotinylated detection antibody (see Reagent Preparation) were added to each well and Incubated for 1 hour at room temperature with gentle shaking then the solution was discarded. The wash was repeated as in step 3 then 100 µl of prepared Streptavidin solution (see Reagent Preparation) was added to each well and incubated for 45 minutes at room temperature with gentle shaking thes the solution was discarded and this step was repeated as in step 3. A 100 µl of TMB

(Item H) were added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Stop Solution (50 µl) (Item I) was added to each well and it was read at 450 nm immediately.

2.6.13. AFP level

This method depends on an immunoenzymometric assay that includes high affinity and specificity of antibodies with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-AFP antibody (Wild, 1994). Simultaneously, the complex was deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. Upon mixing monoclonal biotinylated antibody, the reaction between the enzyme-labeled antibody and a serum containing the native antigen results, without competition or steric hindrance, form a soluble sandwich complex.

Twenty five microliters of the appropriate serum reference, control or specimen were piptted into the assigned well and was added One hundred microliters of the anti-AFP enzyme reagent . The microplate was swirled gently for 20-30 seconds to mix and cover after that the mixture was incubated for 60 min at room temperature. Three hundreds microliters of wash buffer were added Two or three additional times for a total washes . One hundred microliters of working substrate solution was added to all wells. The mixture was incubated at room temperature for 15 minutes. Fifty microliters of stop solution was added to each well and the wells were mixed gently for 15-20 seconds. Then absorbance level was red at 450 nm in a microplate reader. The results were read within thirty minutes of adding the stop solution.

2.6.14. Determination of antioxidants and oxidative stress parameters in liver and kidney homogenate

2.6.14 .1. Lipid peroxidation

Lipid peroxidation is used as an indicator of oxidative stress. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose forming a complex series of compounds e.g. Malondialdehyde (MDA) which can react with thiobarbituric acid. The assay based on reaction between thiobarbituric acid (TBA) and MDA in acidic medium at

temperature of 95°C for 30 minutes to from thiobarbituric acid reactive product. The absorbance of the resultant pink product is measured at 534 nm (Fig.13).

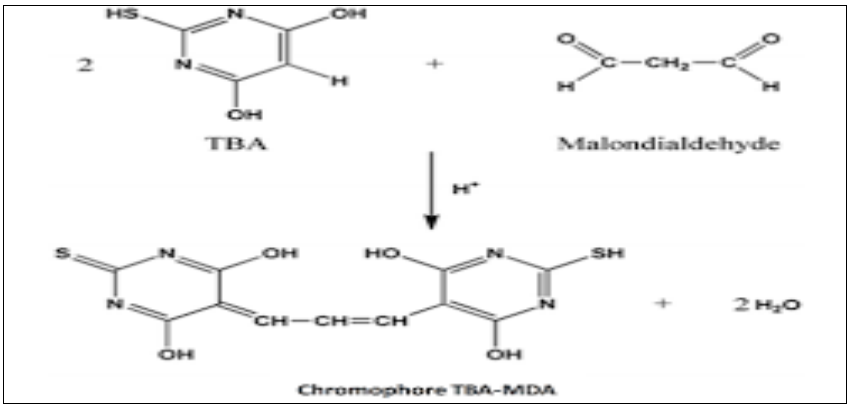

Figure 13. Malondialdehyde formation (Santos-Fandila *et al*., 2014)

Lipid peroxide formation was determined in the liver and kidney homogenate according to the methods described by Satoh (1978) and Ohkawa et al. (1979). In brief, to 0.2 ml of tissue homogenate, 1 ml of TBA (0.67 %) was added. The mixture was incubated for 30 min in boiling water bath. The amount of TBARS formed in each sample was assessed by measuring the optical density of the supernatant at 532 nm against a blank. The absorbance of the sample (A_{Sample}) against blank and standard against distilled water is read at 534 nm. Color stable for 6 hours, linearity up to 100 nmol/ml.

Calculation: Malondialdehyde in the tissue sample= $\frac{A_{sample}}{A_{sandard}} \times \frac{10}{\text{g tissue used}}$ nmol/ g tissue.

2.6.14.2. Determination of TAC level

The determination of the antioxidant capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H_2O_2), the antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual H_2O_2 is determined calorimetrically by an enzymatic reaction which involves the conversion of 3,5,dichloro-2-hydroxy benzensulphonate to colored product.

Total antioxidant capacity was determined in liver and kidney homogenate using a test reagent kit according to the method described by Koracevic *et al.* (2001).Working reagent was prepared by mixing an equal volume of reagents 2 and 3 (Enzyme - Buffer) immediately

before the assay. The reagents 1 (substrate H_2O_2) was diluted 1000 times before use (10 µl reagent 1 + 10 ml distilled water). Then aliquots of 0.5 ml of the substrate were placed into tubes labeled blank and homogenate sample. Then 20µl of distilled water was added into blank tube and 20µl of homogenate was added into homogenate sample tube. This is followed by mixing using a vortex mixer and incubation for 10 min at 37^{o}C. A volume of 0.5ml of the working reagent was added into blank and homogenate sample tubes followed by mixing and incubation for 5 min at 37^{o}C. Finally, the absorbance of blank and homogenate was read at 505 nm against distilled water using a double beam spectrophotometer (UV-150-02, Shimadzu, Japan).

Total antioxidants in tissue homogenate were expressed as nmol/gm tissue and were calculated using the following formula:

$$\text{Total antioxidant conc. (nmol/gm tissue)} = A_{blank} - A_{sample} \times 3.33$$

2.6.14.3. Determination of GPx activity

Glutathione peroxidase (GPx) catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

$$2GSH + ROOH \xrightarrow{GP_X} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

The activity of GP_X was determined in the rat tissues homogenate according to the method of Paglia and Valentine *(1967).*

Ten µl of Cunene hydroperoxide was diluted with 10 mL of distilled water and mixed thoroughly by shaking vigorously as the cumene is difficult to dissolve. This concentrate is stable up to the expiry date when stored at +2 to +8^{o}C. The cumene hydroperoxide volume was measured using a pipette with a positive displacement action, and glass capillaries were used. The contents of a diluting agent vial of diluting agent were reconstituted with 200 ml of redistilled water (stable for 4 weeks when stored at +2 to +8^{o}C or 3 days at +15 to +25^{o}C). The level of glutathione peroxidase was measured at wave length 340 nm at 37°C. In order to measure diluted sample, 0.05ml of diluted sample and 2.5 ml of reagent I were mixed with

0.1ml cummen and all were mixed then add the initial absorbance of sample and reagent blank were read after one minute and again after 2 minutes. The reagent blank value was subtracted from that of the sample. GPx concentration was calculated from the following formula: Unit/L of Haemolysate = 8412 x Ã 340 nm / minute (Ã 340: Absorbance of sample at 340)

2.6.14.4 .Determination of CAT enzyme activity

Catalase reacts with a known quantity of H_2O_2. The reaction is stopped after exactly one min with catalase inhibitor. $2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$

In the presence of peroxidase, remaining H_2O_2 reacts with 3,5-dichloro-2 hydroxybenzene sulfonic acid (DHBS) and 4- aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample.

$$H_2O_2 + \text{DHBS} + \text{AAP} \xrightarrow{\text{HRP}} \text{Quinoneimine Dye} + 4H_2O$$

Catalase was measured in liver and kidney homogenate by using a commercial kit according to the method described by (Aebi, 1984) . Reagent 2 H_2O_2 (substrate and standard) diluted 1000 times immediately before use (10 µl + 10 ml distilled water). Sample and reagents (Buffer : Phosphate buffer, pH 7.0, Chromogen - inhibitor and Enzyme: Peroxidase) were added to test tubes as follow:The test tubes were incubated at 37^{o}C for 10 min.The sample absorbance (A sample) was read against sample blank and standard absorbance (A standard) against standard blank at 510 nm

$$\text{Catalase activity} = \frac{A_{standard} - A_{sample}}{A_{standard}} \times \frac{1}{\text{gm tissue used}}$$

2.7. Histological examination of liver and kidney

Samples of the liver and kidney from each animal were fixed in 10 % neutral formalin for histopathological and histochemical studies. Serial sections of 5µm were made and stained with haemotoxylin and eosin stain for histological examination according to the method described by Drury *et al.* (1976). In brief, deparaffinization of sections was carried out using xylol, while hydration was performed using descending grades of alcohol. The sections were stained in haematoxylin for 10 minutes then counterstained in eosin for 1 minute, followed by rapid rinsing in distilled water. Finally, dehydration, cleaning and mounting in Canada balsam was performed. For histochemical investigations, slides were stained with Bromophenol blue for the determination of protein content in liver and kidney tissue.

2.8. Genotoxicity assays

2.8.1. Micronucleus (MN) assay

The *in vivo* test normally uses mouse bone marrow or mouse peripheral blood. When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualisation of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage (Mavournin et al., 1990).

The MN assay was conducted according to the procedures described previously by Diab *et al*. (2018). Briefly, the bone marrow cells were collected from the femurs using 1.5 mL syringe containing fetal bovine serum (FBS) and then smeared onto a glass microscope slides. The slides were fixed in absolute methanol, air-dried, stained with a mixture of May-Grünwald and Giemsa staining, and analyzed using a light microscope at 1000× magnification. This staining can differentiate between immature (polychromatic erythrocytes, PCE) and mature (normochromic erythrocytes, NCE). Two thousand PCE were scored per animal to determine the frequency of micronucleated polychromatic erythrocytes (MNPCE). The ratio of PCE/ 500 total erythrocytes were calculated per animal to evaluate bone marrow cytotoxicity.

2.8.2. Single cell gel electrophoresis (comet) assay

The comet assay (single-cell gel electrophoresis) is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The likely basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. This can be performed by manual scoring or automatically by imaging software

Bone marrow suspension (10 µl in FBS) was mixed with low-melting-point agarose (0.8%, 100 µL in PBS), and spread onto full-frosted slides precoated with 1% standard agarose. The slides were covered with coverslips and stored at 4 °C for 10 min to solidify the agarose. The coverslips were smoothly removed and the slides immersed in a freshly prepared lysis buffer (2.5M NaCl, 100 mM EDTA,10 mM Tris, pH 10 containing 10% DMSO and 1% Triton X-100) in a refrigerator at 4 °C for 2 h in the dark. Then, the slides were dipped into a freshly prepared chilled alkaline buffer (300 mM NaOH, 1 mM EDTA, pH>13) for 20 min followed by conducting electrophoresis (1 V/cm, 25 V, and 300 mA) for 30 min. The slides were washed with neutralization buffer (0.4 M HCl, pH 7.5), fixed with absolute ethanol, and air-dried overnight. The slides were stained with ethidium bromide (100 µL, 20 µg/mL) and immediately visualized using fluorescence microscope at 400× magnification (Diab et al. 2020, Diab et al. 2018). A total of 150 cells were analyzed per animal using automatic comet score™ software (TriTek Corp, version 2.0.0.0, Sumerduck, VA 22742, United States). The preferred comet parameters included the percentage of DNA in the tail, tail moment (TM), and Olive tail moment (OTM).

2.9. Statistical analysis

The results were given as the mean ± SE for three replicates for each sample. The IC50 (DPPH, ABTS β-carotene bleaching), $A_{0.5}$ (CUPRAC assay, phenanthroline) and EC50 (Chelation of the metal ions) values were calculated by linear regression analysis. The data for *in vivo* were statistically analyzed by analysis of variance (ANOVA) using the general linear model procedure of the Graph Pad Prism (version 5.01 for Windows). Duncan's multiple range tests (Waller and Duncan 1969) was used to clarify the significance between the individual groups at $p \leq 0.05$. The values in this study were expressed as mean ± standard.

1. Extraction

The yield of extracts reported in this study from the initial weight of the used plant powder was affected by the extraction time and the extracting solvent. It was higher in the EE and ME extracts compared to the AE. Moreover, the yield was increased after 36 h in the three extracts as compared to the yield after 24 h; however, there is no significant difference in the extracts yield between 24 and 36 h for both ethanol and methanol extracts (Fig. 14).

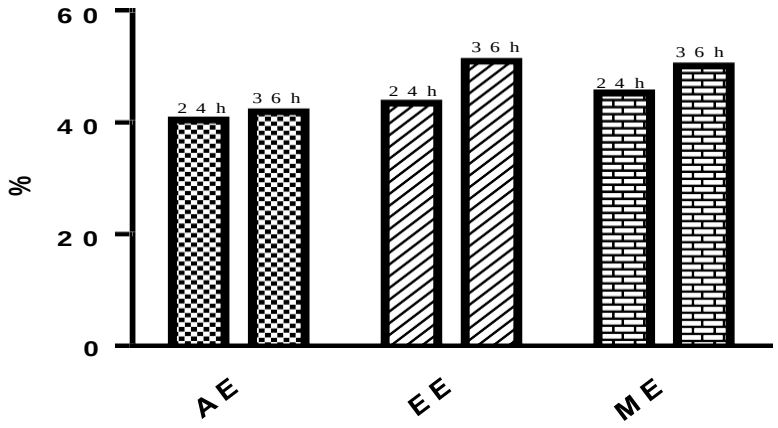

Figure14. Percentage of yield of the three extract of *C. speciosus* after 24 and 36 h

Extraction is a very important step in the isolation, identification and use of phenolic compounds and there is no single and standard extraction method. Solvent extraction is the most common used techniques for the extraction of phenolic compounds due to their ease of use, efficiency, and wide applicability. It, as a function of the biomass status, may be solid-liquid extraction or liquid-liquid extraction (Ignat *et al.*, 2011).

Extraction efficiency is known to be a function of process conditions. Several factors affect the extraction yield and the desired components in the extract: solvent types and concentration, extraction time, agitation speed, sample-solvent ratio, temperature and particle size. However, it is reported that the most influential factor is solvent types (Azwanida, 2015 Wu *et al*., 2007; Gong *et al*., 2012).

2. HPLC analysis of the extracts

The HPLC analysis for the aqueous extract, the ethanolic extract and the methanolic extract compared of the standard (Fig. 15) showed the identification of 13 compounds (Table 03). The aqueous extract contained high concentration of gallic acid, coffeic acid, quercetin and cinnamic acid. white the ethanolic extract contained the higher concentration of

chlorogenic acid only and the methanolic extract was very rich in chlorogenic acid, syringic acid, pyro catechol, ellagic acid, coumaric acid, vanillin, naringenin pyronyl gallate and dihydroxyiso flavones. It is of interest to mention that syringic acid, coumaric acid and vanillin were completely absent in the aqueous extract; however, vanillin, quercetin and cannamic acid were completely absent in the ethanolic extract.

Table (03). HPLC analysis of the total polyphenols of the three extracts of *C. speciosus*

	Concentration (µg/g)		
	Aqueous	**Ethanol (70%)**	**Methanol (70%)**
Gallic acid	2470.21	747.58	689.73
Chlorogenic acid	590.55	1206.48	1175.07
Coffeic acid	154.29	79.25	92.86
Syringic acid	0.00	115.10	136.22
pyro catechol	306.19	300.34	371.77
Ellagic acid	139.69	193.28	265.48
Coumaric acid	0.00	57.58	125.92
Vanillin	0.00	0.00	40.29
Naringenin	873.80	1025.17	1069.65
Propyl Gallate	68.51	65.17	93.79
4`.7-DihydroxyisoFlavone	55.33	46.77	100.54
Querectin	172.35	0.00	127.65
Cinnamic acid	59.00	0.00	49.51

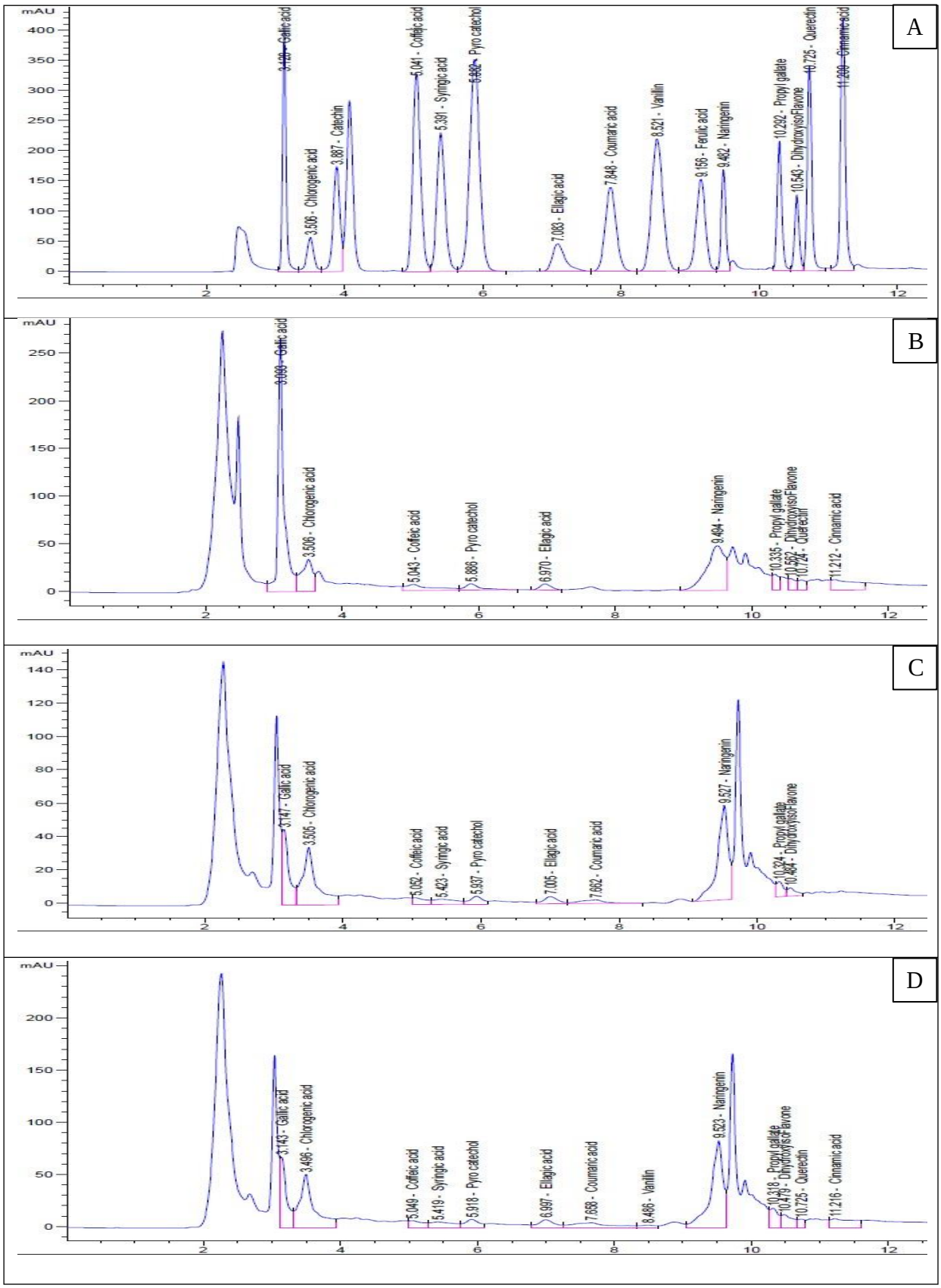

Figure 15 . HPLC chromatograms of the total polyphenols of (A) standard, (B) the aqueous, (C) the ethanol 70% and (D) the methanol 70% extracts of *C. speciosus*.

HPLC is the most used technique for achieving the separation, identification, and quantification of polyphenols from natural complex samples. The chromatographic separation depends on several factors such as stereochemistry, molecular weight, polarity, degree of polymerization of polyphenols (Alonso-Carrillo *et al*., 2017). There is not one single HPLC method that can separate all different types of polyphenols, in fact, the stationary phase, mobile phase, and gradient elution should be optimized for each group of compounds separately. The acidification of the mobile phase is necessary to suppress the ionization of phenolic hydroxyl groups to get shaper peaks and minimized peak tailing (Struck *et al*., 2016).

Depending on the structure, polyphenols can absorb at different wavelengths. For instance, phenolic compounds in general are detected at 240-285 nm, flavones and flavonols at 350-365 nm, and anthocyanins at 460-560 nm (Lorrain *et al*., 2013). To carry out the identification of each polyphenol, its spectral data and retention time are compared with the data obtained from commercial standards. Phenolic compounds are very important plants constituents because of their scavenging ability due to their hydroxyl groups (Uddin *et al*., 2014).

HPLC analysis indicated that several phenolics were found, although the concentrations were differing among the three extracts. Despite no available data about the HPLC analysis of *C. speciosus* extracts, Chang *et al*. (2012) found that the methanol extract contained five flavonoids and the higher one was quercetin, although there were other flavonoids which were not identified in the current study. This may be due to the different method used in the determination (Karthikeyan *et al*., 2012; Abdel-Aziz *et al*., 2014). Additionally, the difference between the results reported previously and the current results may probably be due to the effect of cultivating environment which was reported to affect the chemical composition of the extracts (Abdel-Wahhab *et al*., 2018).

3. Total phenolic content and total flavonoid content

TPC was higher in the ethanolic extract after 24 and 36 h followed by the methanolic extract then the aqueous extract. Meanwhile, TPC was increased by increasing the extraction time within all the three extracts. The results of TFC showed the same trend of TPC and the higher content was found in the ethanolic extract followed by the methanolic then the aqueous extract (Table 04).

Table (04). Total phenolic content (TPC) and total flavonoid content (TFC) the three extracts of *C. speciosus*

Extracts	TPC (mg GAE/g extract)		TFC (mg RE/g extract)	
Time	24 h	36 h	24 h	36 h
Aqueous	43.16 ± 3.05	73.5 ± 0.1	1.93 ± 0.05	2.3 ± 0.02
Ethanolic 70%	98.0 ± 4.24	179.31 ± 2.3	4.33 ± 0.17	5.0 ± 0.3
Methanolic 70%	79.56 ± 3.47	155.83 ± 1.8	3.41 ± 0.01	4.4 ± 0.07

Results are (means ± SE) (n = 3). GAE (gallic acid equivalent, RE (Rutin equivalent)

As phenolic compounds constitute one of the major contributors to the antioxidant capacity of plant, it was reasonable to determine their total amount in the selected plant extracts. The total phenolics content of extracts by the Folin-Ciocalteu method (Li *et al*., 2007), expressed as mg gallic acid equivalents per gram extract (mg GAE/g). Folin- Ciocalteu method is very popular, convenient, simple, reproducible and economic for the measurement of phenolics (Agbor *et al*., 2014).

Flavonoids are the most and important group of phenolic compounds, which are characterized by a benzo-pyrone structure (Bakar *et al*., 2009). Total flavonoids content was determined using the $AlCl_3$ method. Total flavonoids contents were expressed as mg quercetin equivalents per gram of extract (mg QE/g).

Extraction yields of TPC, TFCs and those showing antioxidant properties are influenced by multiple parameters, the polarity of extracting solvents and the solubility of this compound in the solvent used for the extraction process, solvent concentration, extraction temperature and extraction time (Bezerra *et al*., 2008; Wijngaard and Brunton, 2010; Sulaiman *et al*., 2011; Do *et al*., 2014).

4. *In vitro* evaluation of antioxidant activity

4.1. Total antioxidant capacity assay

The TAC of the water extract (120.0 ± 2.30 mg AAE /g extract) was the lowest and is significantly different from that of the other extracts ($p < 0.05$). The TAC of the ethanol extract (190.23 ± 5.11 mg AAE /g extract) is higher than that of the methanolic extract (154.49 ± 4.47 mg AAE /g extract)(Fig.16). However, both extracts show significantly higher TAC than that of the other extracts ($p < 0.05$).

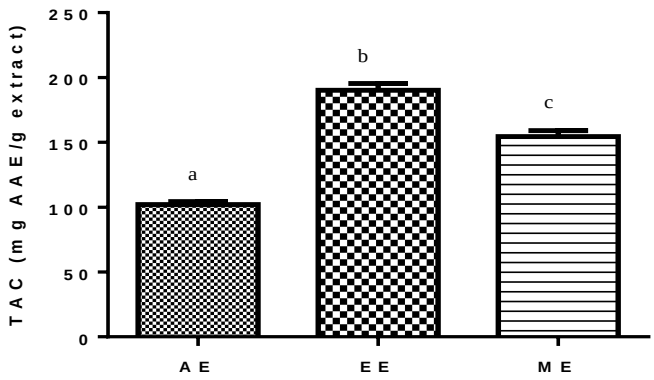

Figure 16: Total antioxidant capacity of different extracts of *C. speciosus.*
Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other

The TAC of the extract was calculated based on the formation of the phosphomolybdenum complex at acidic pH. Singh *et al*. (2018) reported that the total antioxidant capacity depend on the intrinsic levels of polyphenols (phenol and flavonoid) within the plants. In the present study, TAC of extracts had positive relation various phenolic compounds.

4.2. Free radical scavenging assay by DPPH

The antioxidant activities obtained by the DPPH method for extracts are presented as IC_{50} in (Fig.17). The analysis of variance revealed significant differences between various extract. This activity was compared with that of BHT and BHA as a synthetic antioxidant. The results revealed that water extract with an IC_{50} value of 344.18 ± 0.88 µg/ml effective scavenger than other extracts ethanolic and methanolic of *C. speciosus* with an IC_{50} value of 373.00 ± 0.31 and 398.97 ± 1.16 µg/ml respectively.

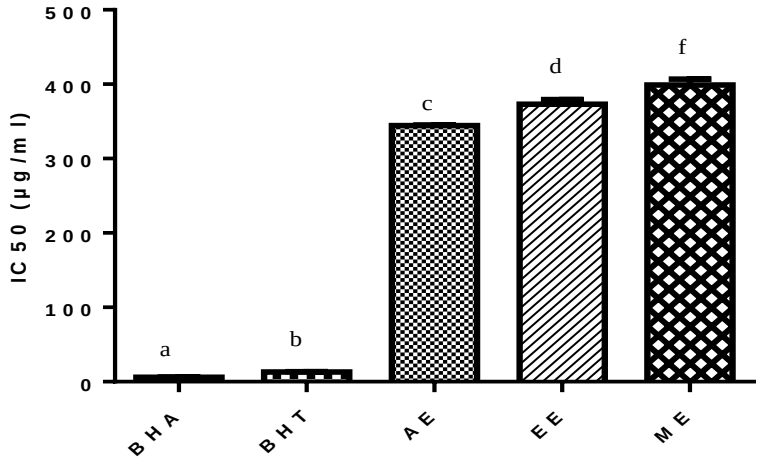

Figure 17. The IC_{50} values in the DPPH radical scavenging activity assay of the extracts. BHA and BHT were used as reference antioxidant .Each value is expressed as a mean ± S.D (n=3).Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other

The IC_{50} of an extract is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant required to decrease the DPPH concentration by 50%, which is obtained by interpolation from a linear regression analysis (Oliveira *et al*., 2016). Results showed that all extracts scavenge DPPH radical in concentration dependent manner.

DPPH scavenging activity of extracts had positive relation with various phenolic compounds and flavonoids (Garg *et al.*, 2012). Generally, the extract with high total phenolic contents had higher scavenging activity (Yardpiroon *et al.*, 2014).

The result revealed in Indian by Gayatri et Rajani., 2011 is found that the ethanol fraction of *C. speciosus* rhizome exhibited the highest radical scavenging activity with 71.61 ± 0.02% with IC_{50} value 210 µg/ml followed by its methanol extract with 66.15 ± 08% and aqueous extract with 60.13 ± 0.07% at 200 µg/ml . From the present result it may be postulated that *C. speciosus* reduces the radical when it react with hydrogen donars in antioxidant principles. DPPH radicals react with suitable reducing agents, the electrons become paired off and solution looses colour stochiometrically depending on the number of electrons taken up (Jeetendra *et al.*, 2010).

4.3. $ABTS^{+\cdot}$ radical cation decolorization assay

The results of ABTS of the extracts are compared with those of BHT and BHT. Results were presented as IC_{50} (Fig.18). The analysis of variance revealed significant differences between various extract. The results revealed that methanolic extract has high scavenging ability of the ABTS radical with an IC_{50} value of 79.36 ± 0.02 µg/ml effective scavenger than

other extracts water and ethanolic of *C. speciosus* with an IC_{50} value of 82.40 ± 0.70 and 84.83 ± 0.51 µg/ml, respectively.

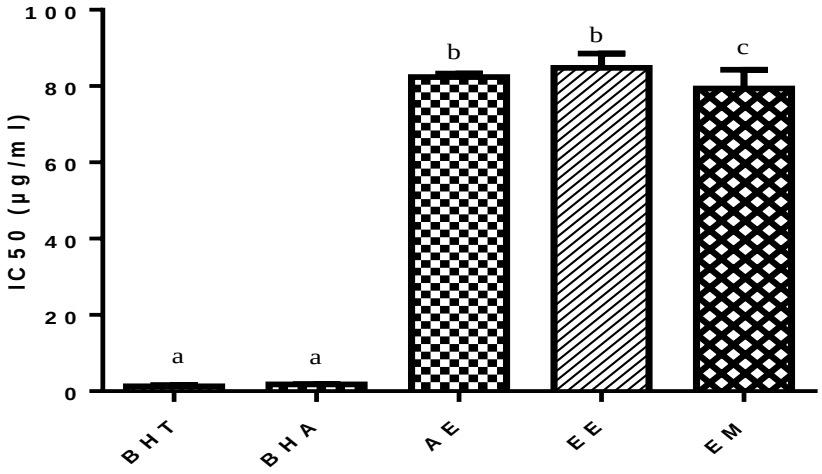

Figure 18. The IC_{50} values in the ABTS radical cation decolorization activity assay of the extracts. BHA and BHT were used as reference antioxidant .Each value is expressed as a mean ± S.D (n=3) Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other.

The $ABTS^{\cdot+}$ radical is one of various radical used for measuring the antioxidant activity of plants (Ch and Meera, 2013). ABTS is a stable organic radical that has gained hydrogen; this method determines the antioxidant activity of hydrogen donating antioxidants in fruit crude extract (Le *et al*., 2007). The radical scavenging capacity measurements using $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays are easy, rapid and sensitive methods and therefore most frequently applied for the preliminary assessment of antioxidant potential of various natural substances. Although the basic principles are similar, the $ABTS^{\cdot+}$ scavenging assay is preferable for its ability to evaluate Radical scavenging capacity of both lipophilic and hydrophilic antioxidants (Dobravalskyte *et al*., 2012). In contrast, DPPH is more selective because it does not react with flavonoids which do not contain hydroxyl groups in cycle B (Yokozawa *et al*., 1998), as well as aromatic acids containing only one hydroxyl group (Von Gadov *et al*., 1997).

4.4. CUPRAC assay

The results of CUPRAC of the extracts are compared with those of BHT and BHT (Fig.19). The analysis of variance revealed significant differences between various extract. Activity (absorbance) increased linearly with the increasing amount of extracts. The aqueous extract exhibited highest show significantly higher activity ($A_{0.50}$ value: 163.35 ± 2.19 µg/mL) than that among the extracts ($p < 0.05$), followed by ethanolic ($A_{0.50}$ value: 171.17 ± 1.25 µg/mL) and methanolic extract ($A_{0.50}$ value: 271.17 ± 0.35 µg/mL). However,

none of the extracts exhibited higher activity than those of antioxidant standard.

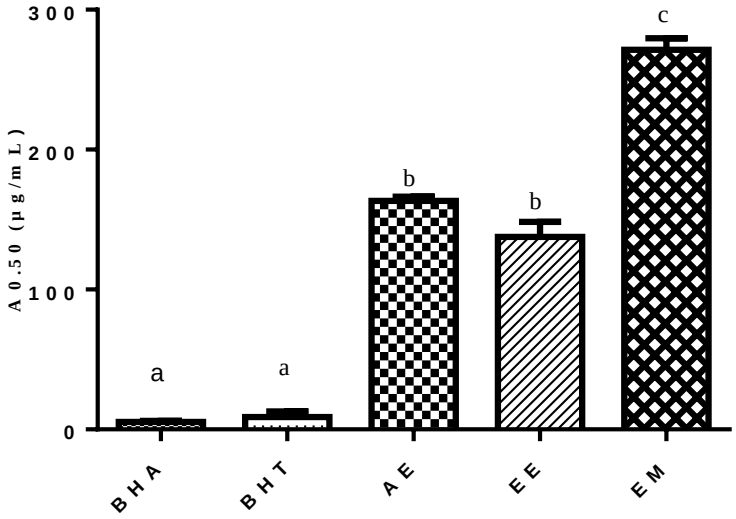

Figure 19. The $A_{0.5}$ values in the Cupric reducing antioxidant capacity of the extracts.
BHA and BHT were used as reference antioxidant .Each value is expressed as a mean ± S.D (n=3).Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other.

The Cuprac method measures electron transferring of the antioxidant. In case of bulky compounds, CUPRAC gives better and accurate results. Since transition metals can accelerate the lipid peroxidation via the Fenton reaction (Tel *et al*., 2013). The Cuprac protocol is applied. The Cuprac reagent is stable, easily accessible, low-cost, and is sensitive toward thiol-type antioxidants. The Reagent Cuprac is $CuCl_2$ which was combined with neocuproine in ammonium acetate buffer pH 7. Cu (II) will be reduced to Cu (I). Complex Cu (I) - neocuproine gives yellow color and show characteristic absorption at wavelength 450 nm 20. Intensity of yellow color depends on amount of Cu (II) that is reduced to Cu (I). Flavonoid which had ortho di OH at C-3'-C4', OH at C-3, oxo function at C-4, double bond at C-2 and C-3 have high antioxidant activity. The ortho di OH at C-3'-C-4' had the highest influence to antioxidant activity of flavonoid. The flavonoid glycosides would give lower antioxidant activity than flavonoid aglycones. Flavonoid had greater antioxidant activity than phenolic acid (Apak *et al*., 2008; Fidrianny *et al*., 2016)

4.5. *β*-carotene bleaching assay

The results of β-carotene bleaching test of the extracts are compared with those of BHT and BHT. The analysis of variance revealed significant differences between various extract. Activity (absorbance) increased linearly with the increasing amount of extracts. The ethanolic extract exhibited highest show significantly higher activity (IC_{50}: 318.65 ± 1.49

µg/mL) than that among the extracts ($p<0.05$), followed by methanolic (IC_{50}: 371.67 ± 5.10 µg/mL). Aqueous extract showed a weak capacity β-carotene bleaching inhibition with IC_{50} values >800 µg/ml (Fig. 20). As described in Figure 20, there were significant differences ($p < 0.05$) between the EE, ME extract, and BHT, BHA standard. From these results, the plant fractions exhibiting the greatest antioxidant potential were those with the highest levels of total polyphénols.

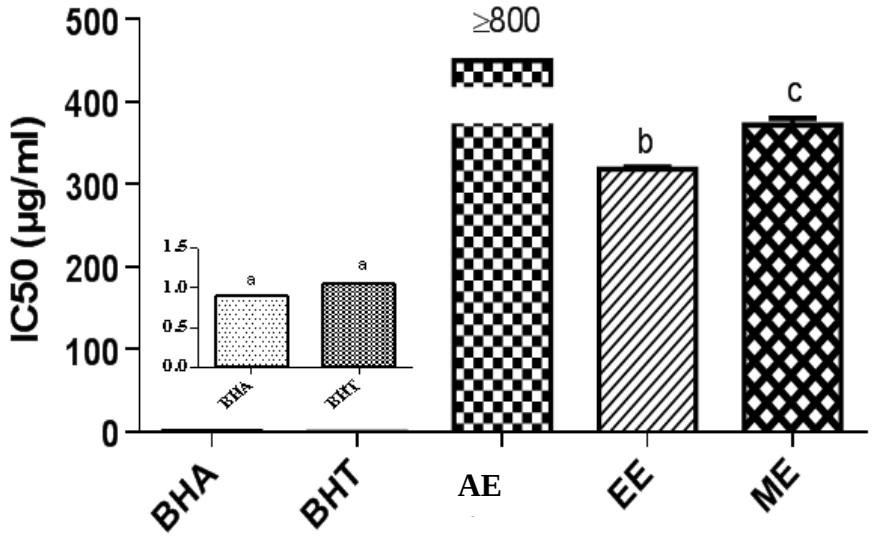

Figure 20. The IC_{50} values in the β -carotene bleaching test antioxidant capacity of the extracts.BHA and BHT were used as reference antioxidant .Each value is expressed as a mean ± S.D (n=3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other

The antioxidant activity of different extracts was also evaluated by the β-carotene-linoleate bleaching assay because β-carotene shows strong biological activity and constitutes physiologically important compound (Msaada *et al.*, 2017). In this system, the peroxyl free radicals were generated due to oxidation of linoleic acid by abstraction of hydrogen atom from diallylic methylene groups of linoleic acid located on carbon-11 between two double bonds. The generated peroxyl radicals decolorize the highly unsaturated β-carotene in the absence of antioxidant (Kumar and Jain, 2015).

Presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. Hydroperoxides formed in this system will be degraded by the antioxidants from the extract/fractions. Accordingly, the absorbance decreased rapidly in samples without antioxidant whereas, in the presence of an antioxidant, they retained their color, and thus absorbance, for a longer period of time (Pandey *et al.*, 2018). The different extracts and standard inhibited the oxidation of β-carotene. This effect is due to either the inhibition of linoleic acid peroxidation or

the radical scavenging of hydroperoxides formed during the peroxidation of linoleic acid.

4.6. Ferrous ions chelating assay

The results of the chelating activity of the extracts are compared with those of EDTA. EDTA is a strong metal chelator, hence, it is used as a standard metal chelator agent in this study. The analysis of variance revealed significant differences between various extract .Activity (absorbance) increased linearly with the increasing amount of extracts. The ethanolic extract also higher chelating activity with (IC_{50}: 356.19 ± 2.64 µg/mL) than that among the extracts ($p < 0.05$), and was similar to IC_{50} value of methanolic extracts (IC_{50}: 365.74 ± 0.47 µg/mL). Aqueous extract showed a weak capacity of the chelating activity with IC_{50} values >800 µg/ml (Fig. 21).

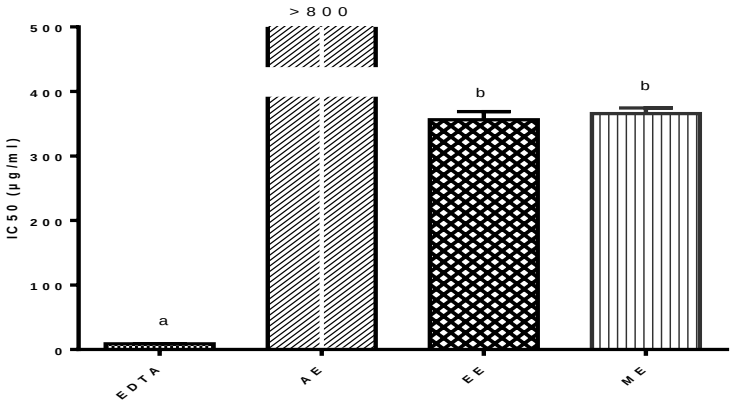

Figure 21. The IC_{50} values in the ferrous ions chelating test antioxidant capacity of the extracts. EDTA were used as reference antioxidant .Each value is expressed as a mean ± S.D (n=3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other.

Ferrozine forms a red colored complex by forming chelates with Fe^{2+}. This reaction is restricted in the presence of other chelating agents and results in a decrease of the red of the ferrozine-Fe^{2+} complexes. A measurement of the color reduction determines the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas *et al*., 2000).

The accumulation of metal ion was considered a source of oxidative stress and leading to occurrence of many diseases. Ferrous ion (Fe^{2+}) is commonly found in food systems. It is involved in the generation of free radicals through Fenton and Haber-Weiss reaction such as hydroxyl radical and accelerates lipid proxidation into alkoxyl radical (Özkan *et al*., 2011) and it is known as an effective prooxidant. They are responsible for DNA damage, lipid peroxidation, protein modification and other effects (Jomova and Valko, 2011). Ferric ions (Fe^{3+}) also produce radicals from peroxides, but the rate is tenfold less than that of ferrous

ion. Ferrous ions (Fe^{2+}) are the most powerful pro-oxidant among the various species of metal ions (Al-Hashimi, 2012).

4.7. Phenanthroline method

The results of phenanthroline test of the extracts are compared with those of BHT and BHT. The analysis of variance revealed significant differences between various extract. Activity (absorbance) increased linearly with the increasing amount of extracts. The aqueous extract exhibited highest show significantly higher activity ($A_{0.5}$: 126.66 ± 0.54 µg/mL) than that among the extracts ($p < 0.05$), followed by ethanolic ($A_{0.5}$: 139.42 ± 1.87 µg/mL) and methanolic extract with $A_{0.5}$ 159.80 ± 0.96 µg/mL. As described in Fig (22), there were significant differences ($p < 0.05$) between the various extract, and BHT, BHA standardeffect.

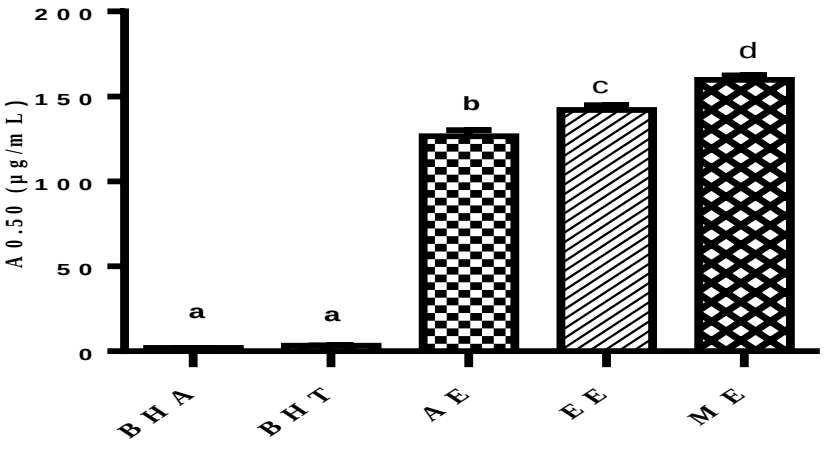

Figure 22. The $A_{0.5}$ values in the Phenanthroline *assay* of the extracts. Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other. BHA and BHT were used as reference antioxidant .Each value is expressed as a mean ± S.D (n=3)

5. Cytotoxicity assay against HePG-2 cell lines

Hep G2 human cell line has been used to study the cytotoxicity of various extracts *C. specieus* organic solvents on HepG2 cells and to evaluate the antiproliferative activity for HepG2 using the MTT colorimetric assay. The cytotoxicity results of the tested extracts compared to doxorubicin (a standard anticancer drug) are given in Figure 23. These results clearly indicated that the methanolic extract of *C. speciosus* had a strong anticancer activity followed by the ethanol extract, then the aqueous extract which has the weakest antiproliferative activity against HePG2 cell lines. The IC_{50} reached 13.87, 24.06 and 53.69 mg/ ml for ethanolic, methanolic and aqueous extracts, respectively, compared to the standard drug doxorubicin which recorded an IC_{50} value of 4.50 mg/ml (Fig. 24).

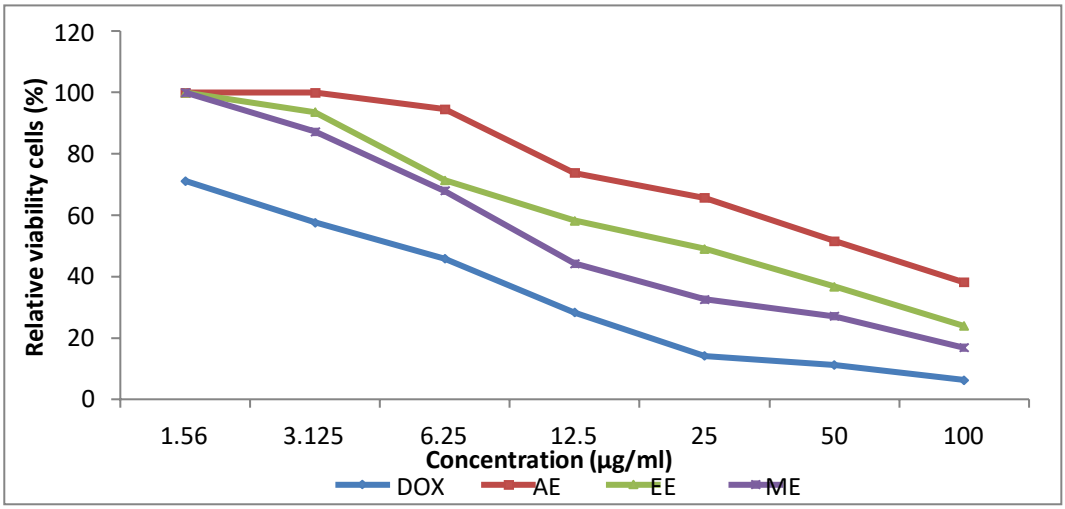

Figure 23 . Relative viability of cells (%) of various extracts and standard (DOX : doxocubicin)

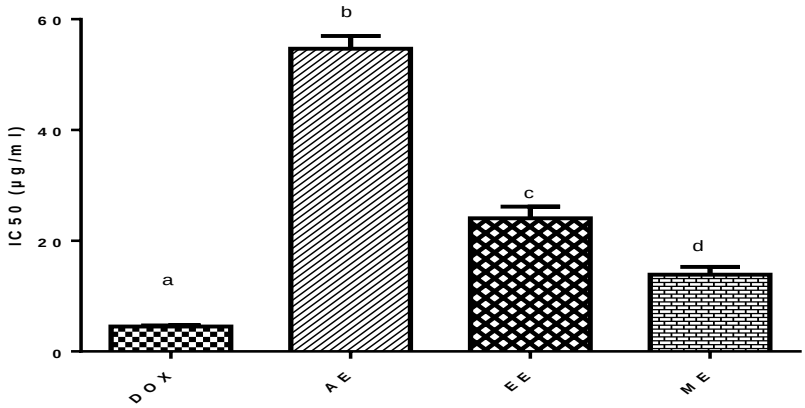

Figure 24. Cytotoxic activity IC_{50} (µg/ml) of some compounds against HePG2 cell line. (DOX: doxocubicin).

In the cytotoxicity study, methanolic extract showed strong cytotoxicity against HePG2 cell lines followed by the ethanol extract which showed moderate cytotoxicity then the aqueous extract whith a weak cytotoxicity. Although the TFC in the methanolic extract was higher than the methanolic extract, the later showed a strong cytotoxicity (Table 4) due to the

presence of certain compounds in the methanolic extract such as vanillin, quercetin and cinnamic acid. These compounds whish are well known to possess anticancer activity (Almeer *et al*., 2019; Ramadoss and Sivalingam, 2019; Rauf *et al*., 2018; Endo *et al*., 2018). Similar to the current results, Jha *et al*. (2010) indicated that the methanolic extract of *C. speciosus* showed strong cytotoxicity against brine shrimp nauplii with an IC_{50} value of 31.55 mg/ml. However, Althubaiti *et al*. (2017) showed that the hexane extract of *C. speciosus* has anticancer activity against human colon tumor cell line HCT-116 with IC_{50} of 160 mg/ml and the cell proliferation was completely suppressed at a concentration of 300 mg/ml. On the other hand, diosgenin is a steroid well known in the pharmaceutical industry for the treatment of anti-proliferation via the induction of apoptosis in cancer cells via caspases (Soares *et al*., 1997; Chang *et al*., 2002).

The antiproliferative activity of the *C. speciosus* extracts mainly due to the presence of the polyphenols reported in the current study as well as several bioactive compounds, including diosgenin which up-regulate COX-2 and 5-LOX (Lepage *et al*., 2010 ; Lepage *et al*., 2011), costunolide which induce intercellular thiols depletion (Hsu *et al*., 2011) and lupeol which up- regulate FADD and down-regulate p13-kinase/Akt (Lepage *et al*., 2010). It is of interest to mention that the methanolic extract showed a potent cytotoxicity after 48 h. in this concern; Diaconeasa (2018) reported that the incubation of phenolic compounds with different cell lines for 24 h is too short to induce significant activity on cell viability. Moreover, longer time also showed low cytotoxicity of polyphenols which may be suggested that these compounds are unstable and highly prone to degradation and/or reaction with some factors such as oxygen and metal ions (Chen *et al*., 2018) which resulting in the decrease of polyphenols biological activities (Giampieri *et al*., 2018). These authors also showed that cell viability was decreased after 72 h after incubation of HepG2 with polyphenols-rich extracts. Consequently the current results and those reported by Giampieri *et al*. (2018) and Diaconeasa (2018) may indicate that the anticancer activities are mainly due to the polyphenols and not to other compounds in the extract.

6. Anticoagulant activity

The anticoagulant activity is habitually estimated by the standard coagulation assays consisting of aPTT, PT and TT activities which are commonly related to the intrinsic and extrinsic pathways of coagulation process.

6.1. Activated partial thromboplastin time (aPPT) test

The results showed that there was significant (P<0.05) prolongation of aPTT with the various extracts at all tested concentrations compared with the control . Longer aPTT times were recorded at concentration of 27 mg/mL compared with other concentrations for all tested extracts (Fig. 25). Highest aPTT prolongation effects were recorded with the ethanolic extract at the tested concentrations with values of 111.0 ± 1.49 ± 1.01, and 39.0 ± 1.0 seconds at 27.0, 13.5 and 6.27 mg/mL respectively, whereas that of the control was 33.62s. The significant effect of EE on aPTT test suggested an inhibition of the intrinsic pathways of blood clots. Therefore, EE of *C. Sepecieus* could be a promising antithrombotic agent. On the other hand, the standardized human plasma from health donors was used as a reservoir of coagulation cascade enzymes. All fractions were measured in different concentrations, in order to evaluate the strength of the biological activity.

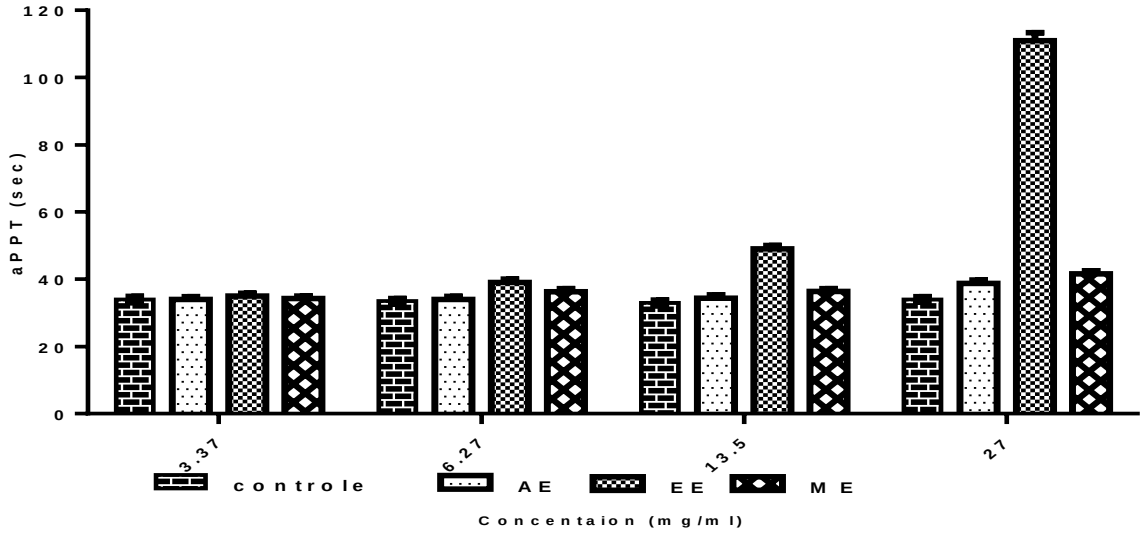

Figure 25. Activated thromboplastin time (aPTT) of various extracts of *C. speciosus* Values are given as mean ± SD (n = 3)

2. Prothrombin Time (PT) test

The current results also showed that there was significant differences (P<0.05) in prolongation of TP with the various extracts at all tested concentrations compared with the control. Longer TP times were recorded at concentration of 27.0 mg/mL compared with other concentrations for all tested extracts. Highest TP prolongation effects were recorded with the methanolic extract at the tested concentrations with values of 47.0 ± 0.9.31 ± 0.93, 24.0 ± 0.84 and 20.0 ± 0.5 seconds at 27.0, 13.5 and 6.27 mg/mL, respectively, whereas that of the control was 13.25 s. (Fig. 26).

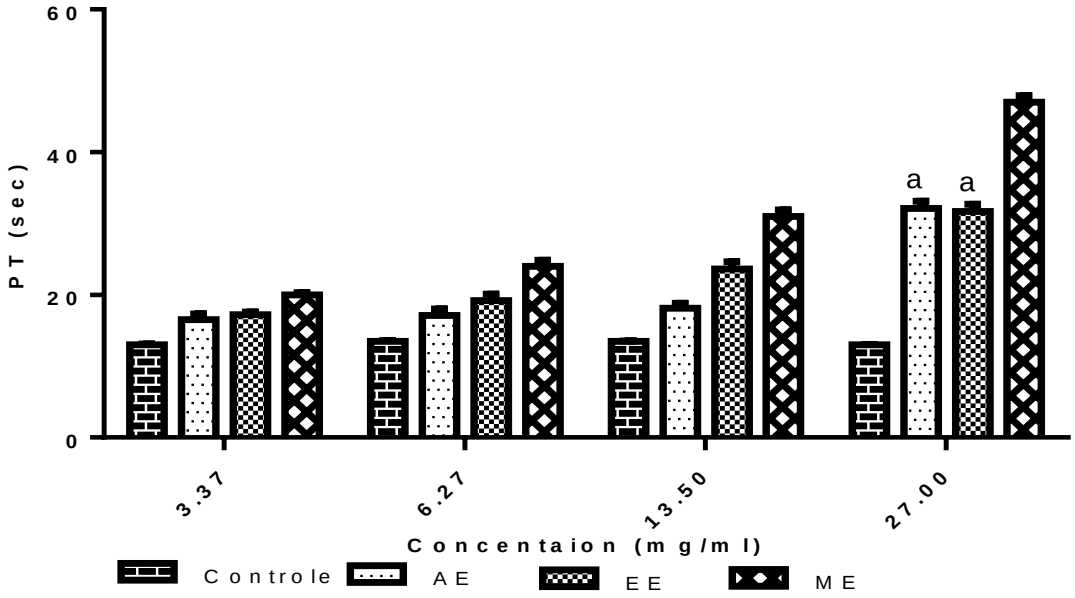

Figure 26. Prothrombin time (PT) of various extracts of *C. speciosus*, values are given as mean ± SD (n = 3)

6.3. Thrombin time (TT)

There was significant differences (P<0.05) in prolongation of TT with the various extracts at all tested concentrations compared with the control. Longer TT times were recorded at concentration of 40.0 mg/mL compared with other concentrations for all tested extracts. Highest TT prolongation effects were recorded with the ethanolic extract at the tested concentrations with values of 162.70 ± 2.06, 57.45 ± 1.90 and 30.75 ± 0. 9 seconds at 40, 20 and 10 mg/mL respectively, whereas that of the control was 24.75s (Fig. 27).

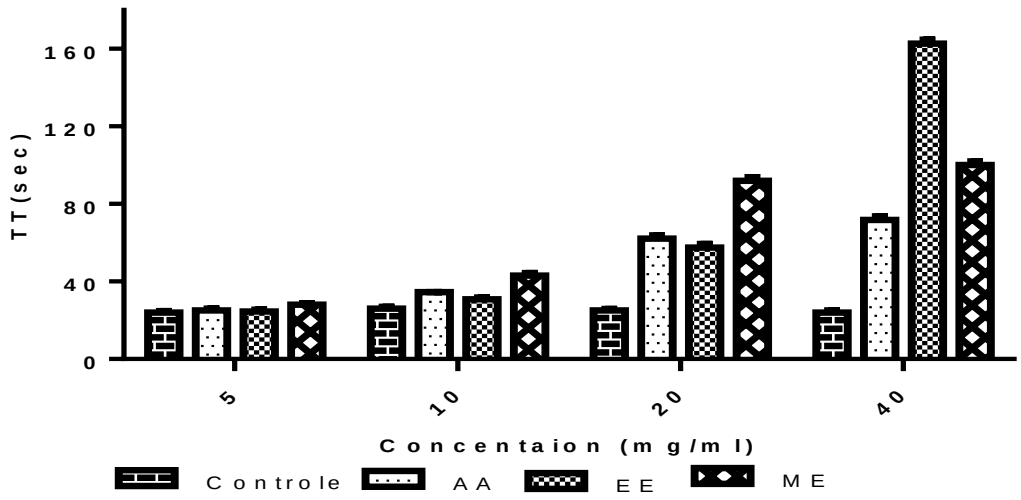

Figure 27. Thrombin time (TT) of various extracts of *C. speciosus* Values are given as mean ± SD (n =3)

6.4.*In vivo* Anticoagulant activity

This *in vitro* anticoagulant result of extracts *C. speciosus* is encouraging to prompted us to study its *in vivo* activity. *In vivo* studies are looking at the actual effect on an organism.

The *in vivo* aPTT, TT and PT variation was concentration dependent (Fig. 28). The aPTT values were 34.0 ± 0.71 s and 55.4 ± 3.03 s at 200 and 400 mg/kg b.w, respectively compared with the control which recorded 26.2 ± 1.02 s (Fig. 28a). As shown in fig 28a, there was a significant difference between test and control groups ($p < 0.05$). The prolongation of aPTT indicates inhibition of the intrinsic and/or common pathways of coagulant.

The effects of both doses of EE on PT were significantly different from that of control ($P < 0.05$) was discovered at 200 and 400 mg/kg values were 16.6 ± 0.81 s and 17.6 ± 0.68 s, respectively; however, comparable with the control groups was than 12.8 ± 0.37s. PT is the extrinsic pathway-dependent clotting time. The positive effect of the anticoagulant on PT suggests that the ethanolic extract from *C. speciosus* inhibit extrinsic pathway of coagulation (Fig. 28b). Thus, EE inhibited the extrinsic, the intrinsic and/or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin.

TT activity of EE of *C. spesiosus* was higher (16.6 ± 0.81 and 17.6 ± 0.68 s) than that of control (12.8 ± 0.37s) at 200 and 400 mg/kg, respectively. As shown in Fig. (28c), there was a significant difference between test and control groups ($p < 0.05$).

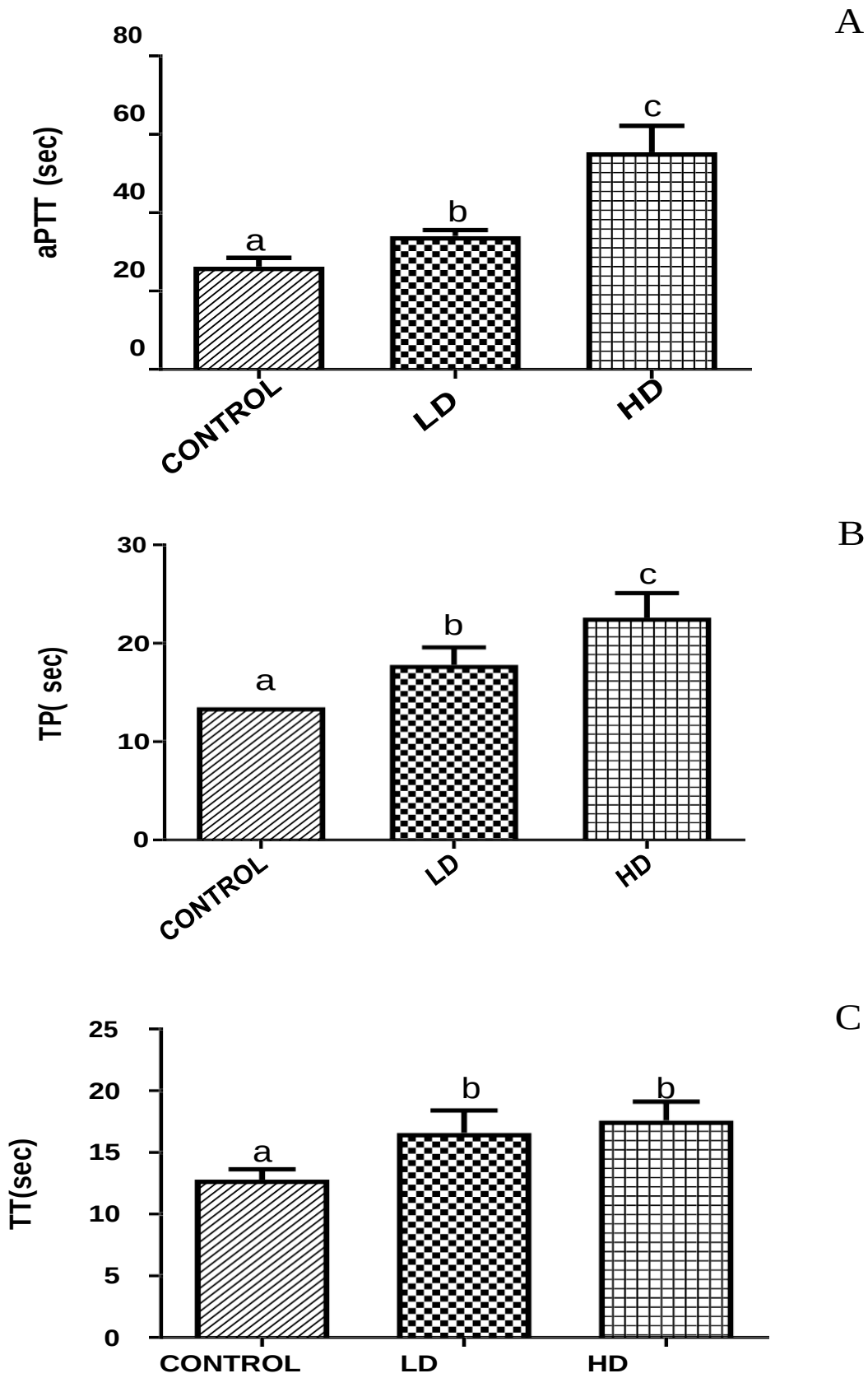

Figure 28. *in vivo* anticoagulant activity of ethanolic extract of *C. Speciesus* evaluated by the measurement of time of activated partial thromboplastin time (aPTT), prothrombin thrombin time (TT)

Blood coagulation is an important part of haemostasis (Omodamiro and Ikekamma, 2016). The indicators of blood coagulation are PT, aPTT and TT (Cordier *et al*., 2012). PT is used to evaluate the coagulation factors V, VII and X in the extrinsic pathway, while aPTT is used to evaluate the coagulation factors such as VIII, IX, XI, XII and prekalikrein in the intrinsic coagulation pathway of the coagulation cascade. The normal value of PT is between 12.5 and 13.7 seconds and between 31 and 39 seconds for aPTT. The PT isa standard test for monitoring coumarin therapy (vitamin K antagonists), while the aPTT is usually used to monitor the effectiveness of heparin treatments(Omar *et al*., 2017).

In our study, the results of aPTT, PT, and TT assays in vitro and in vivo showed that EE of C. spesiosus significantly prolonged APTT, PT, and TT clotting times in a dose-dependent manner However, a possible mechanism of ethanolic extract of *C. speciosus* extract may be through direct inhibition of the common pathway by decreasing thrombin (factor IIa) generation via inhibition of factor Xa and its cofactor Va, or by blocking the interaction of thrombin with substrate fibrinogen thus preventing the formation of fibrin.

These suggest a possible presence of protease inhibitor in *C. speciosus* which may inhibit these proteases, thus preventing the conversion of the zymogens to active factors Xa, Va, and thrombin. C. speciosus active component(s) may also activate the natural anticoagulant pathway by binding to Antithrombin III, causing a conformational change which could result in activation of protein C (PC) to activated protein C (APC), which in conjunction with its cofactor (protein S) inhibit factors Va and VIIIa (cofactors for activation of factors Xa and IIa). This ultimately prevents the activation of factor Xa and thrombin of the common pathway.

The common anticoagulant drugs used in the treatment of thrombotic conditions include heparin, warfarin, all-trans retinoic acid, and recently developed novel anticoagulants (NOACs). Heparin is a short-acting (fast) anticoagulant which action can be reversed, warfarin is long-acting and slow, with also reversible effects, all -trans retinoic acid is slow acting, while, NOACs are fast and cannot be reversed (Wadhera *et al*., 2014; Bryan, 2020) Heparin exerts its anticoagulant action by binding to a specific site of Antithrombin and causing a conformational change which exposes the site that binds and inactivates factor Xa and thrombin (IIa) thus, increasing the anticoagulant activity of antithrombin to about 1,000 folds. Warfarin is a vitamin K antagonist which inhibits the activation or synthesis of vitamin K-dependent proteins of the coagulation pathway. These proteins include factors X, IX, VII, and prothrombin. Vitamin K is a cofactor for the carboxylase enzyme which catalyzes the γ-carboxylation of glutamic acid residue of the polypeptide chain of vitamin K dependent

proteins. γ-carboxylation of the glutamic acid residues of these proteins is required for their calcium-binding ability, and thus their physiological activation (Suttie, 2014; Truong *et al*., 2011). Studies have reported that all-trans retinoic acid exerts its anticoagulant action by downregulating Tissue factor and upregulating thrombomodulin expression, thus increasing the antithrombotic potential of microvascular endothelial cells. Although a number of these drugs have been developed over the years, most are usually accompanied by undesirable side effects (Aoshima *et al*., 1998 ; Ghaffari *et al*., 2019 ; Koyama *et al*., 1994). Ethanolic extract of C. speciosus showed a narrow concentration range of activity peak which indicates fast and reversibility of action similar to heparin. This also corroborates the hypothesized mechanism of action of inhibiting the common coagulation pathway similar to that of heparin.

Several studies (El Haouari *et al*., 2006 ; Gadi *et al*., 2012) have shown the anticoagulant effect of phenolic compounds, including flavonoides and phenols acids that the ethanolic extract of Momordica charantia possesses very mild thrombolytic activity and the activity increases with the increasing of concentrations.

Many researchers study the anticoagulant activity of some plant such as study the anticoagulant activity of *Melastoma malabathricum* Linn. (Aqueous leaf extract), their study revealed the prolonged the coagulation time (Manicam *et al*., 2013). Several plant extracts were found to exhibit antithrombotic and/or anticoagulant activity *Sutherlandia frutescens* leaf extract, *Gloriosa superba* and *Zantedeschia aethiopica* leaf extracts displayed anticoagulant properties, *Leonotis leonurus* root extract (Kee *et al*., 2008 ; Kumar *et al*., 2011)

Coumarin is derived from a variety of plants used as an anticoagulant. In the blood coagulation cascade, coumarin works by inhibiting the calcium activity. Previously, it was been reported that coumarins have antithrombotic and thrombolytic activity, therefore antithrombotic activity of test plant extract might be due to its phenolic coumarins and marked increase of prothrombin time and activated partial thromboplastin time is apparently caused by the interference of procoagulant action of calcium by phenolic compounds found in ethanolic extract of *C. speciosus*. Previously, it has also been reported that flavonoids have thrombolytic activity (Adiba *et al*., 2013) and quercetin, a flavonoid that is also a strong free radical terminator and can aid to reduce atherosclerosis that leads to heart attack and stroke (Koffuor and Amoateng, 2011). Thus, the anticoagulant activities exhibited by *C. speciosus* may be due to the presence of these constituents thus supporting its traditional use in cardiac patients.

The TT revealed the inhibition of thrombin activity or fibrin polymerization as thrombin inhibition-dependent clotting time. TT reflects the blood coagulation status that transforms fibrinogen into fibrin, which is directly induced by the addition of thrombin. The test only detects disturbances in the final stages of coagulation, especially dysfibrinogenemia or the presence of thrombin inhibitors (Blair and Flaumenhaft , 2009)

2. Effect of ethanolic extract on the hepatotoxicity and nephrotoxicity induced by Zeralenone

7.1. Body Weight evolution

The effect of different treatments on body weight is presented in Fig. (29). The analysis of variance revealed significant differences between various treated groups. Duncan's Multiple Range test (LSD) revealed that body weight was significantly decreased inthe group treated with ZN alone. Whereas, the groups treated with CSE at the two tested doses were comparable to control. Moreover, no significant difference was detected in rats treated with the low or high dose of CSE compared to the control group. Animals treated with the CSE at the two tested doses succeeded to restore body weight loss resulted from ZN and thesegroups were comparable to the control group. Data presented in Fig (29) indicated that ZN induced its effect the severe decrease in body weight. Animals treated with CSE alone or plus ZN did not show any significant decrease in body weight throughout the experimental period. Moreover, the body weight was increased dramatically.

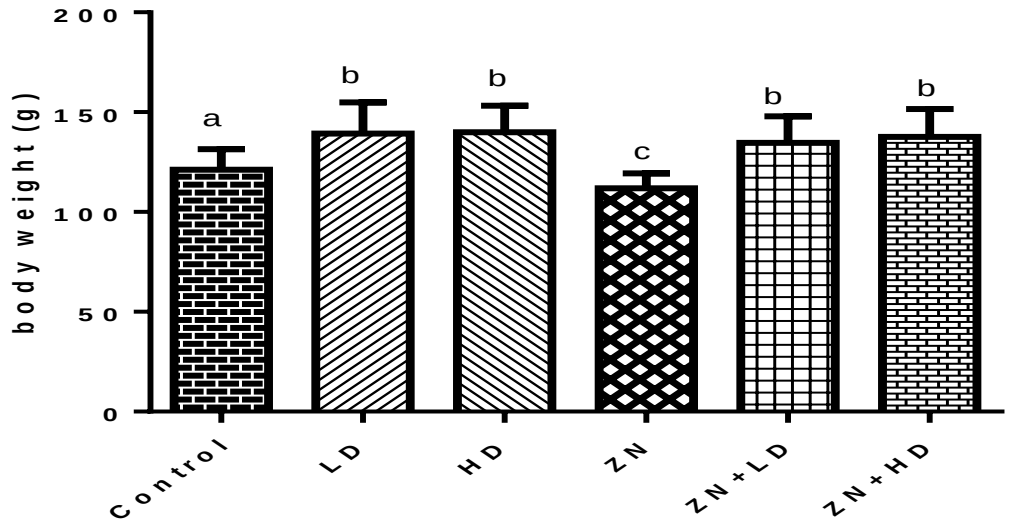

Figure 29 . Effects of ethanolic extract of *C. speciosus* on total body weight gain in rats treated with ZN. Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

The results of study showed that ZN decreased body weight accompanied by severe disturbances in all biochemical parameters of liver and kidney function, lipid profile, oxidative stress markers, genotoxic parameters and the histological pictures typical to the scientific literature of mycotoxicosis (Abdel-Wahhab *et al*., 2012, 2016; Hassan *et al*., 2012, 2015; El-Nekeety *et al*., 2017; Mc-Cullough and Lioyd, 2019; Zhou *et al*., 2019; Engin and Engin, 2019; Rushing and Selim, 2019). This body weight decrease suggested to be due to hepatotoxicity and nephrotoxicity of ZN and supported the finding of Jiang *et al*. (2011) who found a decrease in weight of both liver and kidney of animals after treatment with ZN.

7.2. Serum biochemical parameters

7.2.1. Serum alanine aminotransferase (ALT)

The effects of different treatments on ALT indicated that treatment with ZN resulted in a significant increase. The analysis of variance showed that animals treated with CSE at the low and high doses were comparable to the control. Treatment with ZN resulted in a significant increase in the ALT activity. Animals treated with ZN plus CSE at the two tested doses showed a significant improvement in ALT activity (Fig. 30). This improvement was more pronounced with high doses of CSE.

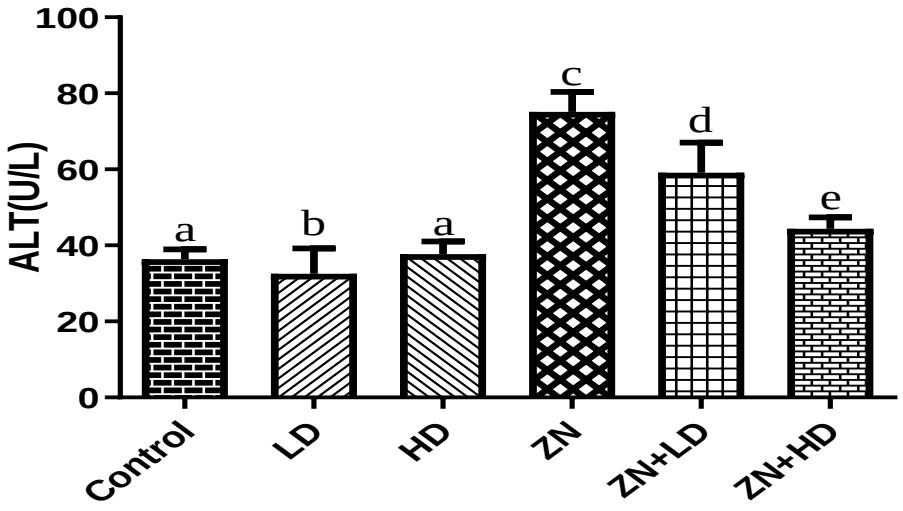

Figure 30. Effect of ethanolic extract *C. speciosus* on serum ALT activity in rats treated with ZN.Within each column, means superscript with different letters are significantly different (P≤0.05).

7.2.2. Serum aspartate aminotransferase (AST)

The effects of different treatments on AST indicated that treatment with ZN resulted in a significant increase in AST. The analysis of variance showed a significant difference between different treatment groups. Treatment with ZN resulted in a significant increase in

the AST activity. Animals treated with ZN plus CSE at the two tested doses showed a significant improvement in AST activity (Fig. 31). This improvement was more pronounced with high doses of CSE.

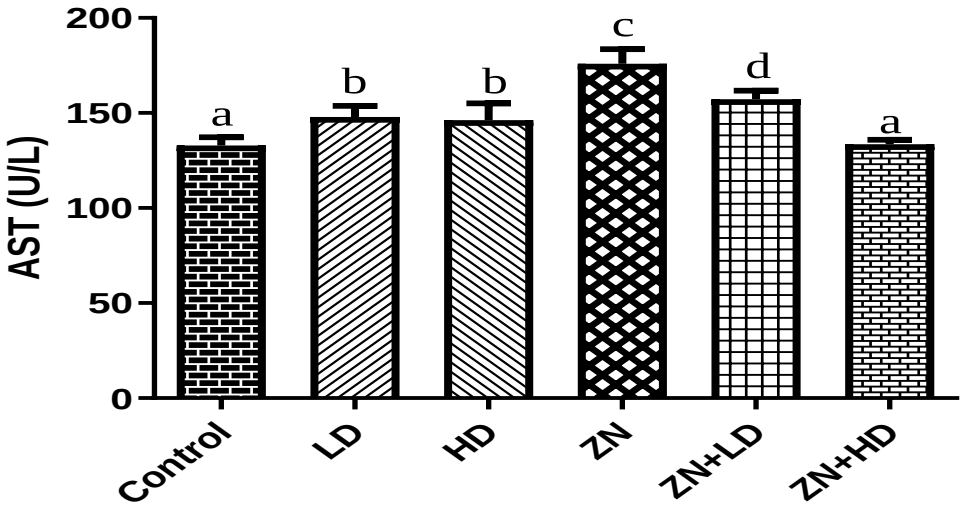

Figure 31. Effect of ethanolic extract *C. speciosus* on serum AST activity in rats treated with ZN.Within each column, means superscript with different letters are significantly different (P≤0.05).

7.2.3. Alkaline Phosphatase (ALP)

The effects of different treatments on ALP indicated that treatment with ZN resulted in a significant increase in ALP. The analysis of variance showed a significant difference between different treatment groups. Treatment with ZN resulted in a significant increase in the ALP activity. Animals treated with ZN plus CSE at the two tested doses showed a significant improvement in ALP activity (Fig.32). This improvement was more pronounced with high doses of CSE.

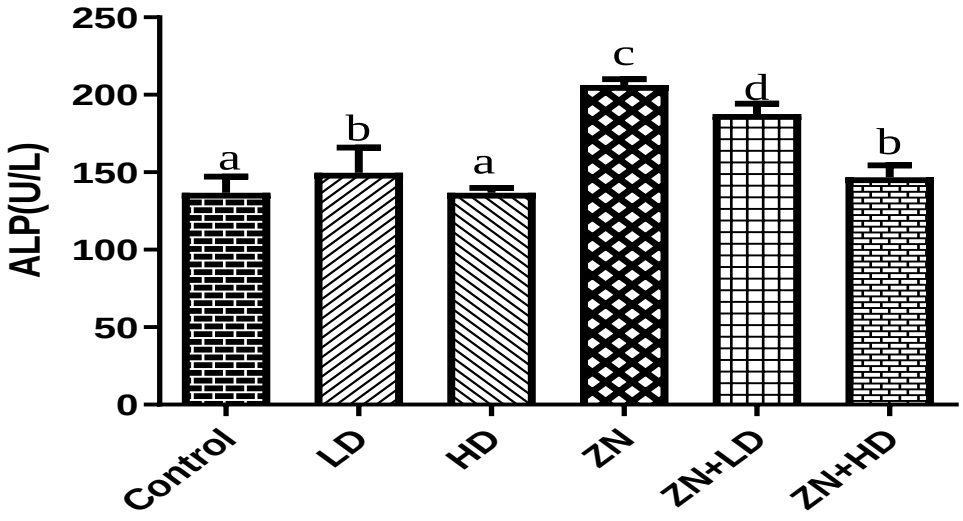

Figure 32. Effect of ethanolic extract *C. speciosus* on serum ALP activity in rats treated with ZN Within each column, means superscript with different letters are significantly different (P≤0.05).

In the current study, the results indicated that ZN induces severe liver toxicity in the experimental rats. Animals treated with ZN showed a significant increase in serum enzymes activities such as ALT, AST and ALP indicating hepatotoxic effects. These results are in agreement with previous researches that proved that ZN has hepatotoxic effects on experimental animals (ConKova *et al.*, 2001; Jiang *et al*., 2011). The elevation of these serum enzymes indicated the disturbance in hepatocytes membrane leading to enzyme leakage into the circulation (Kim *et al.*, 2012 ; Martins *et al*., 2012 ; Abdel-Wahhab *et al*., 2015a ; Salim *et al*., 2018 ; Xiao *et al*., 2015) These results were in accord with numerous studies indicating hepatotoxicity of ZN in rats (Maaroufi *et al*., 1996 ; Čonková *et al*., 2001)

7.2.4. Serum protein profile

The results showed the effect of different treatments on total protein levels. Analysis of variance revealed that there was a significant difference between different treatment groups. LSD test revealed that treatment with ZN resulted in a significant decrease in total protein. The combined treatment with ZN and CSE at the high dose succeeded to normalize total protein level to the normal range of the control (Fig.33).

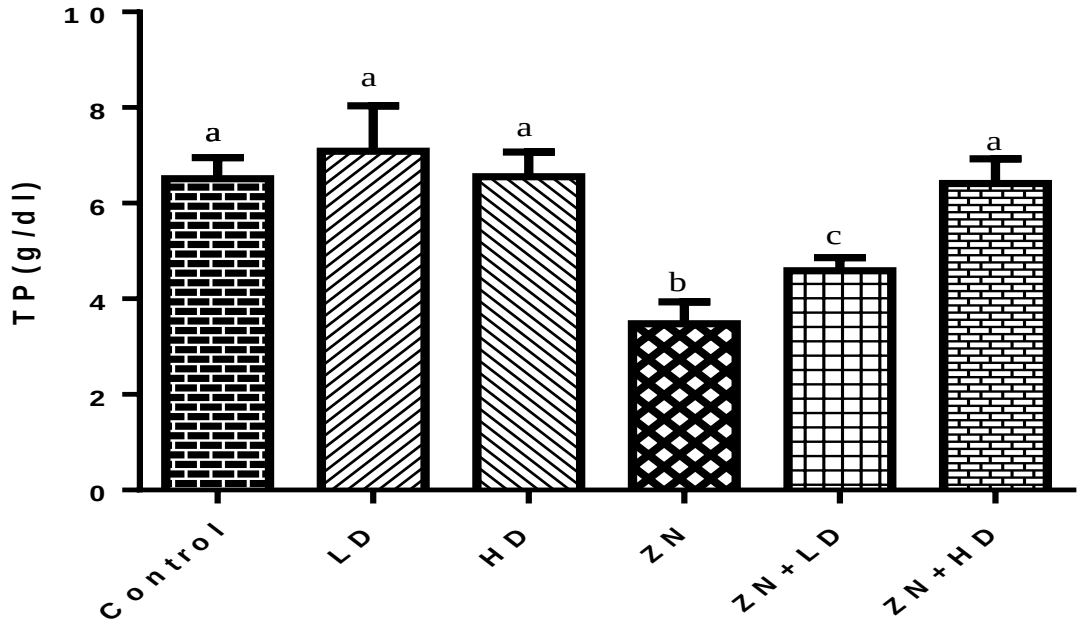

Figure 33. Effects of ethanolic extract *C. speciosus* on total protein level in rats treated withZN. Within each column, means superscript with different letters are significantly different (P≤ 0.05).

7.2.5. Serum albumin level

The results showed the effect of different treatments on albumin levels are presented in Fig. (34). Analysis of variance revealed that there was a significant difference between different treatment groups. LSD test revealed that treatment with ethanolic extract at the tow dose resulted in a significant increase in albumin level. However, CSE did not induce any significant changes. Treatment with ZN showed a significant decrease in albumin. The combined treatment with ZN and CSE at the two tested doses succeeded to normalize albumin level to the normal range of the control (Fig.34).

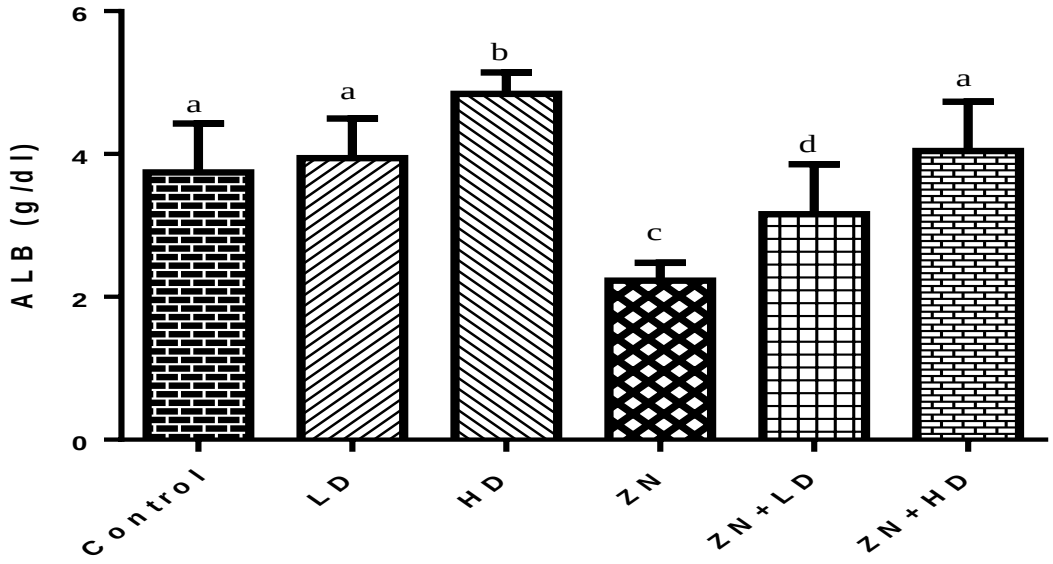

Figure 34. Effects of ethanolic extract *C. speciosus* on albumin level in rats treated with ZN. Within each column, means superscript with different letters are significantly different (P≤ 0.05).

The decrease in total protein and albumin reported in the present study revealed that ZN has a protein catabolism effect and/or kidney dysfunction. Another mechanism involved in the decrease of total protein reported herein may be the effect of ZN and/or its metabolites on the availability of critical amino acid (Abdel-Wahhab *et al.*, 2002, 2007; James *et al.*, 1982; Shyamal *et al.*, 2010).

7.2.6. Serum Urea

The effects of different treatments on serum urea levels indicated that treatment with ZN resulted in a significant increase in serum urea level. Analysis of variance revealed that there was a significant difference between different treatment groups. Animals treated with ZN plus CSE at the low and high dose showed level comparable to control and the extract succeeded to normalize urea level (Fig.35)

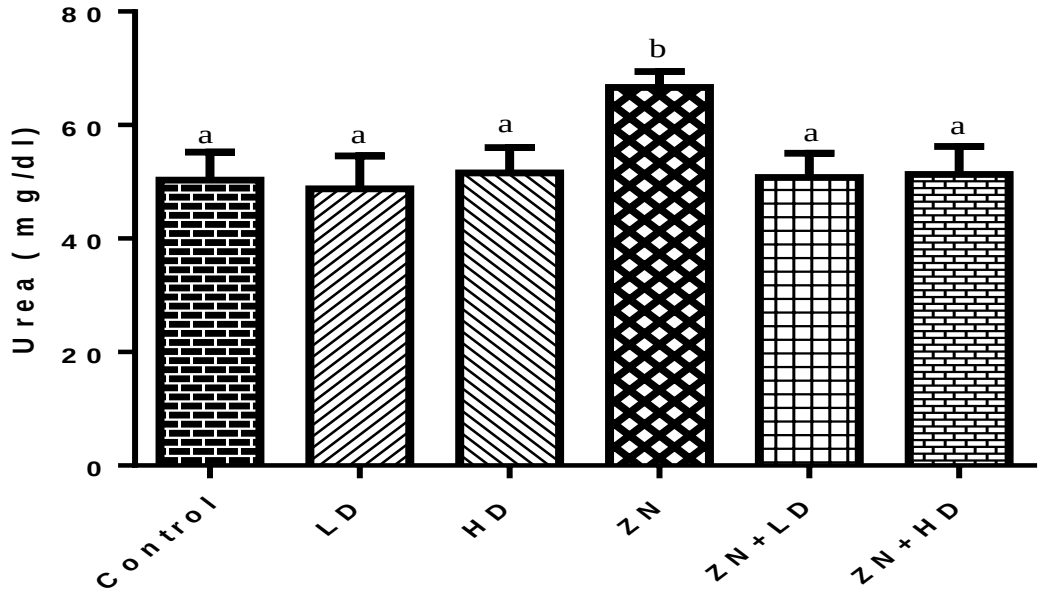

Figure 35. Effects of ethanolic extract *C. speciosus* on urea level in rats treated with ZN. Within each column, means superscript with different letters are significantly different (P≤0.05).

7.2.7.Serum uric acid

The results showed the effect of different treatment on serum uric acid level. The analysis of variance indicated that there was a significant difference between control group and other different groups. It is clear from Fig (36) that treatment with CSE at the low and the high dose of resulted in a significant decrease in serum level of uric acid. Ratstreated with ZN showed significant increase of uric acid level. The combined treatment of ZN with the low dose of CSE resulted in a significant decrease in serum level of uric acid compared to the group treated with ZN alone. The high dose of CSE showed amazing when combined with ZN as it decreased uric acid level.

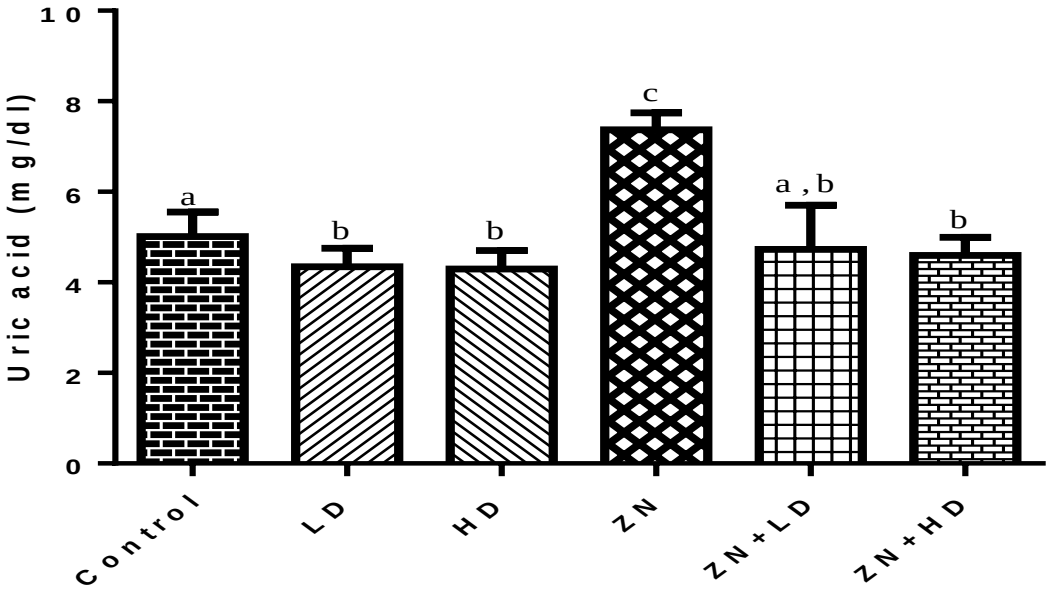

Figure 36. Effects of ethanolic extract of *C speciesus* on uric acid level in rats treated with ZN. Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

7.2.8. Serum creatinine

The results presented in Fig (37) showed effects of different treatments on serum creatinine level. Animals treated with ZN showed a significant increase in creatinine levels, however, animals received the combined treatment of ZN and CSE showed a significant improvement in creatinine level compared to those treated with ZN alone this treatment normalize creatinine level. The improvement in creatinine level in the groups received the combined treatment was more pronounced in the group treated with ZN and the high dose of CSE.

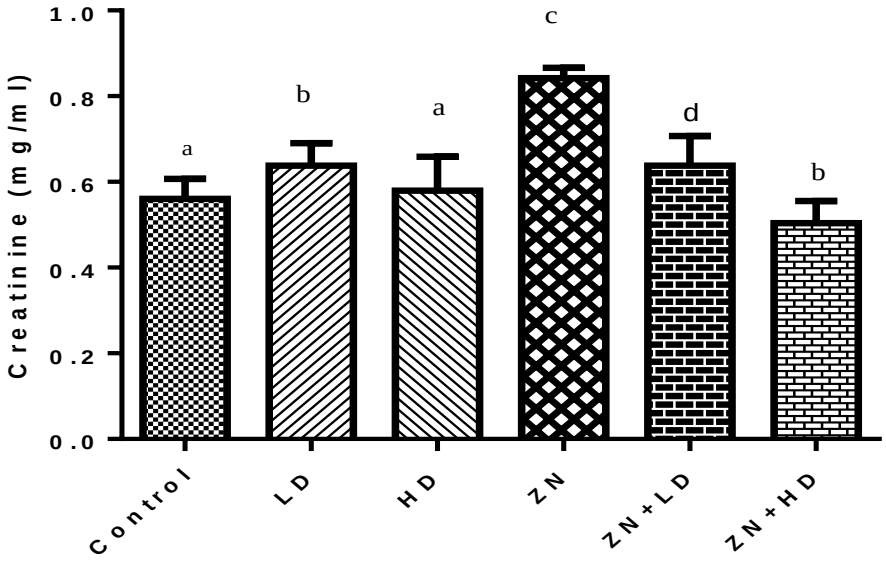

Figure 37. Effects of ethanolic extract of *C speciesus* on serum creatinine level in rats treated with ZN. Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

The current results also revealed that treatment with ZN resulted in a significant increase in serum urea, uric acid and creatinine suggesting toxic effect on kidney. Nephrotoxicity was also reported for ZN in many reports due to the significant increase in kidney marker levels as urea and creatinine (Jiang *et al*., 2011). The result clearly showed that ZN has a harmful and stressful influence on the kidney tissue. On the other hand, the nephroprotective effect of CSE was suggested from the result of current study because it succeeded to decrease the level of kidney markers as urea, creatinine and uric acid which was increased by ZN.

7.3. Serum lipid profile

7.3.1. Serum cholesterol (CHL)

Treatment with ZN induced a significant increase in cholesterol level compared to the control group. A significant difference was observed between different groups as indicated by

the analysis of variance. Animals treated with CSE alone were comparable to the control group however, CSE at the high dose showed a significant decrease in cholesterol level in a dose dependent manner compared to the control group. The treatment with ZN plus CSE at the low or high dose induced more improvement in cholesterol and the high dose was more effective compared to the low dose.

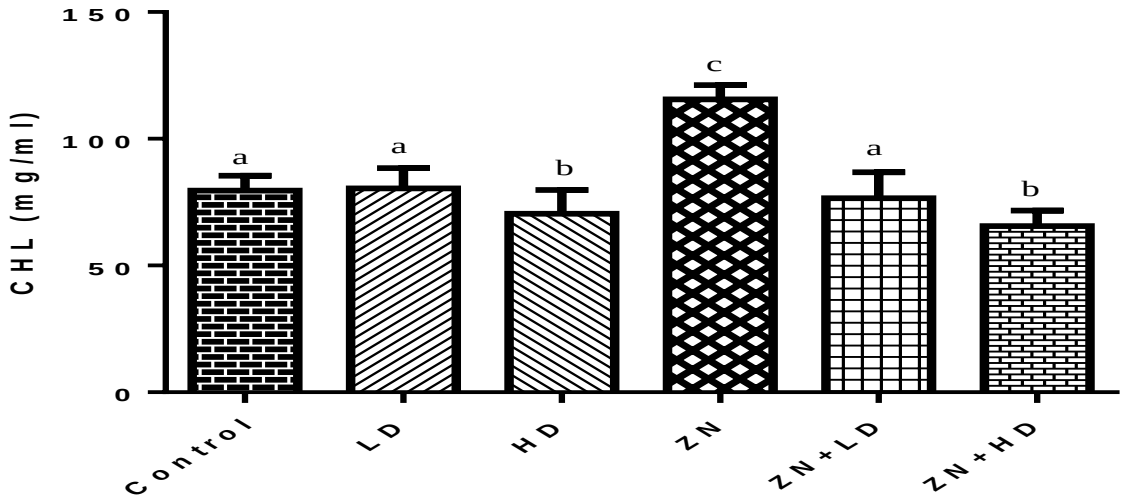

Figure 38. Effect of ethanolic extract of *C sp*eciesus on serum cholesterol level in rats treated with ZN. Within each column, means superscript with different letters are significantly different (P≤ 0.05).

7.3.2. Triglycerides (TG)

Animals treated with ZN showed a significant increase in triglycerides level. The analysis of variance showed a significant difference was observed between different treatments. Animals treated with CSE alone were comparable to the control group however, CSE at the high dose showed a significant decrease in triglycerides level in a dose dependent manner compared to the control group. The treatment with ZN plus CSE at the low or high dose induced more improvement in triglycerides level and the high dose was more effective compared to the low dose (Fig. 39).

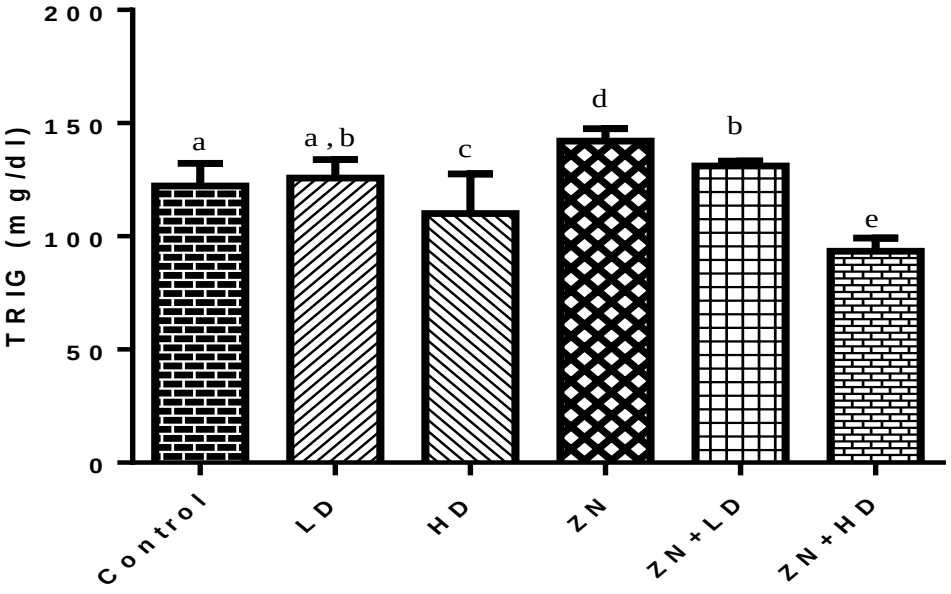

Figure 39. Effect of ethanolic extract of *C speciosus* on serum triglycerides in rats treated with ZN. Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

7.3.3. High-density lipoprotein-cholesterol (HDL-CHL)

The effects of different treatments on HDL-CHL level indicated that treatment with ZN resulted in a significant decrease in the level of HDL. The analysis of variance revealed a significant difference was observed between different treatment groups. The combined treatment with ZN plus CSE at the high dose induced a significant improvement in HDL-CHL level but no significant difference at the low dose and group treated with ZN (Fig. 40).

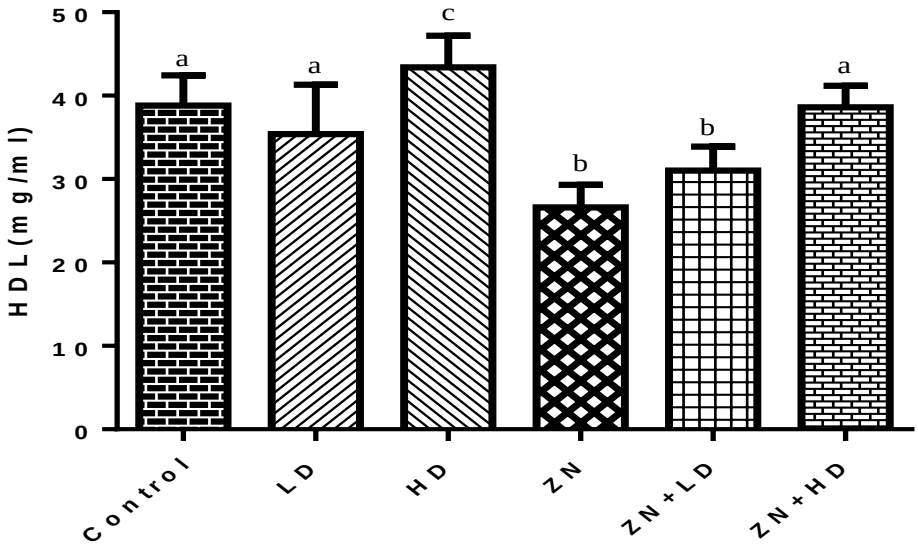

Figure 40. Effect of ethanolic extract of C speciosus on high-density lipoprotein cholesterol (HDL-CHL) level in rats treated with ZN. Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

7.3.4. Low-density lipoprotein cholesterol (LDL-CHL)

The effects of different treatments on LDL-CHL level indicated that treatment with ZN resulted in a significant increase in the level of LDL-CHL. The analysis of variance showed a significant difference was observed between different treatment groups. Animals treated with CSE (LD) and (HD) were comparable to the control group. The combined treatment with ZN plus CSE induced a significant decrease in LDL-CHL level compared to ZN-treated group. Moreover, the combined treatment with ZN plus CSE at the low or high dose induced improvement in LDL-CHL level although these treatments normalize it and CSE (HD) was more effective than CSE (LD) as shown in Fig. (31).

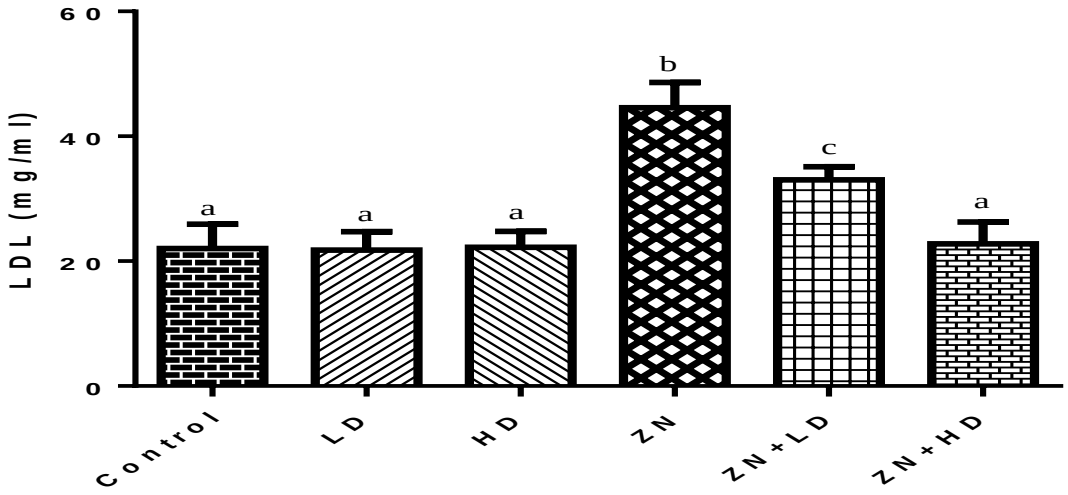

Figure 41. Effect of ethanolic extract of *C. speciosus* (LD) and (HD) on Low-density lipoprotein cholesterol (LDL-CHL) level in rats treated with ZN. Within each column, meanssuperscript with different letters are significantly different ($P \leq 0.05$).

It is well documented that lipids are playing a critical role in the incidence of hepatic disease. In the current study, treatment with ZN resulted in the destruction of lipid metabolism. Animals treated with ZN showed a significant increase in cholesterol, triglycerides and LDL-CHO accompanied with a significant decrease in HDL-CHO. The increase in the cholesterol levels suggested the increased esterification of fatty acids, which decreases the excretion of the cellular lipids and the inhibition of FA b-oxidation (Fernandez and West 2005). Moreover, ZN induced a stimulation of acetate transfer into hepatocytes, increase the synthesis of cholesterol, FA and triglycerides from acetate (Weber *et al*. 2003). Additionally, ZN was also reported to inhibit apo-lipoprotein synthesis consequently, reduces the lipoprotein synthesis (Kamalakkannan *et al*., 2005).

7.4. Serum cytokines

7.4.1.Tumor necrosis factor alfa (TNF-*α)* level in serum

The effect of different treatments indicated that treatment with ZN resulted in a significant increase on serum TNF-α level. The analysis of variance indicated that there is significant difference between groups. However, the group treated with the plant extract alone showed a significant decrease in the TNF-α level. On the other hand, the combined treatment with the plant extract and ZN or in combination resulted in a significant improvement in TNF-α although this high dose was more effective compared to the low dose (Fig. 42).

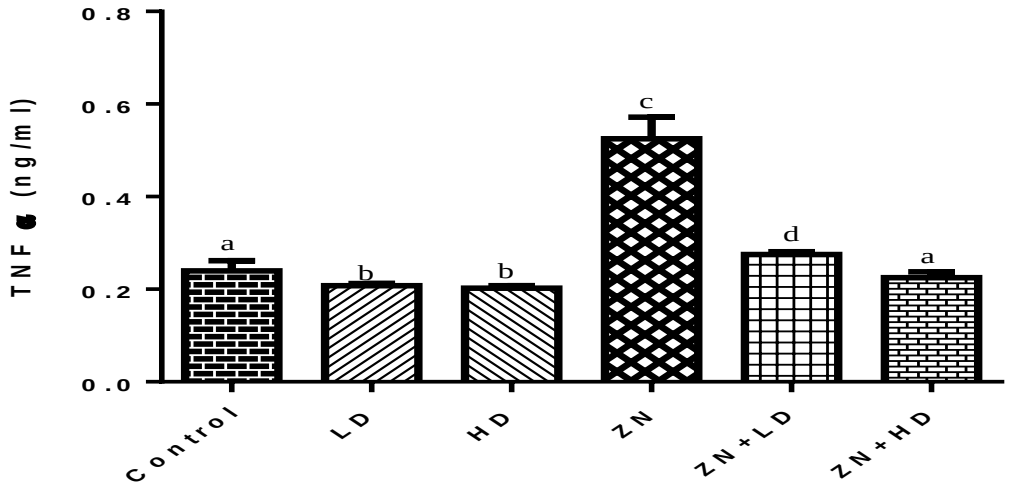

Figure 42. Effect of ethanolic extract of C. speciosus on serum TNF-α level in rats treated withZN.Within each column, means superscript with different letters are significantly different($P \leq 0.05$)

7.4.2. Interleukin 6 (IL-6)

The effect of different treatments indicated that treatment with ZN resulted in a significant increase on serum interleukin-6 level. The analysis of variance indicated that there is significant difference between groups. Animals treated with CSE (LD) or (HD) were comparable to the control group. On the other hand, the combined treatment with the plant extract and ZN or in combination resulted in a significant improvement in interleukin 6 although this high dose was more effective compared to the low dose (Fig 43).

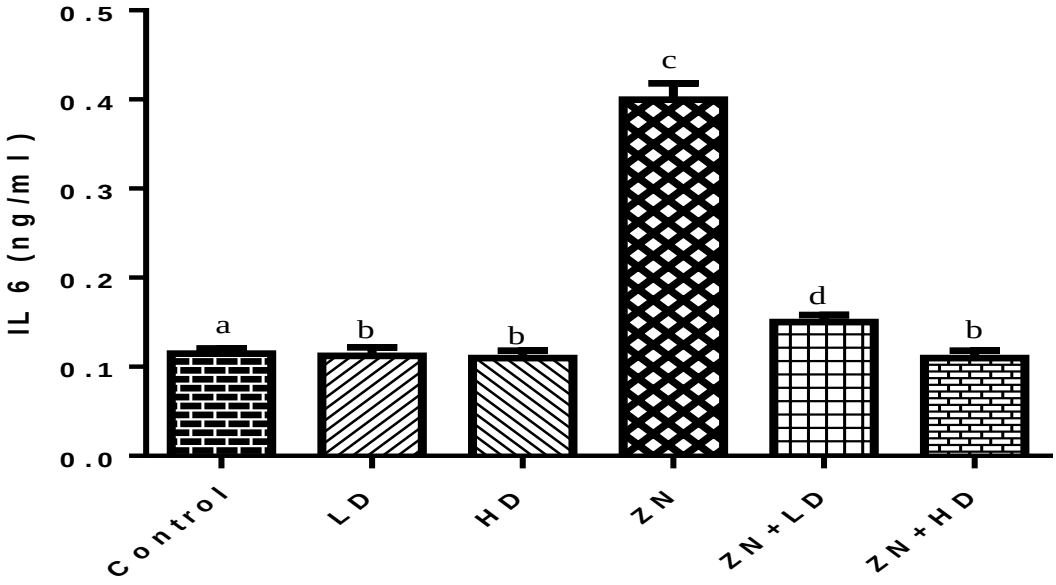

Figure 43. Effect of ethanolic extract of *C. speciesus* on serum in interleukin 6 (IL-6) level inrats treated with ZN. Within each column, means superscript with different letters are significantly different (P≤0.05)

7.4.3. The carceno embryonic antigen (CEA)

The effects of different treatments on CEA level indicated that treatment with ZN resulted in a significant increase in the level of CEA. The analysis of variance revealed a significant difference was observed between the group treated with ZN alone and the other treatment groups. Animals treated with CSE (LD) or (HD) were comparable to the control group. On the other hand, the combined treatment with the plant extract and ZN in combination resulted in a significant improvement in CEA although this high dose was more effective compared to the low dose (Fig. 44).

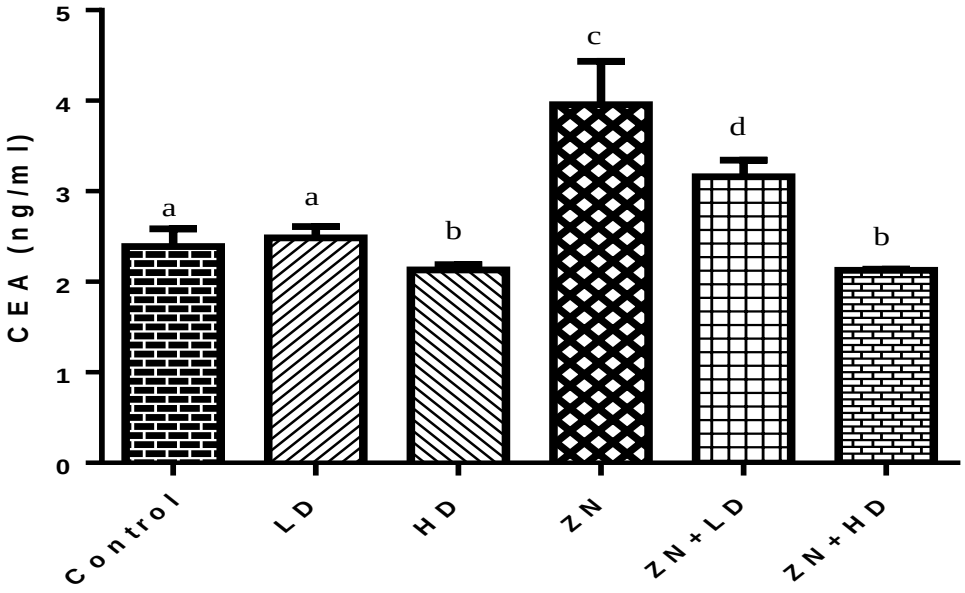

Figure 44. Effect of ethanolic extract of *C. speciosus* on serum in carceno embryonic antigen (CEA) level in rats treated with ZN. Within each column, means superscript withdifferent letters are significantly different (P≤0.05)

Animals treated with ZN alone showed a significant increase in serum CEA, TNF-α and IL-6 suggesting that this mycotoxin impairs the immune defense. Previous reports indicated that ZN can induce inflammatory responses and enhance the expression of several pro-inflammatory cytokine including TNF-α and IL-6 (Cano *et al*., 2013). In general, the cytokine results reported herein indicated that ingestion of ZN increased TNF-α, CEA and IL-6 possibly by affecting macrophage function (Han *et al*., 2016; Abdel- Wahhab *et al*., 2015).

7.4.4. Alpha fetoprotein level (AFP) as a tumor marker

The effects of different treatments on AFP level indicated that treatment with ZN resulted in a significant increase in the level of AFP. The analysis of variance revealed a significant difference was observed between the group treated with ZN alone and the other treatment groups. Animals treated with CSE (LD) of CSE (HD) were comparable to the control group. Moreover, CSE at the low or the high dose induced more improvement andthe group received the high dose was nearly normal (Fig. 45).

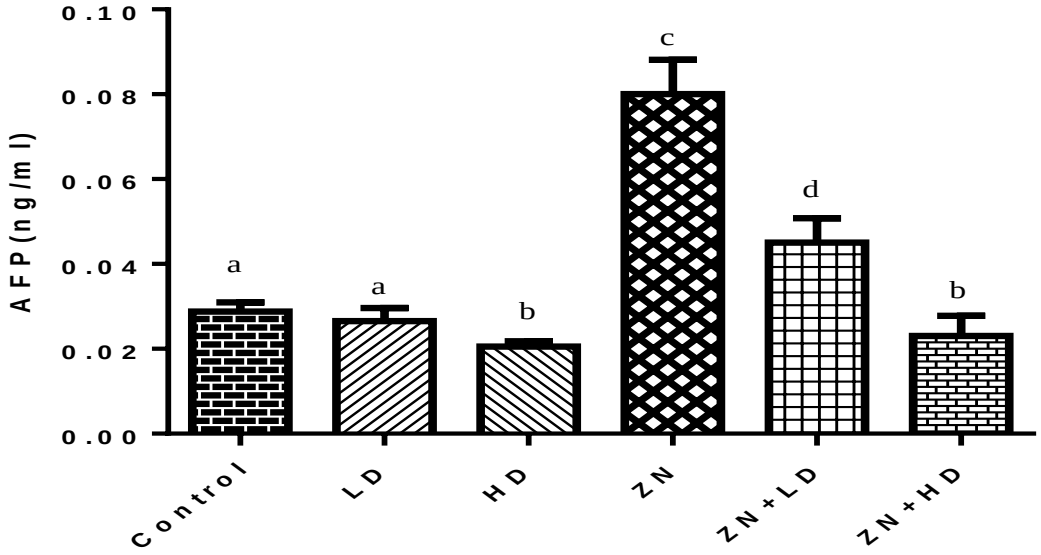

Figure 45. Effect of ethanolic extract of *C. speciosus* on serum in alpha feto protein (AFP) level in rats treated with ZN. Within each column, means superscript with different letters aresignificantly different (P≤0.05)

Animals treated with ZN showed a significant increase in AFP compared to the control group. AFP is a well-known representative tumor marker of hepatocelular carcinoma (HCC). High levels of AFP are believed to be strongly suggestive of HCC (Endo *et al.*, 1975) because greater than 70 % of HCC patients have high serum concentration of AFP due to of the tumor secretion (Xiao *et al.*, 2012). AFP is also most extensively used in the diagnosis of HCC and the increased level of AFP observed in ZN-induced animals is an indicative of HCC (Ramakrishnan *et al.*, 2007). Moreover, the elevation of serum AFP reported herein indicated that ZN is a potent hepatocarcinogen, enhance reactive oxygen species (ROS) formation and causes oxidative DNA damage, which may play a role in its carcinogenicity (Yang *et al.*, 2015; Abdel-Aziz *et al.*, 2005; Abdel-Wahhab *et al.*, 2007; El-Denshary *et al.*, 2012). Therefore, these results affirmed that ZN can induce hepatotoxicity and degeneration in liver

cells in rats and agree with the previous results which reported that AFP was much higher after the exposure to ZN of (Sell *et al.*, 1998; Abdel-Wahhab *et al.*, 2006 ; Madeha *et al*., 2011)

7.5. Tissue biochemical assay

7.5.1. Lipid peroxidation (MDA)

Treatment with ZN induced a significant increase in on oxidative stress marker expressed as malondialdehyde MDA in hepatic (Fig. 46a) and renal (Fig. 46b) tissues compared of different treatment groups. The analysis of variance revealed a significant difference was observed between different treatment groups. CSE at the two tested doses showed a significant decrease in MDA level in comparison with control in a dose dependent manner. The combined treatment with ZN and the low dose of CSE showed remarkable decrease in MDA level but outstanding effect was shown with the high dose of CSE when combined with ZN (Fig. 46) as it succeeded to decrease the level of MDA the control level.

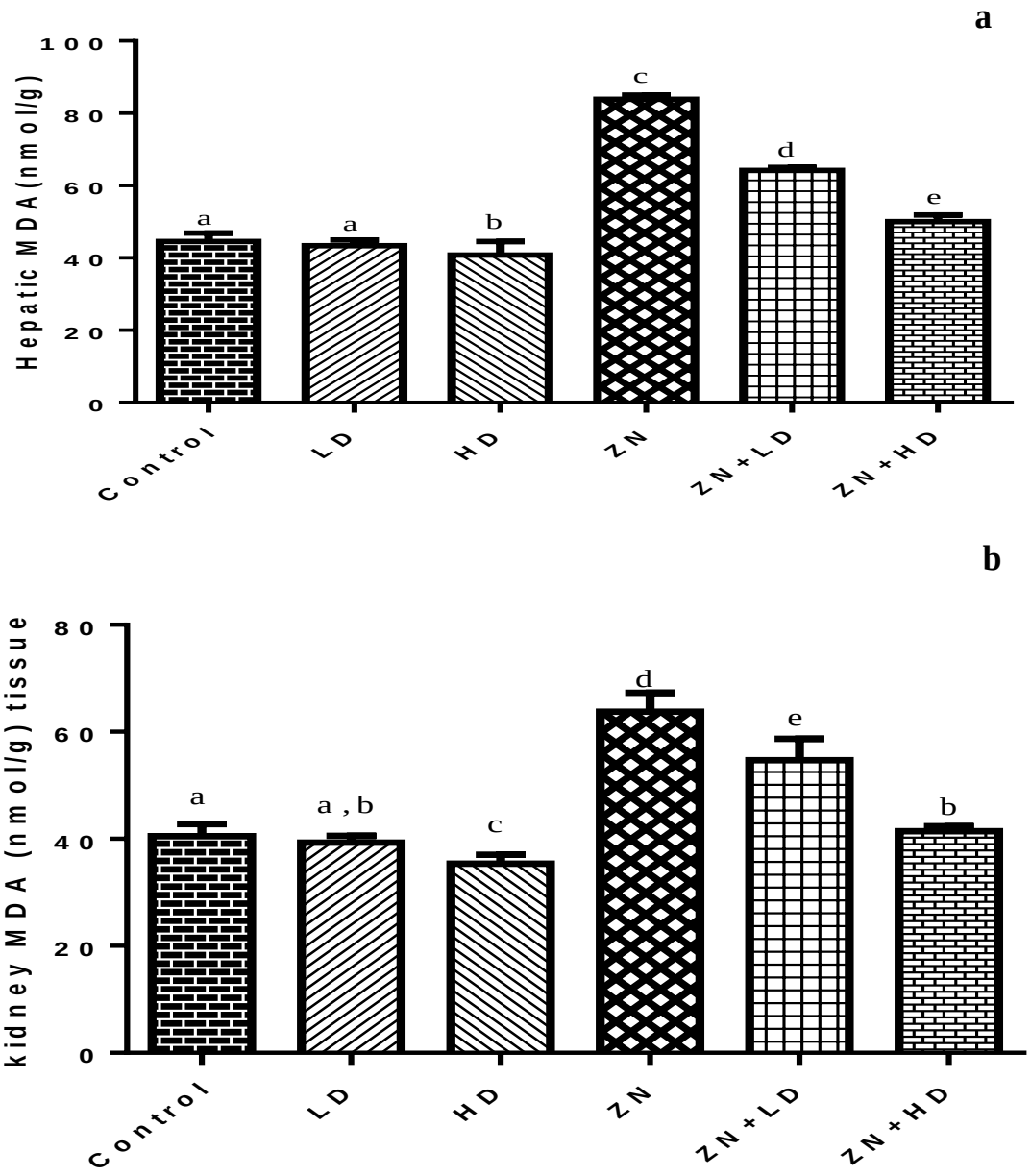

Figure 46. Effect of ethanolic extract of *C. speciosus* on lipid peroxidation in the liver and renal tissues of rats treated with ZN. Within each column, means superscript with different letters are significantly different ($P \leq 0.05$)

Another indicator of liver and kidney damage is lipid peroxidation (Poli *et al*., 1987; Chen *et al*., 2019; Sun *et al*., 2018). In fact, lipid peroxidation level shows the imbalance between the number of free radicals and antioxidants in the body (Dutta *et al*., 2018). That is, if the number of antioxidants in the body is less than the number of oxidants, these extra oxidants can bind with body's critical compounds including the double bonds of membrane and cause damage (Rolo *et al*., 2012). Several reports indicated that lipid peroxidation is the main manifestations of oxidative damage and is one of the important consequences of oxidative stress, playing a principle role in necrosis and apoptosis as well as cellular damage (Abdel-Wahhab *et al*., 2015b, 2019; Hassan *et al*., 2019). In the present study, the level of MDA, as an indicator of lipid peroxidation, in liver and kidney tissues were significantly elevated upon exposure to ZN, the production of MDA induces the damage of mitochondria, suppress the mitochondrial electron transport chain and promote the production of further ROS (Abdel-Wahhab *et al*., 2017).

7.5.2. Total antioxidant capacity (TAC)

The current results showed that ZN administration induced a significant decrease in the hepatic and renal TAC tissue. The analysis of variance showed that a significant difference was found between the treatment groups in the liver and kidney TAC in a dose dependent manner (Tables 34). The treatment of CSE at a low dose the high dose a significant increased in TAC level in the liver (Fig. 47A) and kidney (Fig. 47B) compared to the control group. The combined treatment of ZN and the low dose of CSE showed a significant increase in hepatic and renal TAC levels compared to those treated with ZN alone. However, ZN plus the high dose of CSE succeeded to normalize TAC in hepatic and renal levels (Fig. 47).

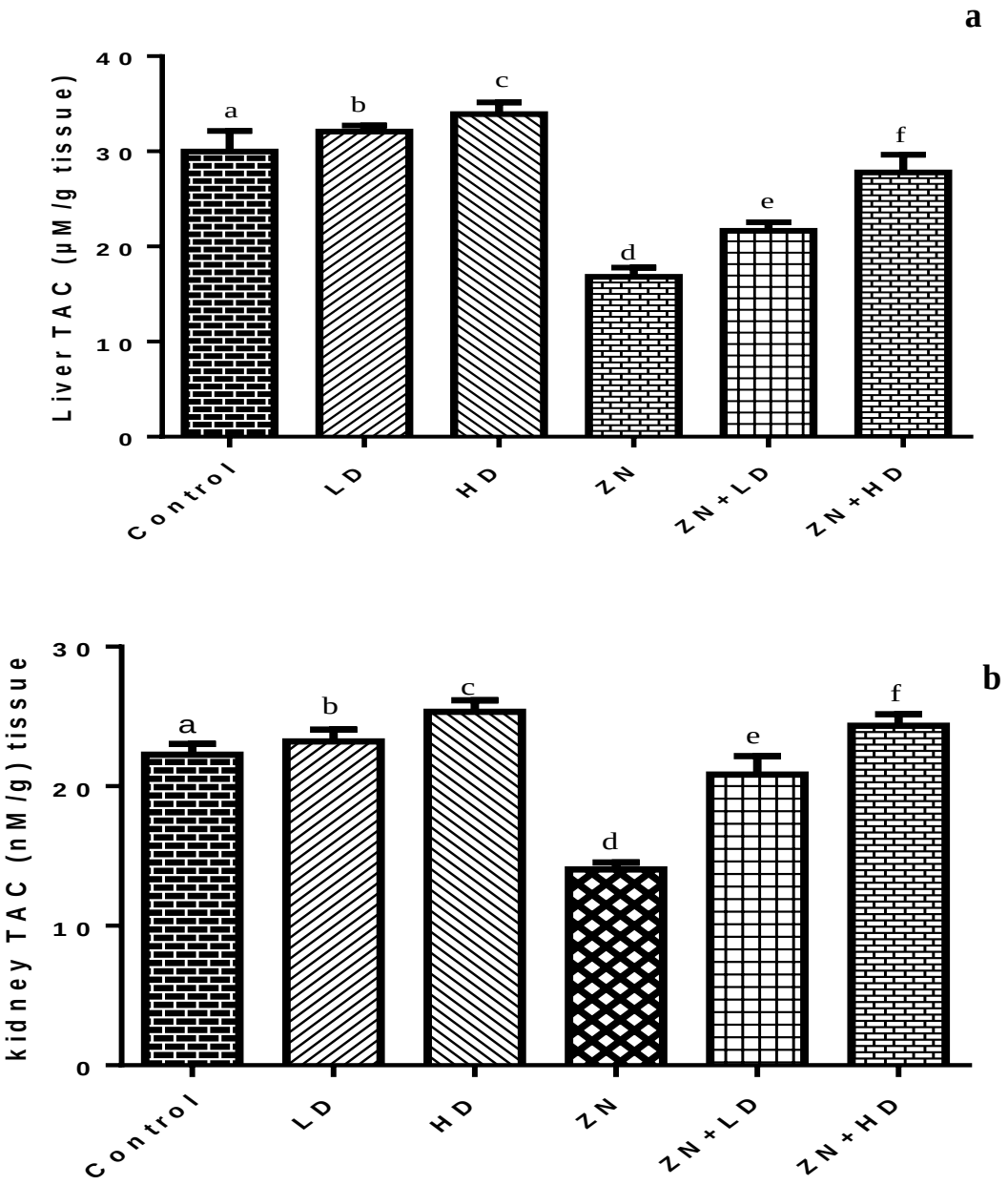

Figure 47. Effect of ethanolic extract of *C speciosus* on hepatic (A) and renal (B) TAC of rats treated with ZN .Column superscripts with different letters are significantly different ($P < 0.05$).

7.6. Antioxidant enzymes

7.6.1. Glutathione peroxidase activity (GPx)

The effects of different treatments on GPx in the hepatic and renal tissue indicated that treatment with ZN induced a significant decrease in the activity of GPx in both organs. The analysis of variance revealed a significant difference was observed between different treatment groups. The

treatment of CSE at a low dose the high dose a significant increased in the liver and kidney GPx level compared to the control group. Treatment with ZN plus CSE (LD) or CSE (HD) induced a significant improvement in hepatic GPx activity in a dose dependent fashion (Fig. 48a.b). However, treatment with ZN in combination with CSE at the low or high dose showed a significant improvement in liver and renal GPx. This improvement was more pronounced in the groups treated with the high dose of CSE (Fig. 48a,b).

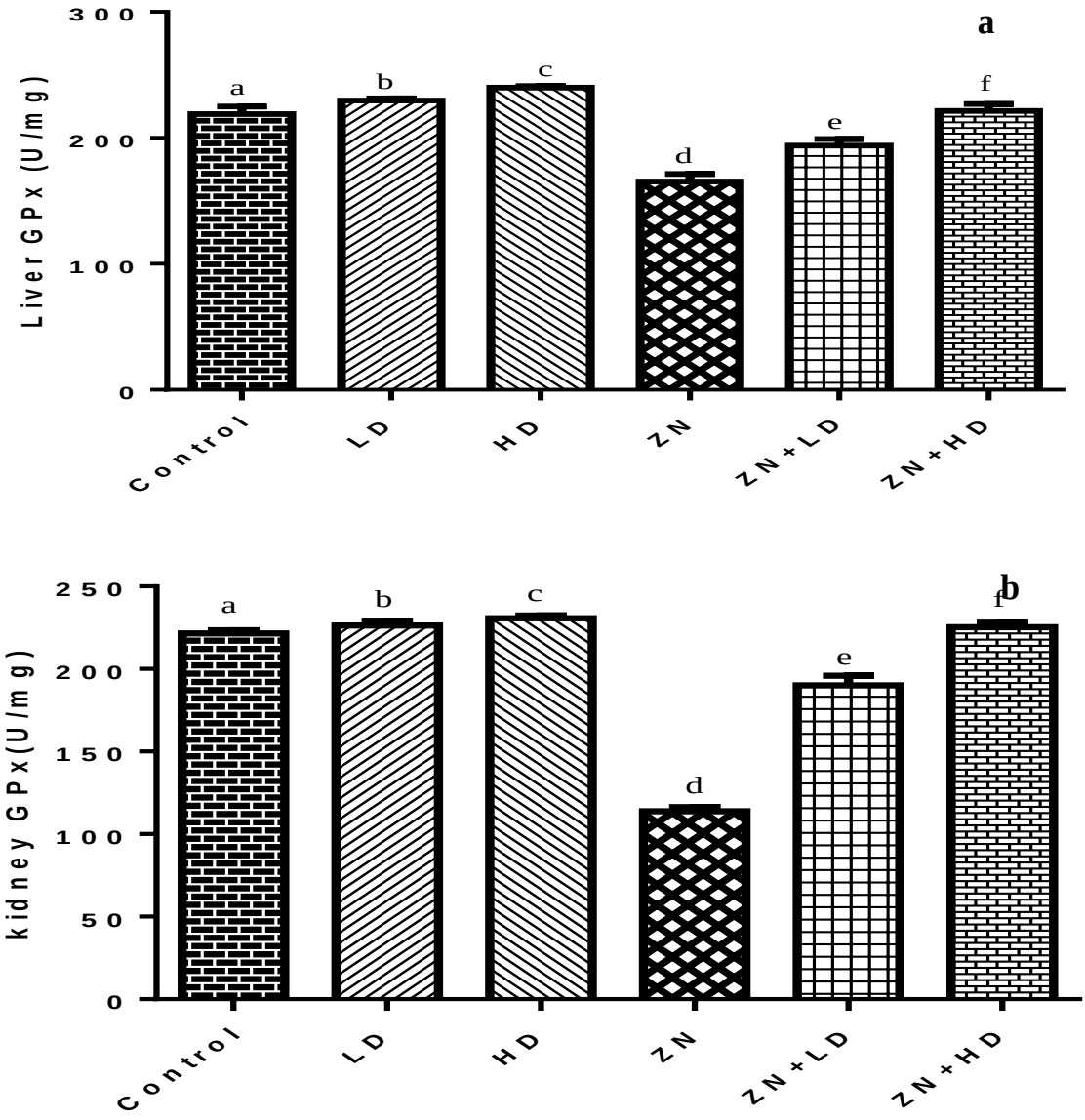

Figure 48. Effect of ethanolic extract of C. speciosus on hepatic and renal GP_X of rats treated with ZN. Column superscripts with different letters are significantly different ($P < 0.05$)

7.6.2. Catalase Activity (CAT)

The effects of different treatments on catalase activity in the hepatic and renal tissue indicated that treatment with ZN induced a significant decrease in catalase activity in both organs. The analysis of variance revealed a significant difference was observed between different treatment groups. The treatment of CSE at a low dose the high dose a significant increased in the liver and kidney catalase activity level compared to the control group. Treatment with ZN plus CSE (LD) or CSE (HD) induced a significant improvement in catalase activity activity in a dose dependent fashion (Fig. 49A,B). However, treatment with ZN in combination with CSE at the low or high dose showed a significant improvement in hepatic and renal catalase activity. This improvement was more pronounced in the groups treated with the High dose of CSE.

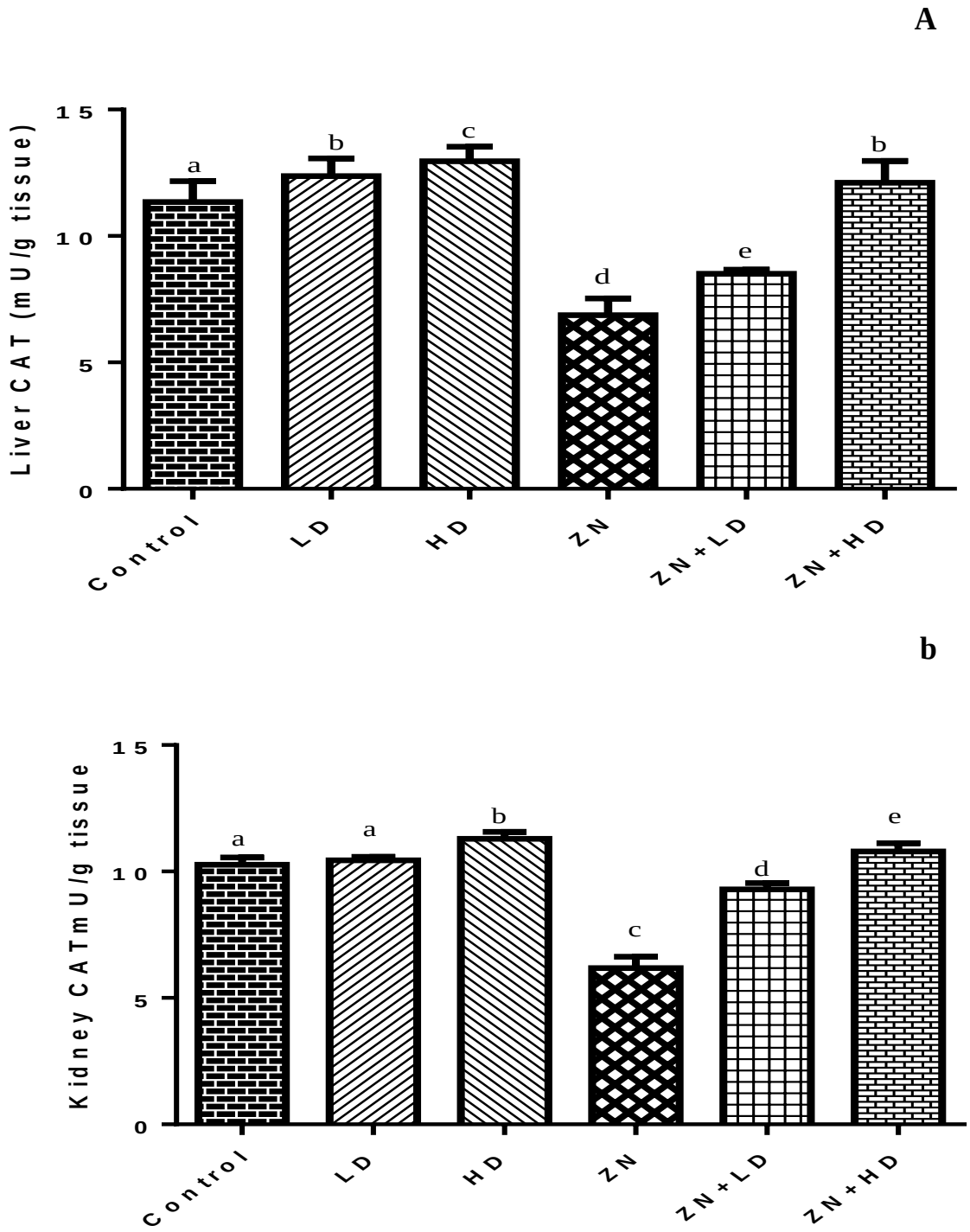

Figure 49. Effect of ethanolic extract of *C. speciosus* on hepatic and renal CAT of rats treated with ZN. Column superscripts with different letters are significantly different ($P < 0.05$)

The decrease in TAC, GPx and catalase in hepatic and renal tissue in ZN-exposed rats indicated the stressful and the oxidative stress in these organs. Furthermore, these disturbances in enzymatic antioxidant system were correlated positively with the impairment of liver or kidney injury (Hassan and Yousef, 2010, Abdel-Wahhab *et al.*, 2016) and resulted

from the increase production of reactive oxygen species (ROS) and the decline of the defense system due to the reduction of antioxidant enzymes activity. Actually, ROS affect the side chains of fatty acids in the lipid molecules present in the cells membrane specially the mitochondrial membranes, which exposed directly and continuously to the superoxide anion ($O2^{\bullet-}$) produced during the respiration of cells. These events as well as the machinery of protein synthesis, lead to the damage of macromolecules inside the mitochondria, especially protein, DNA and lipids; consequently, the mitochondrial dysfunction, energy depletion and the death of cells (Casini *et al*., 1997; Hassan *et al*., 2019). These molecular processes trigger a further increase in the steady concentration of ROS leading to cell death, which eventually damages the organ and its function (Sundaram *et al*., 2014; Abdel-Wahhab *et al*., 2019 ; Freeman and Crapo, 1981; Ben Salah-Abbès *et al*., 2008b, Zourgui *et al*., 2008: Ben Salah-Abbès *et al*., 2009).

7.7. Genotoxicity test

7.7.1. Micronucleus findings

Treatment with ZN alone remarkably increased the frequency of MNPCE in rat bone marrow (1.15%, i.e. a 6.0-fold increase) compared to the control value (Fig. 50). Two-week repeated oral treatment with two doses of *C. speciosus* extract did not induce the frequency of MNPCEs (0.22% and 0.19%, respectively versus 0.19% in the control group, $p>0.05$). When *C. speciosus* extract was given before administration of ZN, the percentage of MNPCEs was decreased in a dose-dependent manner. No significant differences in the ratio of PCE/total erythrocytes were observed in all experimental groups.

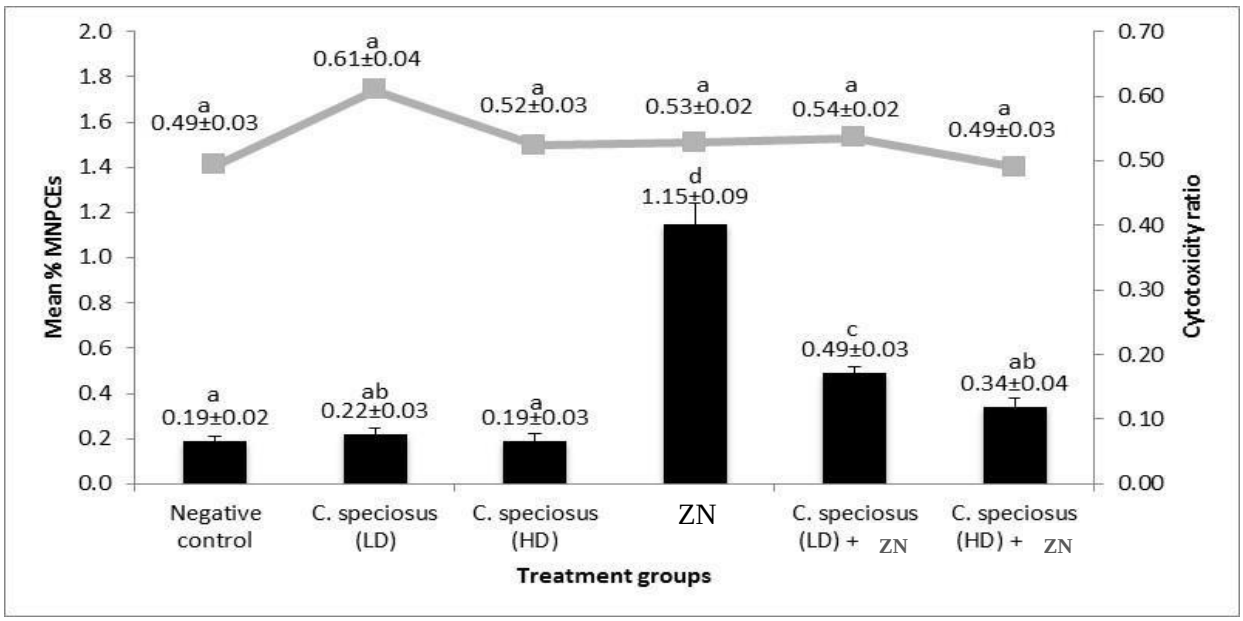

Figure 50. Effect of treatment with *C. speciosus* extract and ZN on the micronucleus formation and cytotoxicity rate in rat bone marrow cells**.** Data expressed as Mean%±S.E. Sum 2000 cells per animal were analyzed to calculate the % MNPEs in the cells. Sum 500 erythrocytes per animal were scored to calculate the cytotoxicity ratio. The data with the identical superscript letters have no statistical significance ($p>0.05$), while the data with different superscript letters have statistical significance (ANOVA, Duncan's multiple comparison test, $p<0.05$)

7.7.2. Comet assay findings

Table (37) and Figure (51) represent the level of DNA breakage in the individual cells treated with C. speciosus and ZN in rat bone marrow cells. As expected, ZN caused a significant increase in the values of the percentage of DNA in comet tail (14.91%, i.e., a 1.9-fold increase), TM (0.96, i.e., a 6-fold increase) and OTM (1.91, i.e., a 2.3-fold-increase) compared to control values. However, oral supplementation with two doses of *C. speciosus* extract alone had no positive effect ($p>0.05$) on the level of comet tail formation compared to the control group (Table 1). Co-treatment with two doses of C. speciosus extract along with ZN dramatically decreased the values of TM, OTM, and the percentage of DNA in the comet tail (11.12% and 10.13%, respectively) compared to the ZN-treated group.

Table (05). Effect of treatment with *C. speciosus* extract and ZN on the comet tail formation in rat bone marrow cells.

Experimental Groups	%DNA in Tail	TM	OTM
	Mean %±SE	Mean %±SE	Mean %±SE
Negative control	7.47±0.48[a]	0.16±0.02[a]	0.83±0.07[a]
***C. speciosus* (LD)**	7.61±0.15[a]	0.20±0.01[a]	0.93±0.03[a]
***C. speciosus* (HD)**	7.54±0.29[a]	0.19±0.02[a]	0.85±0.05[a]
ZN	14.91±0.42[d]	0.96±0.07[c]	1.91±0.06[c]
***C. speciosus* (LD) + ZN**	11.12±0.20[c]	0.41±0.04[b]	1.28±0.05[b]
***C. speciosus* (HD) + ZN**	10.13±0.22[b]	0.40±0.02[b]	1.19±0.04[b]

Data expressed as Mean% ± S.E. One hundred fifty cells analyzed per animal using automatic comet score™ software. In each column, the data with the identical superscript letters have no statistical significance ($p>0.05$), while the data with different superscript letters have statistical significance (ANOVA, Duncan's multiple comparison test, $p<0.05$).

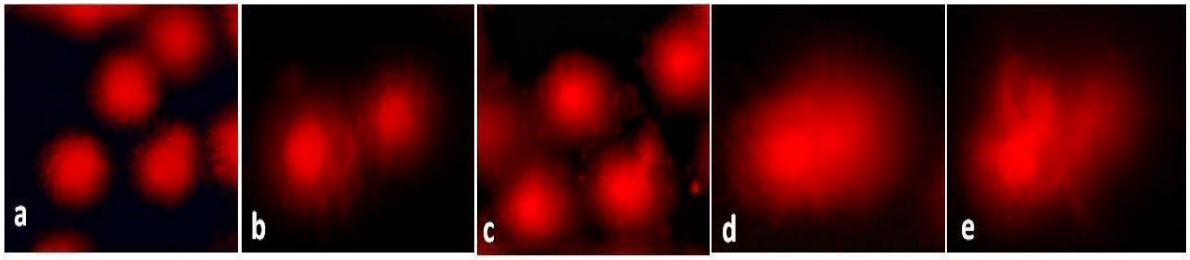

Figure 51. Fluorescence photomicrographs of rat bone marrow cells treated with *C. speciosus* extract and ZN showing (a) intact cells; (b-f) different patterns of comet tail formation (original magnification power, 400×)

For clarifying the genoprotective activity of C. speciosus extract, MN and comet assays were analyzed in rat bone marrow cells. The combined MN and comet assay are globally well-recognized standard methods due to their sensitivity and efficiency to measure DNA breakage in the same animal (Araldi *et al*., 2015). In MN assay, erythroblasts expel their nuclei to originate PCEs which, in turn, undergo the maturation process to originate NCEs. In comet assay, broken cells can visualize by increasing migration of broken DNA fragments resulting in cells similar to the structure of comets (Araldi *et al*., 2015; Kasamoto *et al*., 2017).

In this study, ZN induced genotoxic activity as indicated by increasing the occurrence of MNPCEs and comet tail formation in rat bone marrow cells. Similar studies have proved that ZN causes an increase in the occurrence of chromosomal aberrations and MNPCEs in mouse

bone marrow cells (Ayed *et al*., 2011; Belgacem *et al*., 2019; Ben Salah-Abbès *et al*. 2009, Ouanes *et al*. 2005). Further, in vitro studies have reported that, based on comet assay, ZN induces DNA breakage in a human embryonic kidney cell line (Gao *et al*., 2013), porcine lymphocytes (Kachlek *et al*., 2017), and human hepatocarcinoma cell line (Hassen *et al*., 2007). These results support the fact that catechol metabolites of ZN generate reactive oxygen species (ROS) and causes the formation of DNA adducts which are reliable biomarkers of oxidative DNA damage (Fleck *et al*., 2012). In this regard, ZN induces the formation of DNA-adducts in mouse liver and kidney using a 32P-postlabelling method (Grosse *et al*., 1997).

As highlighted in our study, treatment with two doses of C. speciosus extract caused no detectable DNA damage using MN and comet assays in rat bone marrow. These findings suggest that the selected doses of C. speciosus extract have no genotoxic effect. This is in agreement with Sari and Nurrochmad (2016) who found that 90-days of repeated oral administration of C. speciosus at doses 275-1100 mg/kg did not induce toxic effects on clinical, biochemical, histological aspects in mouse tissues

Interestingly, C. speciosus extract effectively decreased ZN-induced the elevated levels of MNPCEs and comet tail parameters in rat bone marrow cells. These results coincide with those obtained by Girgis *et al*. (2015) who found that water extract of C. speciosus leaves ameliorates streptozotocin-induced MNPCEs and comet tail formation in rat peripheral blood lymphocytes. Similarly, two-months of repeated oral treatment with different extracts of C. speciosus rhizomes successfully restored streptozotocin-inhibited DNA content in rat pancreas. The highest DNA content was observed in hexane extract (74%) group, followed by methanol (67%), then ethyl acetate (62%), and finally aqueous (55%) extract group (Eliza *et al*., 2011). Our results indicate that C. speciosus extract and its constituents can interfere with the biotransformation of ZN and remove ROS before inducing oxidative stress and attacking DNA molecule. As mentioned in our recent study, ethanol extract of *C. speciosus* is a mixture of ten polyphenol compounds including six phenolic acids, mainly chlorogenic acid, and four flavonoids, mainly naringenin. These compounds exert their antigenotoxic activity through two main pathways: (1) their antioxidant capacity as indicated by radical scavenging activity, cupric reducing antioxidant capacity, b-carotene bleaching, and ferrous ions chelating assays (Gheraibia *et al.* 2020); (2) their indirect antioxidant activity through promoting DNA repair pathway, suppression of oxidative stress, stimulation of endogenous antioxidant defense

system, neutralization of lipid peroxidation, and inhibition of apoptotic pathway (Eliza *et al*., 2010 ; Shediwah *et al*., 2019 ; Yi *et al*., 2020)

7.8. Histological studies

The histological examination of the liver sections of the control rats showed normal histological structure, regular distinct hepatocytes with sinusoidal spaces arranged radially around the central vein (Fig. A52). The microscopic examination of the liver section of rats treated with CSE (LD) showed normal histological structure of hepatocytes and blood sinusoid (Fig. B52). Moreover, the liver sections of rats treated with CSE (HD) showed normal hepatocytes around the portal area (Fig. C52). On the other hand, the liver sections of rats treated with ZN showed degeneration of hepatocytes, vacuolization of the cytoplasm, eosinophilic necrosis, congested central vein and fibrosis around the portal tract (Fig. 53). The liver sections of rats treated with ZN lpus CSE (LD) showed restoration of normal liver architecture, central vein; hepatocytes; portal area and sinusoid (Fig. 54).

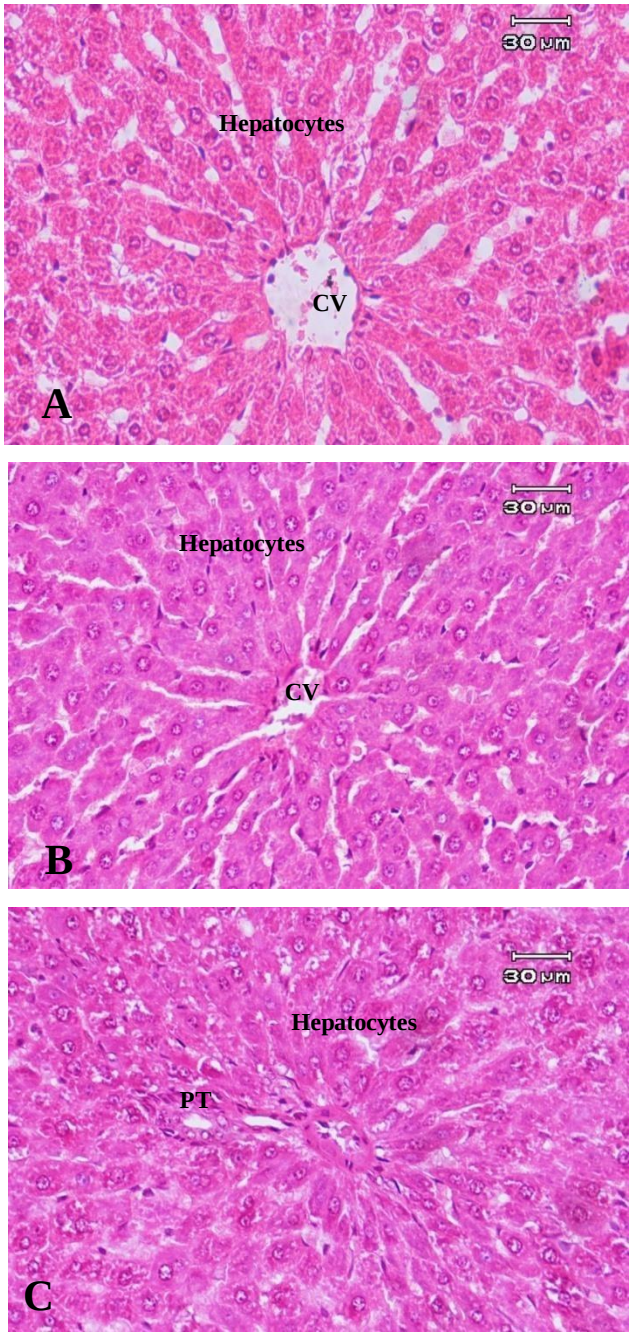

Figure 52. Photomicrograph of liver section of (A) the control rats showing normal histological structure, regular distinct hepatocytes with sinusoidal spaces arranged radially around the central vein, (B) of liver section of the rats treated with CSE (LD) showing normal histological structure of hepatocytes and blood sinusoid and (C) of the rats treated with CSE (HD) showing normal showing normal hepatocytes around the portal area (PT).

(H & E X 400)

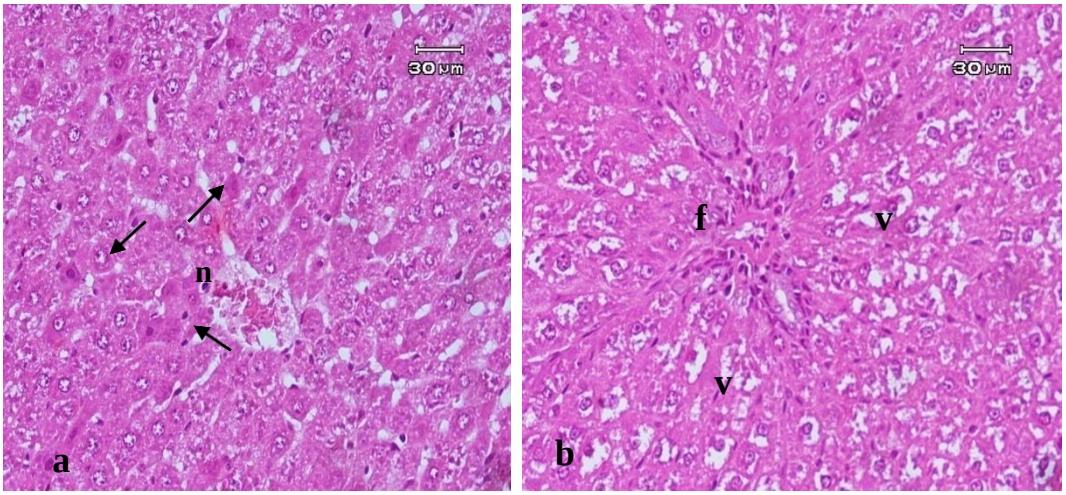

Figure 53. Photomicrographs of liver section of the rats treated with ZN showing degeneration of hepatocytes (n), vacuolization (v) of the cytoplasm, and eosinophilic necrosis (arrow), congested central vein and fibrosis (f) around the portal tract also seen.

(H & E X 400)

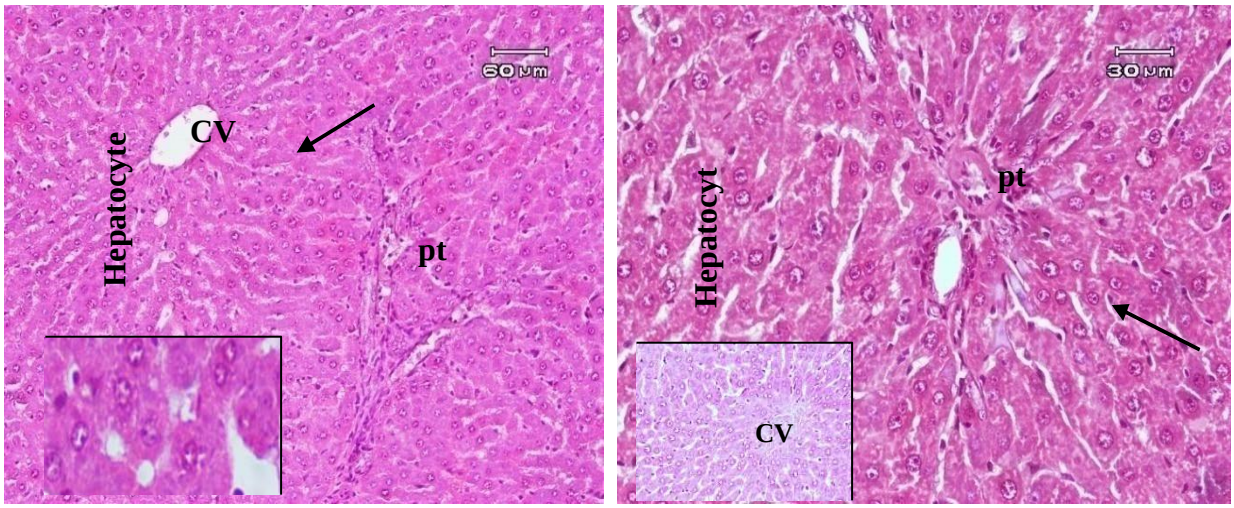

Figure 54. Photomicrographs of liver sections of rats treated with **(a)** ZN plus CSE (LD) and (b) ZN plus CSE (HD) showing restoration of normal liver architecture, central vein (CV), hepatocytes, portal area (pt) and sinusoid. The inset illustrates the normal hepatocytes.

(H & E X400)

The histological examination of the kidney cortex of control rats or those treated with CSE at the low or high dose showed normal kidney histology, the glomeruli are well demonstrated with normal bowman space and the renal tubules filling the bulk of the kidney

parenchyma were clearly observed (Fig. 56 a, b and c). The kidney sections of rats treated with ZN showed atrophy of the glomeruli, the tubules were fairly preserved, cellular regeneration and swelling urinary space (Fig. 57 a, b). The kidney sections of rats treated with ZN plus CSE (LD) showed the improvement of cellular regeneration which is quite prominent (Fig. 58); however, the kidney sections of rats treated with ZN plus CSE (HD) showed atrophy of the glomeruli, the tubules were fairly preserved, cellular regeneration and swelling urinary space (Fig. 59).

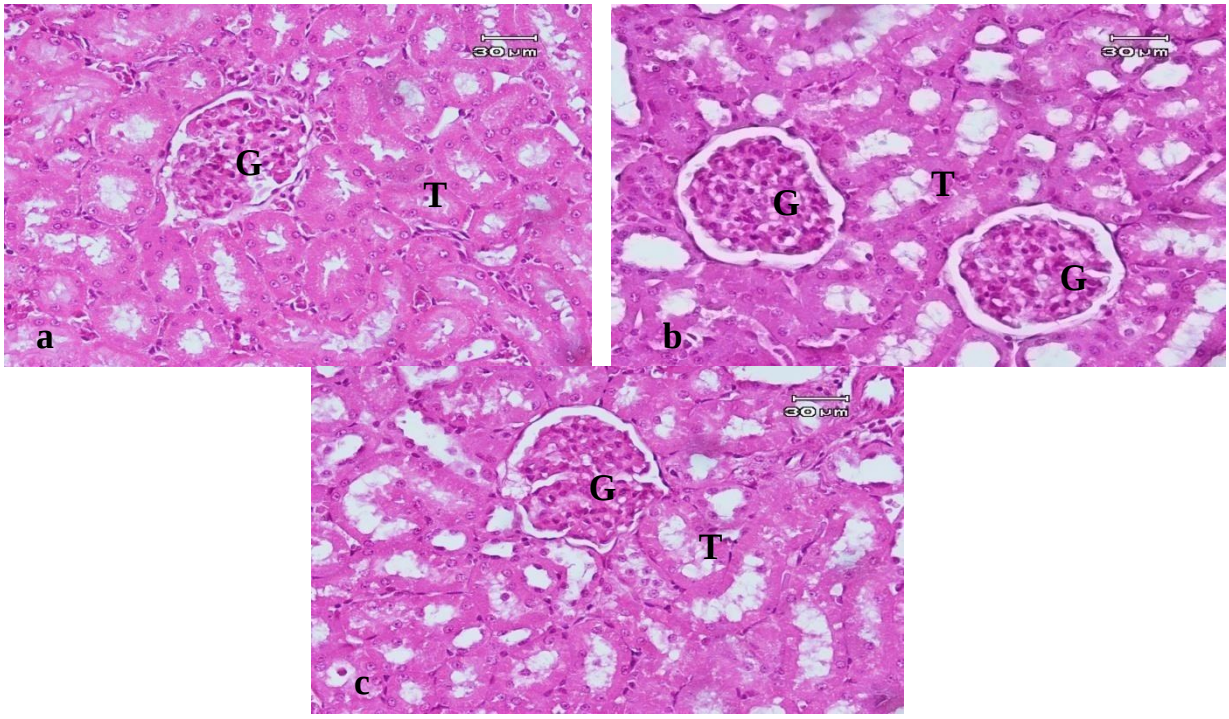

Figure 55. Photomicrographs of kidney section of **(a)** control rat, **(b)** rats treated with CSE (LD) and **(c)** rats treated with CSE (HD) showing normal kidney histology the glomeruli (G) are well demonstrated with normal bowman space. The renal tubules (T) filling the bulk of the kidney parenchyma were clearly observed.

(H & E X 400)

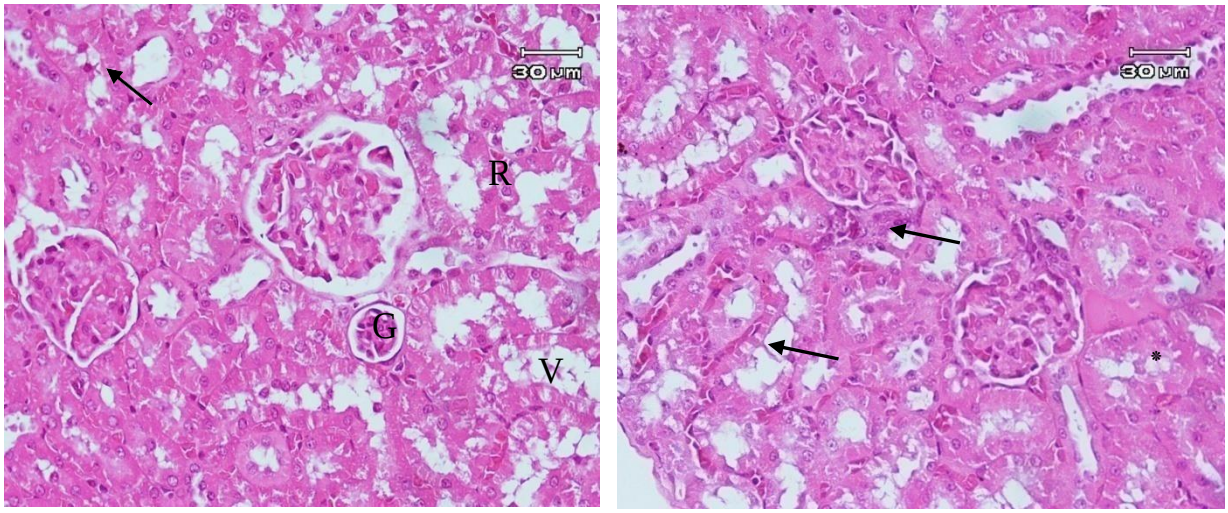

Figure 56. Photomicrographs of kidney sections of rats treated with ZN plus CSE (HD) showing atrophy of the glomeruli (G), the tubules were fairly preserved (R), cellular regeneration (arrow) and swelling urinary space (V).

(H & E X 400)

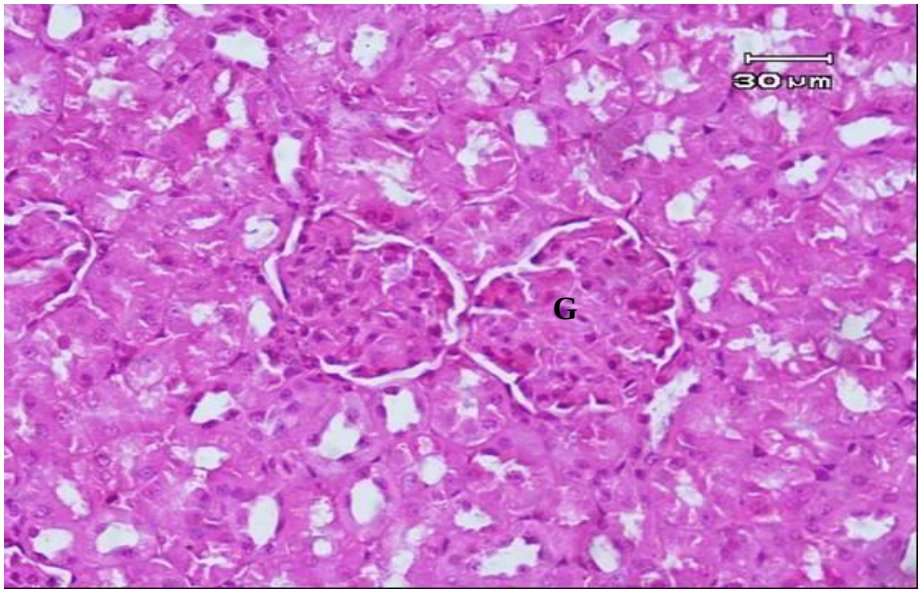

Figure 57. Photomicrographs of kidney section of rats treated with ZN plus CSE (LD) showing the improvement of cellular regeneration which is quite prominent.

(H & E X 400)

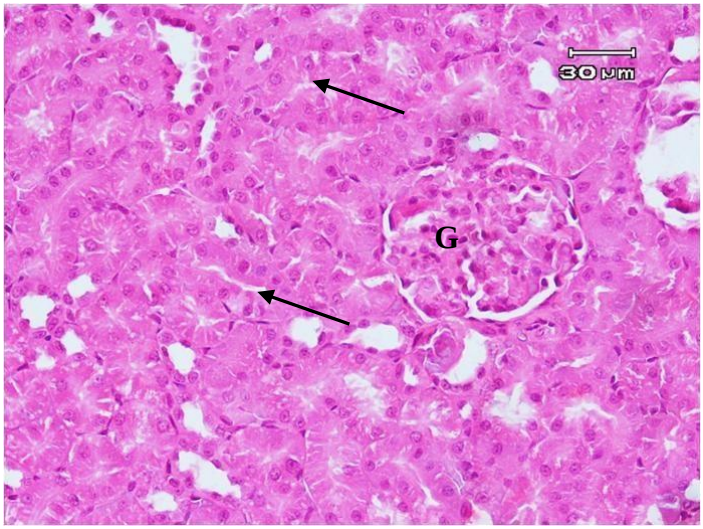

Figure 58. Photomicrographs of kidney section of rats treated with ZN plus CSE (LD) showing the expanded glomeruli with narrow urinary space and swelling in tubular epithelial cells with obliterated in the lumen (arrow).

(H & E X 400)

The histopathological examination of liver and kidney revealed that various alterations denoting the hepatotoxic effect of ZN including vacuolar degeneration of the cytoplasm in the hepatocytes, foci of necrosis, nuclei of different forms of degeneration, pyknosis, kryolysis especially eosnophilic cytoplasm and dark nuclei. Similar hepatocellular degeneration, necrosis and individual apoptosis were reported and confirmed that the liver is the target organ for the effect of zeralenone (Ismaiel *et al*., 2015). Moreover, Skiepko et al. (2020) and Salah-Abbès *et al*. (2020) reported that zeralenone were highly toxic in rat's liver and kidney cells.

Conclusion

The polyphénols has been known for a long time compounds occurring naturally in plants present a broad spectrum of health-promoting properties resulting from their biological activity, especially antioxidant activity. Extracting them from plants in mild and effective conditions assess better understand their mechanisms of biologic activity constitute scientific issues which are the basis of our study.

In the first part, the work focused on studing the effect of extraction method and conditions on the polyphenols content of C. speciosus. Three solvents included aqueous, ethanol and methanol 70% were used under different extraction times and durations. The extraction yields vary according to the time, the nature and the physicochemical characteristics of the solvents used and in particular their polarity. The results reported herein revealed that the yield of C. speciosus extracts were higher in the ethanolic and methanolic extracts compared to the aqueous extract by increasing the extraction time from 24 to 36 h. The increased of extraction time suggesting that the combination of organic solvent and water facilitates the extraction of all compounds that were soluble in both water and organic solvents.

The HPLC analysis revealed the identification of 13 phenolic compounds and the major of these compounds were identified as flavonoides. The ethanolic extraction for 24 h showed the best antioxidant and radical scavenging activity compared to the other extracts. Taken together, the yield and the antioxidant activity of the three extracts indicated that the concentration of TPC and TFC were affected by the solvent used and the extraction time.

The cytotoxic results suggested that the methanolic extract has a potential anticancer property against HePG2 cell line followed by the ethanolic extract, then the aqueous extract which showed a weak anticancer property.

The results of aPTT, PT, and TT assays in vitro and in vivo showed that EE of C. spesiosus significantly prolonged blood clotting time on activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombine time (PT) tests in a dose-dependent.

The results of the current study showed that exposure to zealenone mycotoxin resulted in severe disturbances in liver function and kidney indices, induced cytotoxicity, oxidative stress as indicated by the increased MDA and cytokines. It also decreases the antioxidant capacity, and induced pathological changes in the liver and kidney tissues. The ethanolic extract could mitigate these effects in a dose dependent manner through its rich content of

bioactive compounds via different mechanisms, including the antioxidant activity, the radical scavenging properties and the degradation of ZN.

The present study is the first report on the profile and antioxidants activity of *C. speciosus*. Therefore, more researches and studies are required as there is a large untapped reservoir waiting to be investigated.

- ✓ The future research should include further research aimed to identify, isolate and characterize more active constituents responsible for the strong observed antioxidant activity in order to determine the exact mechanism of the antioxidant activity.
- ✓ Other *in vivo* kinds of antioxidant assays are needed.
- ✓ More work should be carried on both pharmacological and biological activities.
- ✓ Chronic toxicity of the plant should be carried out.
- ✓ The plant should be exploited as natural antioxidants in real food systems.
- ✓ Studies on the pharmacokinetics and pharmacodynamics of plant extracts would be useful before considering further investigations as to their therapeutic use.

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الملخص

القسط الهندي C. speciosus نبات ينتمي إلى أنواع القسط المستخدمة كغذاء وظيفي كما هو موصوف في الطب النبوي ويستخدم على نطاق واسع في الأدوية التقليدية لعدة آلاف من السنين .استهدفت هذه الدراسة التعرف على تحديد المركبات الفينولية للمستخلص المائي والإيثانولي والميثانولي لنبات C. speciosus ، كذلك تحديد المركبات الفينولية بواسطة جهاز التحليل الكروماتورافي السائل عالي الاداء (HPLC) ، وتقييم التأثير المضاد للأكسدة ، التأثير المضادة للسرطان والتحلط وتقييم الدور الوقائي للمستخلص الا يثانولي ضد الإجهاد التأكسدي الناتج عن الزيرالينون (ZN) في الفئران .وكانت اهم النتائج المتحصل عليها هي إجمالي محتوى الفالفونويد والبوليفينول في المستخلص الإيثانولي كان الأعلى يليه المستخلص الميثانول ثم المستخلص المائي وزاد الناتج من هذه المركبات بزيادة وقت الاستخلاص . أظهرت نتائج تحليل HPLC وجود 13 مركبًا في كل مستخلص بتركيز مختلفة وكانت مركبات الكيرسيتين وحمض الكافيك وحمض الغاليك عالية في المستخلص المائي ، بينما كانت المستخلصات الكحولية غنية بحمض السيرنجيك وحمض الكلوروجينيك والنارينجينين. أوضحت النتائج ايضا أن جميع المستخلصات المختبرة لها تأثيرمضاد للأكسدة مع بعض الاختلافات الطفيفة باستثناء اختبار بيتا كاروتين ، reducing power ions, chelating assay حيث أظهرت المستخلصات الكحولية نشاطًا أعلى ب 50٪ مقارنة بالمستخلص المائي.أظهر المستخلص الميثانولي خاصية قويه كمضاد للسرطان ضد الخلايا السرطانيةHePG2 متبوعًا بالمستخلص الإيثانولي، ثم المستخلص المائي الذي أظهر خاصية ضعيفة. أظهرت نتائج الأنشطة المضادة للتحلط مخبريا او في الفئران أن المستخلصات تؤدي الى زيادة زمن التحلط حيث بشكل ملحوظ زمن الثرومبوبلاستين الجزئي المنشط (aPTT) ووقت البروثرومبين (TT) ووقت الثرومبين (PT) مقارنة بالمجموعات المقارنة .أظهر الإجهاد التأكسدي الناجم عن ZN في الفئران إلى زيادة معنوية في نشاطية الأنزيمات ALT ، AST، ALP، ومستويات الكرياتينين ، حمض اليوريك ، اليوريا ، الكوليسترول ، الدهون الثلاثية ، LDL، نشاط عامل الوخز الورمي ، ألفا فيتو بروتين، TNFα، interleukin-6 والمالونالدهايد مصحوبًا بانخفاض كبير في البروتين الكلي ، الألبومين والاحماض الدهنيي منخفضة الكثافة (LD L) ومحتوى الكبد والكلية من الانزيمات لمضادة للاكسده (TAC, GPx, CAT) حيث أستطاع المستخلص الايثانولي احداث تحسنا في جميع القياسات محل الدراسة في اتجاه القيم الطبيعية للحيوانات المقارنة. أدت المعامله بـ ZN في حدوث تغيرات نسيجية كبيرة في الكبد والكلى أثبتت النتائج أن المعالجة بالمستخلص الايثانولي بمفرده لم يكن لها تاثيرات علي التركيب النسيجي للكبد والكلى . كما أشارت النتائج ان المعالجة بجرعة عالية منعت التغيرات النسيجية في الكبد والكلى الناتجة عن الزيرالينون. نستخلص من هذه الدراسة أن مستخلصات جذور القسط أظهرت إمكانات عالية للأنشطة البيولوجية وخصائص وقائية لكبد ولكلى تجاه الكرب التاكسدي المحدث بواسطة الزيرالينون والتي ارتبطت بمحتواها العالي من البوليفينول والفلافونويد.

الكلمات المفتاحية : القسط الهندي, البوليفينول ,HPLC,مضاد للأكسدة ,مضاد لسرطان ,مضاد لتحلط, الزيرالينون, الإجهاد التأكسدي , حماية الكبد والكلية.

Abstract

Costus specious is a plant that belongs to *Costus species*, used as functional food as described in the Prophetic medicine and used widely in traditional medicines for many thousand years. This study aimed to identify the chemical profile of the phenolic compounds of the aqueous, ethanolic and methanolic extracts of C. *speciosus* under different extraction conditions, to determine the phenolic by HPLC, and evaluate, *in vitro* and *in vivo*, their antioxidant, anticoagulant and anticancer activities. The protective role of the ethanolic extract against zearalenone (ZN) as an inductor of oxidative stress in the liver and kidney in rats was also established. The obtained results indicated that : The content of total falvonoids and polyphenol in the ethanolic extract was the highest followed by the methanolic then the aqueous extracts and is was increased by increasing or extraction time. The HPLC analysis identified a total of 13 compounds in each extract with different concentrations. Quercetin, caffeic acid, and gallic acid were high in the aqueous extract, while the alcoholic extracts were rich in syringic acid, chlorogenic acid and naringenin. All the tested extracts have an appreciable antioxidant inhibiting effect with some slight variations except for beta-carotene test, reducing power ions, chelating assay where the extracts showed an activity higher than 50% compared to the aqueous extract. The methanolic extract showed a potential anticancer property against HePG2 cancer cell lines followed by the ethanol extract, then the water extract which showed a weak anticancer property. The *in vitro* and *in vivo* anticoagulant activities tests showed that the extracts prolonged significantly the activated partial thromboplastin time (aPTT), prothrombine time (TT) and thrombin time (PT) compared to the negative control. The oxidative stress induced by ZN in the rat showed a significant increase in serum ALT, AST and ALP activites ,level of creatinine, uric acid, urea, cholesterol, triglycerides, LDL, carcinoembryonic antigen, alpha-fetoprotein, TNFα, interleukin-6, malondialdehyde and a significant decrease in the content of serum TP, albumin, HDL, hepatic and renal TAC, CAT and GPx. Treatment of the stressed rats with the ethanolic extract resulted in improvement of all biochemical markers by restoring their values to normal. Histologically, the induced stress also caused significant tissue changes in the liver and kidneys. However, most of them were counteracted by ethanolic extract administration and the high dose was more effective than the low dose. In conclusion, the extracts of *c. speciosus* have a high potential biological activities and preventive and protective properties towards the liver and the kidneys these activities were linked to their high contents of polyphenols and flavonoids.

Keywords: *Costus speciusus* Phenolic Compounds, HPLC, Antioxidant, Anticoagulant, Anticancer, Zearalenone, Oxidative stress, Hepato-nephroprotectve.

Resumé

Costus speciosus est une plante qui appartient au genre *Costus*, c'est un aliment fonctionnel qui a été décrit en médecine prophétique et qui est largement utilisé dans les médicaments traditionnelle depuis plusieurs milliers d'années. Cette étude vise a établir le profil chimique des composés phénoliques des extraits aqueux, éthanoliques et méthanoliques de *C. speciosus* sous différents conditions d'extraction, à déterminer les composés phénoliques par HPLC, en évaluant, *in vitro* et *in vivo*, leurs activités antioxydantes, anticoagulantes et anticancéreuse. Le rôle protecteur de l'extrait méthanolique contre le stress oxydatif induit par la zéaralénone (ZN) dans le foie et les reins chez le rat a été aussi établi. Les résultats obtenus indiquent que: La teneur totale en polyphénols et flavonoïdes dans l'extrait éthanolique est plus élevée que dans les extraits méthanoliques et aqueux et augmente avec le temps d'extraction. L'analyse par HPLC a permis d'identifier 13 composés dans chaque extrait à des concentrations différentes. La quercétine, l'acide caféique et l'acide gallique sont les principaux composés de l'extrait aqueux, tandis que les extraits alcooliques sont riches en acide syringique, en acide chlorogénique et en naringénine. Tous les extraits testés ont un effet antioxydant significatif avec de légères variations sauf pour les tests de bêta-carotène, du pouvoir réducteur et chélateur où les extraits alcooliques ont montré une activité supérieure de 50% par rapport à l'extrait aqueux. Les extraits méthanoliques et éthanoliques ont montré une propriété anticancéreuse potentielle contre la ligné cellulaire HePG2, tandis que l'extrait aqueux a présenté une faible activité. Les tests des activités anticoagulantes, *in vitro et in vivo*, des extraits ont montré que les extraits prolongent de manière significative le temps de thromboplastine partielle activée (aPTT), le temps de prothrombine (TT) et le temps de thrombine (PT) par rapport au témoin négatif. Le stress oxydatif induit par la ZN chez le rat a montré une augmentation significative de l'activité del' ALT sérique, de l'AST et de l'ALP, de la teneur de la créatinine, de l'acide urique, de l'urée, du cholestérol, des triglycérides, du LDL, de l'antigène carcinoembryonnaire, de l'alpha-foetoprotéine, du TNFα, de l'interleukine-6, du malondialdéhyde et une diminution importante de la teneur en TP sérique, albumine, HDL, TAC hépatique et rénale, CAT et GPx. Le traitement des rats stressés avec l'extrait éthanolique a amélioré tous les marqueurs biochimiques en rétablissant leurs valeurs à la normal. Par ailleurs et sur le plan histologique, le stress induit a entrainé des modifications tissulaires importantes au niveau du foie et des reins. L'extrait éthanolique a exercé un effet préventif et protecteur du foie et des reins contre toute altération à la dose élevée.En conclusion, les extraits de *C. speciosus* possèdent un fort potentiel d'activités biologiques et des propriétés préventives et protectrices vis-à-vis du foie et des reins qui étaient liées à leurs teneurs élevées en polyphénols et des flavonoïdes.

Keywords: *Costus specious*, Polyphénols, Analyse HPLC, Antioxydantes, Anticoagulantes, Anticancéreuse, zéaralénone, stress oxydant, hepato-néphroprotecteur.