An-Najah National University

Faculty of Graduate Studies

Biochemical and Molecular Evaluation of the Plant *Ecballium elaterium* Extract Effects on *Escherichia coli*

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Dedication

This thesis is dedicated to my father Mr Jarrah Abdat whose strength inspired me to continue and never give up till reach. To my mother lady Nada Abdat who cultivated ambition and confidence inside me since childhood. To my beloved sisters, brothers, nephews and pretty nieces who made me discover that love is fuel. My precious family members, may Allah bless you and keep you.

To all the martyrs who died defending Al-Aqsa mosque and to all the prisoners in Israeli jails.

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الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Biochemical and Molecular Evaluation of the Plant *Ecballium elaterium* Extract Effects on *Escherichia coli*

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

E. coli	*Escherichia coli*
E. elaterium	*Ecballium elaterium*
Cuc	Cucurbitacin
ERIC	Enterobacterial repetitive intergenic consensus
TSI	Triple Sugar Iron test
PCR	Polymerase chain reaction
EMB	Eosin methylene blue
MHA	Mueller Hinton agar
SIM	Sulfide Indole Motility
NB	Nutrient broth
MR-VP	Methyl red-Voges Proskauer
IMViC	Indole production, Methyl red test, Voges-Proskauer test and Citrate utilization test
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
C. longa	*Curcuma longa*
M. azedarach	*Melia azedarach*
C. quinquefasciatus	*Culex quinquefasciatus*
RFLP	Restriction fragment length polymorphism
RAPD	Random amplification of polymorphic DNA
SDS-PAGE	Sodium dodecyl sulfate-polyacrilamide gel electrophoresis
R. stricta	*Rhazya stricta*
MIC	Minimal inhibitory concentration
TEMED	Tetra methyl ethylene diamine
APS	Amonuim per sulfate
Rf	Relative mobility
ATCC	American Type Culture Collection

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Abstract

Ecballium elaterium plant is traditionally used in several countries around the world for the treatment of various illnesses including jaundice and rhinosinusitis. The present study was conducted to investigate the antimicrobial activity of fruit and leaf ethanolic extracts of *E. elateruim* on clinical and reference strains of *E. coli*. Microbroth dilution method indicated that ethanolic leaf and fruit extracts of *E. elaterium* exhibited the same degree of antimicrobial activity against both tested *E. coli* strains with an MIC value of 25mg/ml. The effect of *E. elaterium* ethanolic extracts on these strains was also examined at the molecular level using ERIC-PCR. SDS-PAGE technique was also carried out to examine gene expression of total cellular proteins under the same conditions. Results of ERIC-PCR and SDS-PAGE showed alterations in DNA and protein profiles of both tested *E. coli* strains treated with fruit and leaf extracts compared with untreated control. The alterations ranged between decreased or increased intensity of some bands, absence or appearance of new amplified

fragments. Among the changes that occurred in ERIC-PCR profile, an amplified fragment with amplicon size 450-bp disappeared after 6 and 24h from reference strain treated with 6 mg/ml fruit extract compared with untreated control. The band which had an amplicon size of about 550-bp was faint after 2h in clinical strain treated with 25 mg/ml ethanolic leaf extract in comparison with untreated control. In addition, after 6h treatment of reference *E. coli* with 25 mg/ml leaf extract appearance of extra new bands at Rf values of approximately 0.45 and 0.47 was recorded compared with untreated control in SDS-PAGE profile. SDS-PAGE profile also revealed the increase in the intensity of bands at approximate Rf values of 0.15 and 0.7 compared after 6 h treatment of clinical *E. coli* with 10 and 6 mg/ml fruit extract with untreated control. Moreover, increased concentrations of *E. elaterium* extracts and increased time intervals seems to yield a more profound increase in total protein concentrations in both tested *E. coli* strains. Such findings strongly indicate the genotoxic effects of *E. elaterium* extracts on both *E. coli* strains. The findings draw attention to the unsafe improper use of *E. elaterium* extracts in folkloric medicine and also point out the capability of using *E. elaterium* to treat *E. coli* infections. More studies are required to find out the exact mechanisms responsible for the observed genotoxicity.

Chapter One
Introduction

1.1. General Background

Plants produce a diverse range of bioactive molecules, making them rich sources of different types of medicines. Bioactive compounds usually extracted from plants are used as medicines, food additives, dyes, insecticides, cosmetics, perfumes and fine chemicals. In some countries, 80% of the population are depending on medicinal plants to maintain their health and to cure their diseases (Hajar and Gumgumjee, 2014).

The plant *Ecballium elaterium* (*E. elaterium*), bears the common name squirting cucumber, is a weed which belongs to Cucurbitaceae family. Its detailed classification is shown in table 1.1. It is perennial, fleshy, rough hairy with stems 30 - 100 cm long. The flowers are greenish-yellow and the fruit is large juicy berry, 3 - 4 cm, ovate-oblong, detaching itself explosively at maturity scattering seeds and juice and when unripe of a pale green color and covered with numerous hairs. It is a plant indigenous to the Mediterranean countries and cultivated in central Europe and England. This plant grows wild in many places including the roadsides and cultivated areas (Abu-Irmeileh, 1982; Brouzas *et al*., 2012).

Bioactive compounds of *E. elaterium* juice have been reported to possess different pharmacological activities for example: purgatives, analgesics, hemorrhoids, varicose veins, and nose bleeding so a fresh juice is applied locally to treat these diseases (Lavi and Willner, 1985; Yesilada *et al*.,

1988; Greige-Gerges *et al*., 2007; Jaradat *et al*., 2012). The fruit of this plant is poisonous, and it was used in the ancient world as an abortifacient (Jaradat *et al*., 2012). Nowadays, people in different countries are using the juice in the treatment of acute and chronic jaundice, rhinosinusitis, sinusitis, and other ailments of inflammations (Salhab *et al*., 1986; Yesilada *et al*., 1988; Sezik, 1997; Greige-Gerges *et al*., 2007; Salhab, 2013). Other traditional uses include the treatment of fever, cancer, liver cirrhosis, constipation, hypertension, rheumatic disease (Sezik *et al*., 2001), and malaria in humans (di Tizio *et al*., 2012).

The fruit juice is known to be rich in cucurbitacins, phenolics and glycosylated compounds, which have numerous bioactivities (Rao *et al*, 1974; Greige-Gerges *et al*., 2007; Jaradat *et al*., 2012). Cucurbitacins are tetracyclic triterpenoids A, E, D, I and are of interest medicinally because of their cytotoxic, antitumor and anti-jaundice properties (Salhab, 2013). Cucurbitacin E tested on peripheral human lymphocytes and showed an immunomodulatory activity (Attard *et al*., 2005), and had a cytotoxic effect against prostate and breast cancer cells in vitro (Attard and Cuschieri, 2004).

Cucurbitacin I showed cytotoxic effects against four human cancer cell lines and significant activity against HIV replication in H9 lymphocyte cells (Wu *et al*., 2004). Cucurbitacin B has a potent antiproliferative effect on breast cancer (Wakimoto *et al*., 2008). Cucurbitacins E and I showed antitumor activities on different carcinoma cell lines (Saker *et al*., 2012).

Freeze-dried aqueous extracts of *E. elaterium* fruit were also reported to haves cytotoxic effects on gastric and esophageal cancer cell lines (Bohlooli *et al*., 2012). A recent study showed that *E. elaterium* juice extract had antioxidant and hepatoprotective activity against paracetamol induced hepatotoxicity in male albino rats (Elmhdwi *et al*., 2014).

In addition, the leaves, fruits, and flowers of *E. elaterium* are rich with flavonoids (phytomelin), which may have antioxidant, anti-inflammatory, anticarcinogen, antithrombotic, cytoprotective and vasoprotective activities (El-Haci and Bekkara, 2011; Jaradat *et al*., 2012). Furthermore, *Ecballium* extract has antimicrobial effect (Oskay and Sari, 2007; Oskay *et al*., 2009; Adwan *et al*., 2011; El-Haci and Bekkara, 2011; Abbassi *et al*., 2014).

Table 1.1. Classification of *Ecballium elaterium* (Biology-Flora):

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Violales
Family	Cucurbitaceae
Genus	Ecballium
Species	*Ecballium elaterium*

1.2. Literature Review

1.2.1. Antimicrobial activity

Ecballium elaterium extract showed antimicrobial activity against the food pathogens at concentrations ranging from 0.004 to 2.5 mg/ml (Abbassi *et al*., 2014). Ethanolic extract of *E. elaterium* showed a high antibacterial activity against different bacterial species (Oskay and Sari, 2007; Oskay *et al*., 2009). Roots, fruits and flowers of *Ecballium elaterium* extracted using several different solvents showed antimicrobial activity against some of the representative food-borne pathogenic bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Escherichia coli* (*E. coli* 25922). *Pseudomonas aeruginosa* and *E. coli* (25922) were found to be the most resistant to *E. elaterium* extracts (Abbassi *et al*., 2014). Fungicidal activity of the leaf extract and/or fruit juice was also studied. Leaves and fruits (2:1, v/v) Ethanolic extract of *E. elaterium* were among the 10 plant extracts out of 19 tested that showed anti-candidal activity using agar well diffusion assay (Oskay and Sari, 2007). Adwan *et al*. (2011) also studied the activity of *E. elaterium* ethanolic fruit extract against *Candida albicans* strains and reported an MIC value between 0.048 8 to 6.250 0 mg/mL. In another study, The ethyl acetate extract from the ethanolic extract of the whole *E. elaterium* plant exhibited significant level of activity against *Aspergillus orizea* and *Aspergillus niger*, moderate activity against *Penicillium* sp. and

Saccharomyces cerevisiae was completely resistant against the extract (El Sayed and Badr, 2012).

1.2.2. Biochemical and molecular evaluation of genetic effects of plant extracts

Many studies revealed that some plants extracts can modify the genotoxicity of some other known potent mutagens by acting as inhibitors of mutagenesis and/or clastogenesis or even they can be themselves genotoxic agents (Sarkar *et al.*, 1996).

Genotoxic effects of *E. elaterium* juice based on Allium test was studied. Inhibition of root growth and mitodepressive effects on cell division in *Allium cepa* root tip cells was reported after exposure to *E. elaterium* fruit juice. It was also reported that exposure to fruit juice significantly increased the dose-dependent frequency of chromosome aberrations in root tip cells and micronucleus formations. There were no dividing cells in the undiluted *E. elaterium* fruit juice treated group, but there were pyknotic/apoptotic cells with varying frequency (Celik and Aslantürk, 2009). *E. elaterium* juice was also reported to have a mutagenic and a cytotoxic effects in human peripheral lymphocytes (Rencüzogullari *et al*., 2006). Moreover, *E. elaterium* fruit extract increased comet tail moments in human lymphocytes in a manner that is not dose-dependent. This increase indicates the ability of the fruit to cause strand breakage in DNA. On the other hand, the fruit failed to produce reverse mutations in two strains of *Salmonella typhimurium* TA98 and TA100 (Basaran *et al*., 1996).

Furthermore, the fruit juice of *E. elaterium* significantly increased the formation of micronuclei in mice peripheral red blood cells (RBCs) in a dose-dependent manner. The juice also resulted in a dose-dependent increase in DNA fragmentation in mice liver DNA only after 1h of administration (Shabbar and Maslat, 2006).

The genetic effects of acetone and chloroform *Curcuma longa* and *Melia azedarach* leaves extract on Culex *quinquefasciatus* larvae were evaluated by random amplification of polymorphic DNA (RAPD – PCR) assay to assess the level of DNA damage at different concentrations (Lalrotluanga *et al*., 2011). DNA changes in comparison with untreated control larvae were evaluated. It was observed that the number of bands increased in the *M. azedarach* (acetone extracts) treatment whereas the number of bands decreased in *C. longa* acetone and chloroform treatments. Further, the results showed that DNA strand breakage was more in the larvae of *C. quinquefasciatus* treated with plant extracts (Lalrotluanga *et al*., 2011).

The biochemical and molecular evaluation of genetic effects of aqueous *Rhazya stricta* (Decne) leaves extract on three different bacterial species included *E. coli*, *Aeromonas hydrophila* and *Staphylococcus aureus* was evaluated by restriction fragment length polymorphism (RFLP), RAPD analysis and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique. The study results based on observations by scanning electron microscopy showed bacterial cell damage after the exposure and treatment with plant extracts. Results of RFLP analysis of

the plasmid DNA and RAPD analysis of the genomic DNA extracted from treated and untreated bacterial strains with *R. stricta* leaf extract demonstrated polymorphic band pattern for most treated microbes compared with the wild type (untreated) strain. Concerning gene expression under the same conditions, total protein contents of the three treated bacteria showed significantly gradual increase in all of the treatment doses compared to control. In addition, the SDS-PAGE of the bacterial cellular proteins resulted in the induction of some protein bands under the treatment conditions (El-Tarras *et al*., 2013).

In other study, the genotoxic effect of the *R. stricta* leaf aqueous extract as antifungal agent was demonstrated against *Saccharomyces cerevicae* auxotrophic mutant. The results showed that the extract had potent lethal and mutagenic activities. It was found that with increased concentration of extract or time of exposure, the survival percentage decreased and auxotrophic mutants increased. Most auxotrophic mutants were amino acid requiring mutants, less abundant were the nitrogen bases requiring ones and come last the vitamins requiring mutants (Baeshin *et al*., 2005).

Moreover, the genotoxic and biochemical effect of the *R. stricta* leaf aqueous extract as antifungal agent was demonstrated against *Aspergillus terreus*. Survival assay, quantities of DNA and total protein were further subjected to RAPD and SDS-PAGR tests. It was found that as concentration of extract or time of exposure increased, survival percentage decreased and auxotrophic mutants increased. Most auxotrophic mutants

were amino acid requiring mutants. Quantity of DNA of each mutant was significantly less than in corresponding control and so on for total protein. The RAPD results demonstrated polymorphic numbers of genetic bands, which were the electrophoretic products of PCR for all mutants compared with the wild type strain. SDS-PAGE results expressed a polymorphism of protein bands as well. All these results strongly point out the mutagenicity of the leaves extract of *R. stricta* (Baeshin *et al*., 2008).

Abulyazid *et al*. (2013) evaluated the effect of *Lawsonia inermis* leaves on proteins of clinical *E. coli* strain using SDS-PAGE. The protein pattern of *E. coli* treated with various concentrations of *L. inermis* leaves powder (1%, 2%, 3%, 4%, 5%, 10% and 15%) showed the disappearance of several bands when compared with untreated *E. coli*. Most bands disappeared upon higher concentrations treatment. Effect of *L. inermis* leaves powder on amylase enzyme of treated *E. coli* was also assessed using SDS-PAGE and activity staining. At all doses used, the results showed the disappearance of 2 bands out of three in treated *E. coli* compared with un-treated controls.

In another study, methanolic extract of *Melastoma candidum* flowers was evaluated for its effects on protein expression profile of *E. coli* ATCC 35218 and *S. aureus* ATCC 6538. The proteins of bacterial strains, exposed to the extract for different time intervals (5, 30, 60 and 90 min), were extracted and separated using SDS-PAGE. Reduced expression of four proteins in case of *E. coli* ATCC 35218, and only one protein in case

of *S. aureus* ATCC 6538 was identified from the SDS-PAGE profiles. These proteins were analyzed using mass spectrometry technique to recognize their identity and characteristics. Analysis revealed that the differentially expressed proteins in *M. candidum* treated *E.coli* are involved in glucose and pyruvate metabolism, cabability of *E. coli* in surviving in acidic environments by preventing protons influx from the surrounding, and protein elongation process. The pore-forming exotoxin α-hemolysin was down-regulated upon *M. candidum* treatment of *S. aureus* (Wong *et al*., 2014).

1.2.3. Methods for evaluating the gentoxic activity of plant products

Several tests were used to evaluate the genotoxicity and anti-genotoxicity of plants or their extracts. Examples of tests used include Comet assay (single-cell gel electrophoresis) (Grollino *et al*., 2017; Cigerci *et al*., 2016), Ames test (bacterial mutagenesis test) (Park *et al*., 2017), micronucleus assay (de Araújo *et al*., 2018; Park *et al*., 2017; Ojo *et al*., 2017) and DNA-based assays such as RAPD-PCR (Hajar and Gumgumjee, 2014; Lalrotluanga *et al*., 2011; Qari and Abdel-Fattah, 2017; El-Tarras *et al*., 2013; Cigerci *et al*., 2016).

Ames test is based on the ability of the tested substance to induce a reverse mutation in an auxotrophic mutant. Although this test is laborious, but it can detect point and frame shift mutations, as well as base substitutions sensitively. On the other hand, Comet assay detects DNA damage in eukaryotic cells via employing the fact that the firm association between

DNA and matrix proteins is disrupted when DNA is damaged. Thus, if an electric field is applied, the loosely attached damaged DNA travels toward the positive anode, while the firmly attached intact DNA fragments do not migrate. DNA migration appears as a comet. The extent of DNA damage is correlated to the size and shape of the comet as well as the distribution of DNA within it. Comet assay detects with high sensitivity and low costs single- and double-strand breaks, alkali labile sites, abasic sites, oxidative damage, and cross-linking of DNA with DNA, protein, or drug (Ranganatha *et al*., 2016; Kumari *et al*., 2008).

Micronucleus test is one of the most reliably used tests for assessing the genotoxicity. It detects the formation of micronuclei in eukaryotic cells either in vitro or in vivo. Micronuclei are formed due to anaphase failure during mitosis or meiosis. Anaphase failure results when a chromosome lacking the centromere region (acentric) can not bind to the mitotic spindle and thus cannot be pulled into the other pole of the cell. As a result, these acentric chromosomes, either entirely or portions of them, form a small extra nucleus (Ranganatha *et al*., 2016).

DNA-based assays such as RAPD-PCR are the most widely used tools for assessment of the genetic variation and detection of DNA damage (Qari and Abdel-Fattah, 2017). Moreover, PCR-based method is regarded to be one of the most reliable methods used for detection of DNA damage (Kumari *et al*., 2008). Polymerase progression through the DNA template is halted by presence of damage (Mutlu, 2012; Kumari *et al*., 2008).

It is important to note that classifying a particular substance as genotoxic cannot be made based only on a single test. Thus, it is recommended to use a combination of in vitro and in vivo tests (Poivre *et al*., 2017; Ranganatha *et al*., 2016).

1.3. Aims of the Study

This study aimed to:

1. Explore the antimicrobial activity of ethanolic fruit and leaf extracts of *E. elaterium* using broth microdilution method.

2. Evaluate the genotoxic effect of these extracts on *E. coli* using enterobacterial repetitive intergenic consensus ERIC-PCR and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Chapter Two
Materials and Methods

2.1. Plant Material: Collection and Extract Preparation

The ripe fruits and mature leaves of *E. elaterium* were collected from Nablus, West Bank-Palestine, during August and September 2017. Ethanolic fruit and leaf extracts of *E. elaterium* were prepared by cold maceration method. The collected fruits and leaves were washed with water to remove soil and dust particles, then they were dried. Exposure to light was avoided to prevent the loss of effective ingredients. After that, the fresh fruits (20g) were homogenized finely using blender after addition 80 ml of absolute ethanol. The dried leaves were powdered finely using blender. Approximately 30-40g of dried leaf material was mixed thoroughly with magnetic stirrer in 150 ml of 80% ethanol. Both ethanol-fruit mixture and ethanol-leaf mixture were left on shaker at room temperature for 48h. The mixtures then were filtered using muslin cloth to remove large particle and insoluble materials. After that, to remove fine particles mixtures were centrifuged at 5,000 rpm for 15 min at 4°C. Then, the extracts were dried and concentrated using rotary evaporator at 50°C, and freeze dryer (lyophilizer). The dried extract obtained was stored at 4°C. Before starting the experiments, plant extracts were dissolved in 10% Dimethyl sulfoxide (DMSO) to obtain a concentration of 200 mg/ml and stored at 4^{o}C for further assays.

2.2. Bacterial Isolates Collection

Two strains of *E. coli* were used in this study, *E. coli* ATCC 25922 and a clinical strain. *E. coli* ATCC 25922 was kindly provided by Mr. N. R. Jarrar Department of Biology Biotechnology, Faculty of Science, An-Najah National University. The clinical strain was isolated from urine sample obtained from Rafidia Hospital-Nablus, West Bank-Palestine.

Both strains were identified depending on morphological and biochemical tests in the Microbiology laboratory at An-Najah National University-Nablus, Palestine. The isolates were cultured on EMB agar and MacConkey agar. Gram stain and other biochemical tests were carried out such as IMViC Tests (Indole production, Methyl red test, Voges-Proskauer test and Citrate utilization) and H_2S production.

2.3. Media Preparation

2.3.1. Eosin methylene blue (EMB) agar

Following the instructions of the manufacturer, EMB (Himedia, India) was prepared. To about 96.3 ml deionized water (d. H_2O) present in 250 ml flask, 3.75 g EMB agar were added. The flask content was then heated and stirred until agar dissolved. The content in the flask was then covered with a piece of aluminum foil that overlies a plug of cotton. Then autoclaved at 121ºC for 15 min, cooled to 50ºC. After that, about each 25 ml of the melted EMB agar was poured into sterile Petri dish. The dishes were then

left overnight at room temperature, and in the next day stored in refrigerator at 4ºC.

2.3.2. MacConkey agar

The manufacurer's instructions were followed to prepare MacConkey agar (Himedia, India). To a 250ml flask containing 95 ml deionized water, 5g MacConkey agar were added. To dissolve the agar, the flask content was heated with stirring. After plugging with cotton and covering with a piece of aluminum foil, the content in the flask was autoclaved at 121ºC for 15 min. Then allowed to cool to nearly 50ºC. After that, about each 25 ml of the melted MacConkey agar was poured into sterile Petri dish. The dishes were then left overnight at room temperature, and in the next day stored in refrigerator at 4ºC.

2.3.3. Sulfide indole motility (SIM) medium

SIM medium (Acumedia, USA) was prepared according to the manufacturer's instructions. A 250 ml flask containing 97 ml deionized water and 3 g of SIM agar were heated and stirred until dissolved. Medium was dispensed into tubes to give a depth of about 7-10 cm. The tubes were plugged tightly with cotton then covered with a piece of aluminum foil, then autoclaved at 121°C for 15 min, allowed to cool by leaving the tubes on a rack to form deep agar, and then stored in refrigerator at 4°C.

2.3.4. Methyl red-voges proskauer (MR-VP)

MR-VP medium (Himedia, India) was prepared according to the manufacturer's instructions. A 250 ml flask containing 98.3 ml deionized water and 1.7 g of MR-VP medium were mixed thoroughly. Then, MR-VP broth (5 ml) was dispensed into tubes, plugged with cotton and covered with aluminum foil. Then, the tubes were autoclaved at 121°C for 15 min, allowed to cool and stored in refrigerator at 4°C.

2.3.5. Simmons citrate agar

Simmons citrate agar (Acumedia, USA) was prepared according to the manufacturer's instructions. A 250 ml flask containing 97.8 ml deionized water and 2.25 g of Simmons citrate agar were heated and stirred until dissolved. Ten ml of Simmons citrate agar was dispensed into tubes. The tubes were then plugged tightly with a piece of cotton and covered with aluminum foil. Then, the tubes were autoclaved at 121°C for 15 min. After that, the tubes were allowed to cool in a slant position and later were stored in refrigerator at 4°C.

2.3.6. Triple sugar iron (TSI) agar

TSI agar (Acumedia, USA) was prepared according to the manufacturer's instructions. A 250 ml flask containing 93.5 ml deionized water and 6.5 g of TSI agar were mixed thoroughly and heated to dissolve. Ten ml of Triple sugar iron medium was dispensed into tubes, plugged with a piece of cotton which then covered with aluminum foil. The tubes were autoclaved

at 121°C for 15 min, and then the tubes were left to cool and solidify in a slant position. Finally, the tubes were stored in refrigerator at 4°C.

2.3.7. Mueller-Hinton agar (MHA)

Mueller-Hinton agar (Acumedia, USA) was prepared according to the manufacturer's instructions. A 1000 ml flask containing 336.7 ml deionized water and 13.3 g Mueller-Hinton agar were mixed thoroughly, heated and stirred until the agar dissolved. The flask was then plugged tightly with a piece of cotton and covered with a piece of aluminum foil. Then the media was autoclaved at 121°C for 15 min. After that, media was allowed to cool to about 50°C, then a total of 25 ml of Mueller-Hinton agar was poured into sterile Petri dishes. The Petri dishes were left to cool at room temperature and then stored in refrigerator at 4°C.

2.3.8. Nutrient broth (NB) media

Nutrient broth (Acumedia, USA) was prepared according to manufacturer's instructions. A 2 L flask containing 793.6 ml deionized water and 6.4 g nutrient broth were mixed to dissolve. The flask was then plugged with a piece of cotton and covered with aluminum foil. Then, autoclaved at 121°C for 15 min, allowed to cool then stored in refrigerator at 4°C.

2.4. *Ecsherichia coli* Identification

2.4.1. Gram staining

Gram staining was performed to distinguish Gram-positive bacteria from Gram-negative bacteria. A thin smear of bacteria was made on a clean glass slide by picking the isolates from marked colonies after 24 h incubation on Meuller-Hinton agar plate and mixed with a drop of distilled water. The smear was heat fixed by passing through a flame 2 or 3 times. Care was taken to avoid air bubbles formation and overheating to prevent distortions of the glass slide. After cooling, the slide was flooded with primary stain (crystal violet) and left for one minute then washed with tap water. Gram's iodine solution was then added for one minute and again washed off with tap water, decolorized with acetone alcohol from 10 to 20 seconds and then again washed with tap water. Finally, the slide was flooded with a counter stain (safranin) for 1 minute then washed with tap water and let to dry. The smear was observed under 100x objective lens of the light microscope (Cappuccino and Sherman, 1996).

2.4.2. Motility test

In SIM deep agar tube, the tested bacteria picked up with a sterile needle, inoculated by stabbing the bottom of SIM tube with a single in-and-out motion in the same line, then the tube was incubated at 37 °C for 18-24 h. Growth of non-motile organisms occurs only along the line of inoculation, while growth of motile organisms occurs throughout the tube (Cappuccino and Sherman, 1996).

2.4.3. Indole Production

The same SIM tube used for motility test and inoculated by mean of stab method was also used to detect the presence of tryptophanase enzyme that catabolizes tryptophan producing indole. This was carried out by adding 10 drops of Kovac's reagent after 24 h of inoculation. Incase of indole positive bacteria, Kovac's reagent will combine with indole producing a red color at the top of the agar tube. The absence of red color change after the addition of Kovac's reagent, indicates an indole negative bacteria (Cappuccino and Sherman, 1996).

2.4.4. MR-VP test

MRVP broth was inoculated with the tested bacterium, then incubated at 37°C for 24 h. The broth was then divided into two tubes one for MR test and the other for VP test. MR test was carried out by the addition of 5 drops of methyl red indicator, while VP test was carried out by adding 10 drops of Barritt’s reagent A, the culture was shaken, then immediately 10 drops of Barritt’s reagent B was added, then culture was shaken and re-shaken after every 3-4 min. The appearance of immediate a red color after the addition of MR reagent indicates a MR positive result, while no development of a pink-red color within 10-60 minutes was indicative of a negative VP reaction (Cappuccino and Sherman, 1996).

2.4.5. Citrate utilization test

Citrate utilization test was carried out by stabbing the butt and streaking the slant of Simmons citrate agar. The tube was then incubated at 37°C for 24 h. Citrate positive result was indicated by either change in the color of the medium from green to blue or growth of bacteria in the medium or both. Lack of growth on citrate medium and lack of color change indicates a citrate negative result (Cappuccino and Sherman, 1996).

2.4.6. Triple sugar iron test

This test was carried out by stabbing the butt and streaking the slant of TSI agar tube by a zig-zag pattern. The tube was then incubated at 37°C for 24 h. *Escherichia coli* sub-cultured on TSI agar showed the whole media had a yellow color due to fermentation of glucose and lactose (acid/acid), splitting of the agar (gas production), and no blackening of the agar (No H_2S production) (Cappuccino and Sherman, 1996).

2.5. Antibiotic Resistance

2.5.1. Disk diffusion method

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method (CLSI, 2015). *E. coli* strains were examined for resistance to Amikacin (AK) 30 µg, cefuroxime (CXM) 30 µg, tetracycline (TE) 30 µg, kanamycin (K) 30 µg, meropenem (MEM) 10 µg, nalidixic acid (NA) 30 µg, norfloxacin (NOR) 10 µg, ceftriaxone (CRO) 30 µg, and trimethoprim/

sulfamethoxazole (SXT) 1.25/23.75 µg. Two to three of 24 h-old colonies of each *E. coli* strain were sub-cultured into nutrient broth media and incubated for 4-5 h with frequent shaking. Mueller Hinton agar (MHA) plates were swabbed with a 4-5 h-old culture of the bacterial strains; antibiotic disks were placed on the MHA plates containing the inoculum. The plates were incubated at 37ºC for 24 h. Following incubation, diameter of inhibition zones were measured in millimeters. Interpretation of inhibition zones was determined in accordance with procedures of the Clinical and Laboratory Standard Institute (CLSI, 2015).

2.5.2. Minimal inhibitory concentration (MIC)

2.5.2.1. Preparation of McFarland turbidity standard No. 0.5

McFarland 0.5 turbidity standard was prepared by adding 50 µl of a 1.175% (w/v) barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$) solution to 9.95 ml of 1% (v/v) sulfuric acid. McFarland standard tube was then sealed with parafilm to prevent evaporation and stored in the dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1-cm light path cuvette; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm and water as a blank standard will be between 0.08 to 0.13. The 0.5 McFarland standard was vigorously agitated on a vortex mixer before use. As with the barium sulfate standards, a 0.5 McFarland Standard is comparable to a bacterial suspension of 1.5 X 10^8 colony-forming units (CFU)/ml (Andrews, 2006).

2.5.2.2. Preparation of inoculum for MIC determination

Three to four isolated overnight cultured colonies were transferred to a 5-10 ml of sterile saline. The bacterial suspension was compared to the 0.5 McFarland standard against a sheet of white paper on which sharp black lines drawn. The bacterial suspension was adjusted the proper density as the McFarland 0.5 by adding sterile saline or more bacterial growth. Then bacterial suspension was diluted with normal saline to obtain 10^6 CFU/ml. (CLSI, 2015)

2.5.2.3. Determination of MIC by broth microdilution method

MIC of plant extracts was determined by the microbroth dilution method in sterile 96- wells microtiter plates according to the CLSI (CLSI, 2015). The plant extract (200 mg/ml of 10% DMSO) and 10% DMSO (negative control) were two fold-serially diluted in nutrient broth directly in the wells of the plates in a final volume of 100µL. The final concentrations of plant extracts achieved after dilution were 100, 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 mg/ml. The highest percentage of DMSO being used in the wells was 5 %. After that, a bacterial inoculum size of 10^4 CFU/ml was added to each well. Negative control wells containing either 100µL NB only, or 100µL DMSO with bacterial inoculum, or plant extracts and nutrient broth without bacteria were included in this experiment. Each plant extract was run in duplicate. The microtiter plates were then covered and incubated at 37°C for 24 h. The MIC was taken as the minimum concentration of the

dilutions that inhibited the growth of the test microorganism. MIC was determined by visual inspection.

2.6. Evaluation of the Genotoxic Potential of *Ecballium elaterium* Ethanolic Extracts on DNA extracts of *Escherichia coli*

2.6.1. Inoculation of *Escherichia coli*

From 24h old colonies of both *E. coli* strains plated on Meuller-Hinton agar media, three to four colonies were sub-cultured under sterile conditions into bottles containing 25 ml nutrient broth for 2h with continuous shaking. In the next day, constant volumes of nutrient broth (25 ml) were inoculated with 3.5 ml of both *E. coli* strains (from the previously prepared bacterial suspension) under sterile conditions using laminar flow, a sterile pipette and ethanol 70% and then incubated at 37°C for 1h with continuous shaking. Then different concentrations of ethanolic leaf extract and ethanolic fruit extract of *E. elaterium* (25mg/ml, 10mg/ml and 6 mg/ml of nutrient broth) were added to bacterial broth culture. Samples of 3 and 6 ml size were taken from the growth after 2h, 6h, and 24h, centrifuged for 10 minutes at 14.000 rpm, then the supernatant was discarded and the pellet was saved at -20°C. The 3 ml sample pellet was used to isolate bacterial DNA for ERIC-PCR and the 6 ml sample pellet to isolate bacterial protein for SDS-PAGE. A broth sub-cultured with *E. coli* strain and treated with 10% Dimethyl sulfoxide (DMSO) was used as a negative control. This experiment with the subsequent DNA and protein assays were carried out

in duplicate. This method is the same used by Abulyazid *et al*. (2013) with some modifications.

2.6.2. DNA extraction

Escherichia coli DNA genome was prepared for PCR according to method described previously (Adwan *et al*., 2013). Each frozen bacterial sample pellet was re-suspended in 750µl of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1mM EDTA [pH 8]), centrifuged for 5 minutes at 14.000 rpm, then the supernatant was discarded, this step was repeated twice. After that, the pellet was re-suspended in 350µl of sterile distilled H_2O, boiled for 15 min, the mixture was incubated on ice for 5 min. The debris was pelleted by centrifugation at 14.000 rpm for 5 min, and the supernatant was transferred into a new eppendorf tube. Equal volume of chloroform was added to the supernatant, centrifuged for 10 minutes at 4.000 rpm. The uppermost layer of supernatant was transferred into new eppendorf tube. DNA concentration was determined using nanodrop spectrophotometer (Genova Nano, Jenway). The samples were stored at -20ºC for further DNA analysis.

2.6.3. ERIC-PCR assay

Enterobacterial repetitive intergenic consensus PCR was performed using primer ERIC1: 5`-ATG TAA GCT CCT GGG GAT TCA C-3` and primer ERIC2 : 5`-AAG TAA GTG ACT GGG GTG AGC G-3`, described previously (Meacham *et al*., 2003). Each PCR reaction mix (25 µL) was performed using 12.5 µL of PCR premix (ReadyMix™ Taq PCR Reaction

Mix with $MgCl_2$, Sigma), 0.8 μM of each primer, 52 ng DNA template, concentration of dNTPs was modified to 0.4 mM, $MgCl_2$ to 3 mM and Taq DNA polymerase to 1.5 U. The reaction mixture was given a short spin for complete mixing of the components. DNA amplification was then carried out using the thermal cycler (Mastercycler personal, Eppendorf) according to the following thermal conditions: initial denaturation for 3 min at 94ºC was followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 40ºC for 1 min and extension at 72ºC for 2 min, with a final extension step at 72ºC for 5 min (Adwan *et al*., 2016).

2.6.4. Agarose gel electrophoresis

PCR products were analyzed by 2% agarose gels. A DNA ladder of 100-bp subjected to electrophoresis on the same gel including the ERIC-PCR amplified products. Electrophoresis was run using 1X TAE working buffer (50x: 242g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA [pH 8.0]) for 1.5 h at 80 V, then the gel was stained with Ethidium bromide. The amplified pattern was visualized on a UV trans-illuminator and photographed. The changes occurring in ERIC banding pattern profiles following plant extract treatments including, variations in band intensity as well as gain or loss of bands, were taken into consideration (Lalrotluanga *et al*., 2011; Atienzar *et al*., 2002).

2.7. Protein Assay

2.7.1. Protein isolation

To each previously taken 6 ml *E. coli* sample, 300 µl of lysis buffer (25 mM Tris-HCL, 100 mM NaCl [pH 8]) were added then vortexed. The samples were then left on ice for 15-20 minutes. After that, each sample was sonicated using sonicator (Q55 Sonicator, QSonica, CT, U.S.A.) at 35% power for 10 minutes divided as 10 seconds on and 10 seconds off. This process was carried out on ice. After that, samples were centrifuged for 10 minutes at 16100Xg speed. The resulting supernatant was then aspirated and stored at -20°C.

2.7.2. Determination of protein concentration

2.7.2.1. Preparation of Bradford reagent

Bradford reagent was prepared by dissolving 0.1 g Commassie Brilliant Blue G-250 (CBB-G250) in 50 ml ethanol (95%). Then 100 ml of phosphoric acid (85%) were added. Finally, the solution was completed to 1 L with d. H_2O The dye solution was filtrated before use and stored at 4°C.

2.7.2.2. Bradford method

The protein content in the culture filtrates was estimated by the dye binding method of (Bradford, 1976). From each protein sample, 5 µl were added to 745 µl d. H_2O. To this mixture, 750 µl of Bradford reagent was added.

After that, the samples were vortexed and left at room temperature for 10 minutes. Next, the absorbance at 595 nm was measured using spectrophotometer (JENWAY 6405 UV/Vis. Spectrophotometer) for each sample. The protein concentrations were calculated using bovine serum albumin (BSA) as standard.

2.7.3. SDS-PAGE procedure

Total extracted protein was separated by denatured polyacrylamide gel electrophoresis (SDS-PAGE) with 8% stacking gel and 12% separating gel according to Baeshin *et al*. (2008). Vertical slab gel electrophoresis was performed using the ENDURO PAGE System (E2010-P0, Labnet Int., Inc., NJ, U.S.A.) that can be used to run two gels at the same time. The assembly of the glass plate cassettes (10 X 10 cm, 1 mm thick) and the process of gel casting were done according to instruction manual provided with the apparatus. The preparation of the separating and stacking gels was started after the cassettes were properly assembled and mounted.

Preparation of 12 % separating gel and 8 % stacking gel polymerizing solutions was carried out just before use by mixing the following volumes of stock solutions in the provided order as given in **Table 2.1**. The separating gel solution was first prepared with the TEMED added immediately before casting the gel into the glass assembly from the edge of one of the spacers until the required height of the solution was obtained. After that, the liquid gel was covered with a thin layer of distilled water (about 0.5 ml), without disturbing the gel surface, to have a regular

interface between the separating gel and the stacking gel. The gel was then left to polymerize at room temperature for at least 30 minutes. Following polymerization, filter paper was used for complete elimination of the layer of water without disturbing the gel surface. The stacking gel was then poured on the top of the separating gel and the comb was inserted into the layer of the stacking gel without trapping air bubbles under the teeth of the comb. The gel was then allowed to polymerize for around 30 minutes. The comb was removed carefully after the gel was polymerized, and the wells were washed with distilled water and filled with electrode buffer. At this point, the gel cassettes were removed from the casting stand, mounted and clamped onto the running frame with the notched glass plate of each cassette facing inside.

The protein samples to be tested were diluted 3:1 with the 4X sample buffer (3 parts sample and 1 part sample buffer), to have the samples in 62.5 mM Tris-HCl buffer, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue. The samples were then boiled at 100 ºC for 5-10 minutes. Afterwards, protein samples were loaded using a 25 µl Hamilton syringe.

After loading the samples, the compartment created by the running frame and the cassettes was filled with the running buffer (25 mM Tris, 192 mM Glycine, pH 8.3) and the system was checked for leakage. The outer tank was also filled with the running buffer. Thereafter, the running frame was inserted into the outer tank, the safety cover was replaced and the leads

were plugged into the electrophoresis power supply (PS 251-2, Sigma-Aldrich techware). The power supply was adjusted to give a constant voltage of 85 V at the beginning and elevated up to 200 V at the end of the run that took a total of about 1.5 h.

The power supply was switched off, when the dye front is just 0.5 cm from the lower end of the glass plates, the running frame was taken out and the buffer was removed from the upper buffer compartment. Afterwards, the clamps were detached and the cassettes were removed from the running frame. To gain access to the gels in the cassette, the glass plates were pried apart using a spatula taking care not to chip the edges of the glass plates. The left-top corner of each gel was cut to indicate the order of wells.

Table 2.1: Formulations for SDS-PAGE separating and stacking gels

	Separating Gel	**Stacking Gel**
Monomer Concentration	**12 %**	**8 %**
Acrylamide/bis	12.0 ml	4 ml
Distilled water	10.0 ml	7 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	----
0.5 M Tris-HCl, pH 6.8	----	3.75 ml
10% (w/v) SDS	300 µl	150 µl
10 % APS	185 µl	22.5 µl
TEMED	15 µl	7.5 µl
Total monomer	30 ml	15 ml

2.7.4. Silver staining of SDS-PAGE gels

Silver staining of the SDS-PAGE gels was carried out according to the method of Blum *et al.* (1987) as explained in Table 2.2.

The protein profile was analyzed by SDS-PAGE. Changes occurring in protein banding profiles, following plant extract treatments, include

variation in band intensity as well as gain or loss of bands were taken in consideration (El-Tarras *et al*., 2013).

Table 2.2: Procedure of silver staining for polyacrylamide gels

Steps	Solution[a]	Time of Treatment
1) Fix	50 % Methanol; 12 % Acetic acid; 0.5 ml 37 % HCOH /liter	≥ 1 h
2) Wash	50 % Ethanol	3 X 20 min
3) Pretreat	$Na_2S_2O_3.5H_2O$ (0.2 g/liter)	1 min[c]
4) Rinse	H_2O	3 X 20 sec[c]
5) Impregnate	$AgNO_3$ (2 g/liter)	20 min
6) Rinse	H_2O	2 X 20 sec[c]
7) Develop	Na_2CO_3 (60 g/liter); 0.5 ml 37 % HCOH /liter; $Na_2S_2O_3.5H_2O$ (4 mg/liter)[d]	10 min
8) Wash	H_2O	2 X 2 min
9) Stop	50 % Methanol; 12 % Acetic acid	10 min
10) Wash	50 % Methanol	≥ 20 min[e]

[a] Solutions freshly prepared in a quantity that is 10-fold larger than the volume of the gel.

[b] Steps 1-10 were carried out on a shaker at room temperature (20-25^{3}C).

[c] The times indicated here should be observed exactly in order to ensure a reproducible image development.

[d] (20 ml/ liter) from the solution prepared in step 3 are added instead.

[e] After step 10, gel is transferred to 4^{3}C for storage.

Chapter Three
Results

3.1. Identification of *E. coli* Strains

Tow strains were used in this study, one was isolated from clinical urine sample and the other is a reference strain *E. coli* ATCC 25922. Colonies of these strains had typical green metallic sheen after inoculation on EMB medium, and bright pink or red colonies after inoculation on MacConkey agar. Gram stain showed that both *E. coli* strains are Gram-negative bacteria, short rod, single pair or in short chain. Both tested strains had acid butt and acid slant in TSI medium, with gas but no H_2S production, Positive Methyl-Red test**,** negative Voges-Proskauer test, Indole test was positive, citrate utilization was negative and motile.

3.2. Antibiotic Resistance Test and MIC Assay

Antibiotic sensitivity test showed that both ATCC 25922 and clinical *E. coli* strains were susceptible to all antibiotics used in this study. The MIC value of both ethanolic fruit and ethanolic leaf extracts of *E. elaterium* on both *E. coli* strains were found to be 25 mg/ml. The highest percentage of DMSO (negative control) which had a concentration of 5 %, showed no antibacterial activity against these strains.

3.3. Genotoxic Potential of Ethanolic *E. elaterium* Extract on DNA of *E. coli* Strains

DNA genome that was extracted from each *E. coli* strain, was exposed to different concentrations of ethanolic leaf and fruit extracts of *E. elaterium* for various time intervals. Changes in extracted DNA genome from these strains were evaluated in comparison with untreated controls at the same time interval. In the current study, only major obvious changes in DNA banding profile were taken into consideration. The results of three DNA samples in this study were excluded, because of their low DNA concentrations. These samples included *E. coli* ATCC 25922 treated with 10 mg of ethanolic fruit extract/ml for 2 h (Fig 3.1 A lane 2); clinical *E. coli* strain treated with 25 mg of ethanolic leaf extract/ml and 25 mg of ethanolic fruit extract/ml for 24 h (Fig 3.2 A lane 1 and Fig 3.2 B lane 1).

3.3.1. Evaluation of the genotoxic potential of ethanolic extracts of *E. elaterium* on DNA of *E. coli* reference strain

The effect of fruit ethanolic extract on *E. coli* reference strain was evaluated using different extract concentrations at different time intervals. ERIC-PCR profile showed that a band with an amplicon length of about 450-bp was more intense in *E. coli* reference strain treated with 6 mg/ml of fruit ethanolic extract for 2h in (Fig. 3.1 A lane 3) comparison with the same band appeared in un-treated control (Fig. 3.1 A C1). This band disappeared after 6 and 24h from *reference strain* treated with 6 mg/ml fruit extract (Fig. 3.1 A lanes 6 and 9) in comparison with the non-treated

controls (Fig. 3.1 A lanes C2 and C3), respectively. Moreover, two additional bands with an amplicon lengths of approximately 800-bp and 300-bp, appeared after 6h in the same strain treated with 10 mg/ml fruit ethanolic extract (Fig. 3.1 well 5), while these bands were not detected in non-treated control C2. ERIC-PCR profiles for treated and untreated *E. coli* reference strain under different conditions are shown in Figure 3.1 A.

Regarding the effect of ethanolic leaf extract on *E. coli* reference strain, the absence of the band which had an amplicon size close to 750-bp length was noticed in the DNA extracted after 24h from *E. coli* reference strain treated with 25 mg/ml ethanolic leaf extract (Fig. 3.1 B lane 7) compared with the non-treated control (Fig. 3.1 B lane C3). This band was also found in samples treated with extract concentrations of 6 and 10 mg/ml and incubated for 24h (Fig. 3.1 B lanes 9 and 8). Moreover, it was found that the band which had an amplicon size of approximately 450-bp was more intense in DNA extracted after 6h from the same strain treated with 10 and 6 mg/ml ethanolic leaf extract (Fig. 3.1 B lanes 5 and 6) compared to samples incubated with extract concentration of 25 mg/ml and with un-treated control (Fig. 3.1 B lanes 4 and C2). ERIC-PCR profile for treated and untreated *E. coli* tested strains with different concentrations of ethanolic leaf extract of *E. elaterium* at different time intervals is shown in Figure 3.1 B.

Fruit and leaf ethanolic extracts of *E. elaterium* showed different effects on genomic DNA of treated-reference *E. coli* strain at the same time interval.

For example, it was observed that after 24h treatment with 25 mg/ml fruit extract (Fig. 3.1 A lane 7), the band of about 450-bp amplicon size was almost having the same intensity as the control C3 (Fig. 3.1 A lane C3). On the other hand, the same band was fainter after 24h treatment with 25 mg/ml leaf extract (Fig. 3.1 B lane 7) compared with the control C3 (Fig. 3.1 B lane C3). Absence of bands with amplicon sizes of about 300 and 750-bp was also observed upon 24h treatment with 25 mg/ml leaf extract (Fig. 3.1 B lane 7) compared with control C3 (Fig. 3.1 B lane C3).

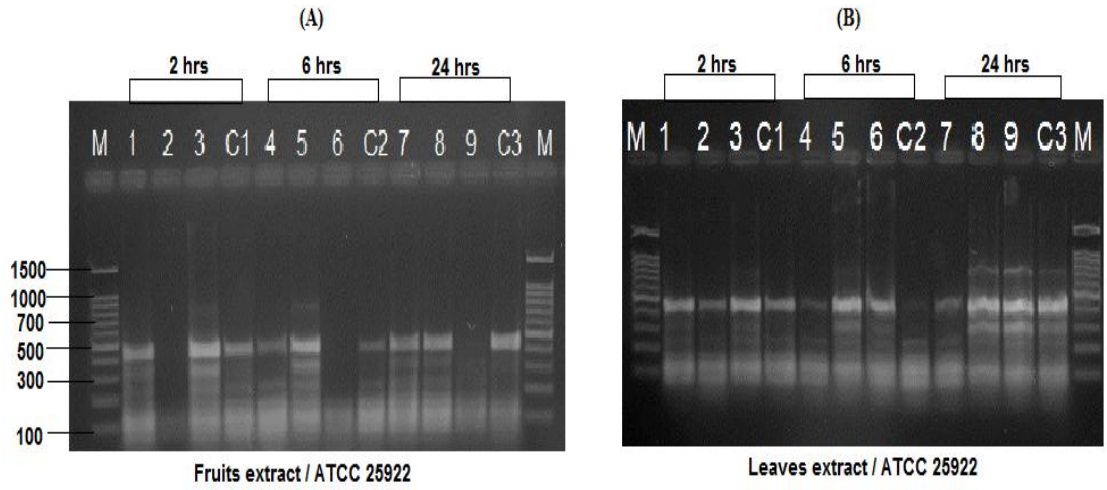

Figure 3.1: ERIC-PCR profile of *E. coli* ATCC 25922 strain treated and untreated with different fruit extract concentrations (A) and leaf (B) ethanolic extracts of *E. elaterium* at different time intervals. Lanes C1, C2 and C3 are untreated (negative controls); lanes 1, 4 and 7 treated with 25 mg/ml; Lanes 2, 5 and 8 treated with 10 mg/ml; Lanes 3, 6 and 9 treated with 6 mg/ml.

3.3.2. Evaluation of the genotoxic potential of ethanolic extracts of *E. elaterium* on DNA of clinical *E. coli* strain

Treatment of clinical *E. coli* strain with ethanolic fruit extract resulted in the loss of several bands from ERIC-PCR profile of this strain compared with the profile of untreated control samples. ERIC-PCR showed the

absence of two major bands with amplicon sizes of about 1000-bp and 800-bp from clinical *E. coli* strain treated with 25 mg/ml, 10 mg/ml and 6 mg/ml ethanolic fruit extract for 2h (Fig. 3.2 A lanes 1, 2 and 3), compared with untreated control C1(Fig. 3.2 A lane C1). Another major band with approximately 550-bp amplicon size is also missing from the profile of the DNA extracted from the clinical strain treated with 25 mg/ml and 10 mg/ml after 2h (Fig. 3.2 A lanes 1 and 2), compared to the profile of the sample treated with 6 mg/ml (Fig. 3.2 A lane 3) and untreated control (Fig. 3.2 A lane C1). Moreover, the band which had an amplicon size of approximately 350-bp was faint and absent after 2h from the DNA profile of clinical *E. coli* strain treated with ethanolic fruit extracts of 25 mg/ml and 10 mg/ml (Fig. 3.2 A lanes 1 and 2), Compared with the sample treated with 6 mg/ml (Fig. 3.2 A lane 3) and untreated control (Fig. 3.2 A lane C1). The bands which had amplicon sizes of approximately 1500-bp and 600-bp, amplified from clinical *E. coli* strain treated with 25 mg/ml of fruit extract disappeared (Fig. 3.2 A lane 1), while the band which had amplicon size of about 100-bp length was faint in comparison with untreated control C2 after 2h (Fig. 3.2 A lane C2). Finally, ERIC-PCR showed that all bands were absent from clinical strain treated with 6 mg/ml and 10 mg/ml fruit extract for 24 h (Fig. 3.2 A lanes 8 and 9) compared with untreated control C3 (Fig. 3.2 A lane C3). ERIC-PCR profile for treated and untreated clinical *E. coli* strain with different concentrations of ethanolic fruit extract of *E. elaterium* at different time intervals is shown in Figure 3.2 A.

Loss of bands was also observed upon treatment of clinical *E. coli* strain with ethanolic leaf extract. Bands with amplicon sizes around 1000-bp, 800-bp and 550-bp lengths were found in controls C1 and C2, but disappeared after 2 and 6h in *E. coli* strain treated with 10 and 6 mg/ml ethanolic leaf extract (Fig. 3.2 B lanes 2, 3, 5 and 6), and after 6 h in strain treated with 25 mg/ml ethanolic leaf extract (Fig. 3.2 B lane 4). Moreover, ERIC-PCR showed that the band which had amplicon size about to 550-bp length was faint after 2h in clinical strain treated with 25 mg/ml ethanolic leaf extract in comparison with control C1 (Fig. 3.2 B lane 1). On the other hand, incubation for 24h for samples treated with 10 and 6 mg/ml leaf extract showed identical banding pattern compared with control C3 (Fig. 3.2 B lanes 8 and 9) with the exception of the presence of the band with amplicon size of about 1300 bp was faint in samples treated with 10 and 6 mg/ml leaf extract compared with control C3 (Fig. 3.2 B Lane 7). ERIC-PCR profile for treated and untreated clinical *E. coli* strain with different concentrations of ethanolic leaf extract of *E. elaterium* at different time intervals is shown in Figure 3.2 B.

Fruit and leaf ethanolic extracts of *E. elaterium* showed different effects on genomic DNA of clinical *E. coli* strain at the same time interval. For example, 24h treatment of clinical E. coli with 10 and 6mg/ml fruit extract (Fig. 3.2 A lanes 8 and 9) resulted in loss of all bands compared with the untreated control C3 (Fig. 3.2 A lane C3). On the other hand, 24h treatment of clinical *E. coli* with 10 and 6mg/ml leaf extract (Fig. 3.2 B lanes 8 and 9) resulted in neither loss nor appearance of extra bands

compared with control C3 (Fig. 3.2 B lane C3), but the band with an amplicon size of about 1000-bp was faint upon treatment.

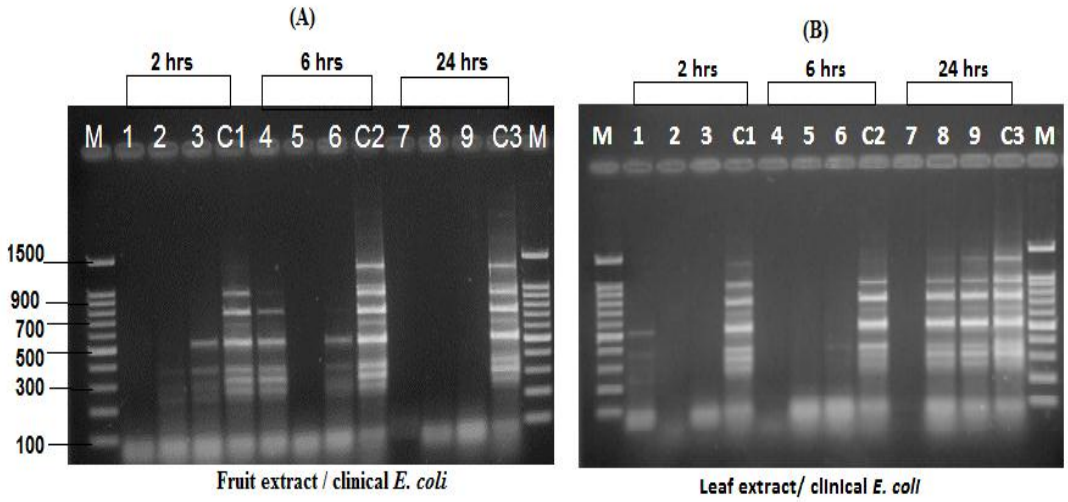

Figure 3.2: ERIC-PCR profile of clinical *E. coli* strain treated and untreated with different concentrations of fruit (A) and leaf (B) ethanolic extracts of *E. elaterium* at different time intervals. Lanes C1, C2 and C3 are untreated (negative controls); lanes 1, 4 and 7 treated with 25 mg/ml; Lanes 2, 5 and 8 treated with 10 mg/ml; Lanes 3, 6 and 9 treated with 6 mg/ml.

3.4. Effect of Ethanolic Extracts of *E. elaterium* on Total Protein Concentration of Both *E. coli* Strains

Treatment of both *E. coli* strains with fruit and leaf ethanolic extracts of *E. elaterium* resulted in noticeable elevation in total protein concentration under different experimental conditions, especially in bacterial strains exposed to fruit extracts. This increase was not reported when *E. coli* ATCC 25922 reference strain was treated with 25, 10 and 6 mg/ml fruit extract for 24 h, and when clinical *E. coli* was treated for 24 h with 25 and 10 mg/ml fruit extract.

3.5. Effect of Ethanolic Extracts of *E. elaterium* on Protein Profile of *E. coli* Strains

Total protein was extracted from both *E. coli* strains that were exposed to different concentrations (6, 10 and 25 mg/ml) of ethanolic leaf and fruit extracts of *E. elaterium* for different time intervals (2, 6 and 24h). Changes in extracted protein from treated strains were evaluated in comparison with untreated control samples at the same interval time. In the present study, only major obvious changes were taken into consideration.

3.5.1. Effects of ethanolic extracts of *E. elaterium* on protein profile of reference *E. coli* strain

Several changes in reference *E. coli* protein profile were observed upon treatment with *E. elaterium* fruit extracts. Treatment with 25 mg/ml fruit extract for 6 h (Fig. 3.3 lane 4) caused several bands in untreated control C2 (Fig. 3.3 lane C2) to disappear including those with Rf values 0.1, 0.15, 0.25, 0.3 and 0.36. Moreover, the band at Rf 0.29 was very faint after 6 h treatment with 6 mg/ml fruit extract (Fig. 3.3 lane 6) and absent after 6 h treatment with 10 mg/ml fruit extract (Fig. 3.3 lane 5) compared with control C2 (Fig. 3.3 lane C2). Absence of bands with Rf values 0.04, 0.05 and 0.9 was observed at 24 h treatment with 25, 10 and 6 mg/ml fruit extract (Fig. 3.3 lanes 7, 8 and 9) compared with control C3 (Fig. 3.3 lane C3). Protein profile for treated and untreated reference *E. coli* strain with different concentrations of *E. elaterium* ethanolic fruit extract is shown in Figure 3.3.

Leaf extract treatment of refernce *E. coli* strain also resulted in several modifications in the protein profile. For example, upon 6 h treatment with 25 mg/ml leaf extract appearance of 2 new bands at Rf values of approximately 0.45 and 0.47 was recorded compared with untreated control C2. On the other hand, disappearance of bands with 0.14, 0.15, 0.24 and 0.65 Rf values was observed after 24 h treatment with 25 mg/ml leaf extract compared with control C3 (Figure is not shown).

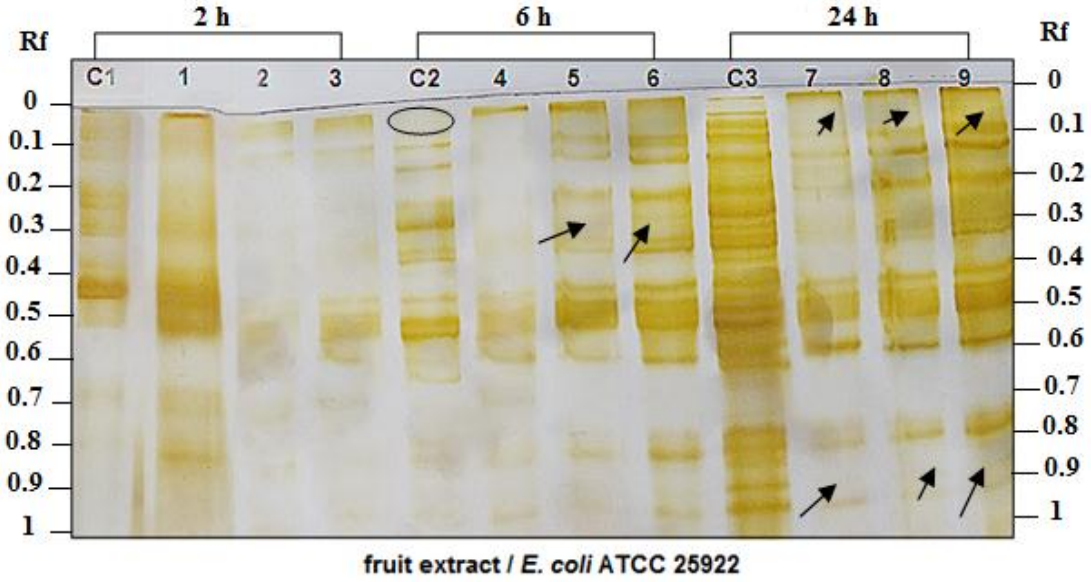

Figure 3.3: Protein profile of *E. coli* ATCC 25922 reference strain treated and untreated with different concentrations of fruit ethanolic extract of *E. elaterium* at different time intervals. Lanes C1, C2 and C3 are untreated (negative controls); Lanes 1,4 and 7 are treated with 25 mg/ml; Lanes 2, 5 and 8 are treated with 10 mg/ml; Lanes 3, 6 and 9 are treated with 6 mg/ml. Arrows and ovals indicate some of the treatment-affected bands mentioned in the text.

3.5.2. Effects of ethanolic extracts of *E. elaterium* on protein profile of clinical *E. coli* strain

Ethanolic fruit extract of *E. elaterium* resulted in differences in the protein profile of the treated clinical *E. coli*. The observed differences varied between either up regulation, down regulation or even complete

disappearance of particular protein fractions. After 2 h of application of 25 mg/ml fruit extract (Fig. 3.4 A lane 1), bands at Rf values of about 0.22 and 0.42 were faint compared with control C1 (Fig. 3.4 A lane C1). The band at Rf 0.42 was also faint upon 2 h treatment with 10 and 6 mg/ml fruit extract compared with control C1 (Fig. 3.4 A lane C1). On the other hand, 6 h treatment with 10 and 6 mg/ml fruit extract (Fig. 3.4 A lanes 5 and 6) resulted in an increase in the intensity of bands at approximate Rf of 0.15 and 0.7 compared with untreated control C2 (Fig. 3.4 A lane C2). Decreasing the intensity of bands at Rf 0.53 and 0.7 was noticed after 24 h treatment with 25, 10 and 6 mg/ml fruit extract (Fig. 3.4 A lanes 7, 8 and 9) compared with control C3 (Fig. 3.4 A lane C3). Moreover, the band at Rf 0.3 was pale after 24 h treatment with 10 and 6 mg/ml fruit extract (Fig. 3.4 A lanes 8 and 9) and completely disappeared upon 24 h treatment with 25 mg/ml fruit extract (Fig. 3.4 A lane 7) compared with control C3 (Fig. 3.4 A lane C3). Protein profile for treated and untreated clinical *E. coli* strain with different concentrations of *E. elaterium* ethanolic fruit extract at different time intervals is shown in Figure 3.4 A.

Treatment of clinical *E. coli* strain with leaf extract also resulted in several modifications. The SDS-PAGE protein profile revealed that 6 h treatment with 10 mg/ml and 6 mg/ml leaf extract (Fig. 3.4 B lanes 5 and 6) resulted in increasing the intensity of bands at relative mobility (Rf) of 0.06 and 0.1 compared with the control C2 (Fig. 3.4 B lane C2). On the hand, 6h treatment with 25 mg/ml leaf extract resulted in the disappearance of band at Rf 0.06 compared with control C2 (Fig. 3.4 B lane C2). Moreover,

appearance of new band with nearly 0.8 Rf value was recorded after 6 h treatment with 10 mg/ml leaf extract (Fig. 3.4 B lane 5) compared with control C2 (Fig. 3.4 B lane C2). Treatment with 25 mg/ml leaf extract for 24h (Fig. 3.4 B lane 7) caused the absence of band at approximate Rf value of 0.33, while the band at Rf 0.68 was very faint compared with the control C3 (Fig. 3.4 B lane C3). Protein profile for treated and untreated clinical *E. coli* strain with different concentrations of *E. elaterium* ethanolic leaf extract at different time intervals is presented in Figure 3.6B.

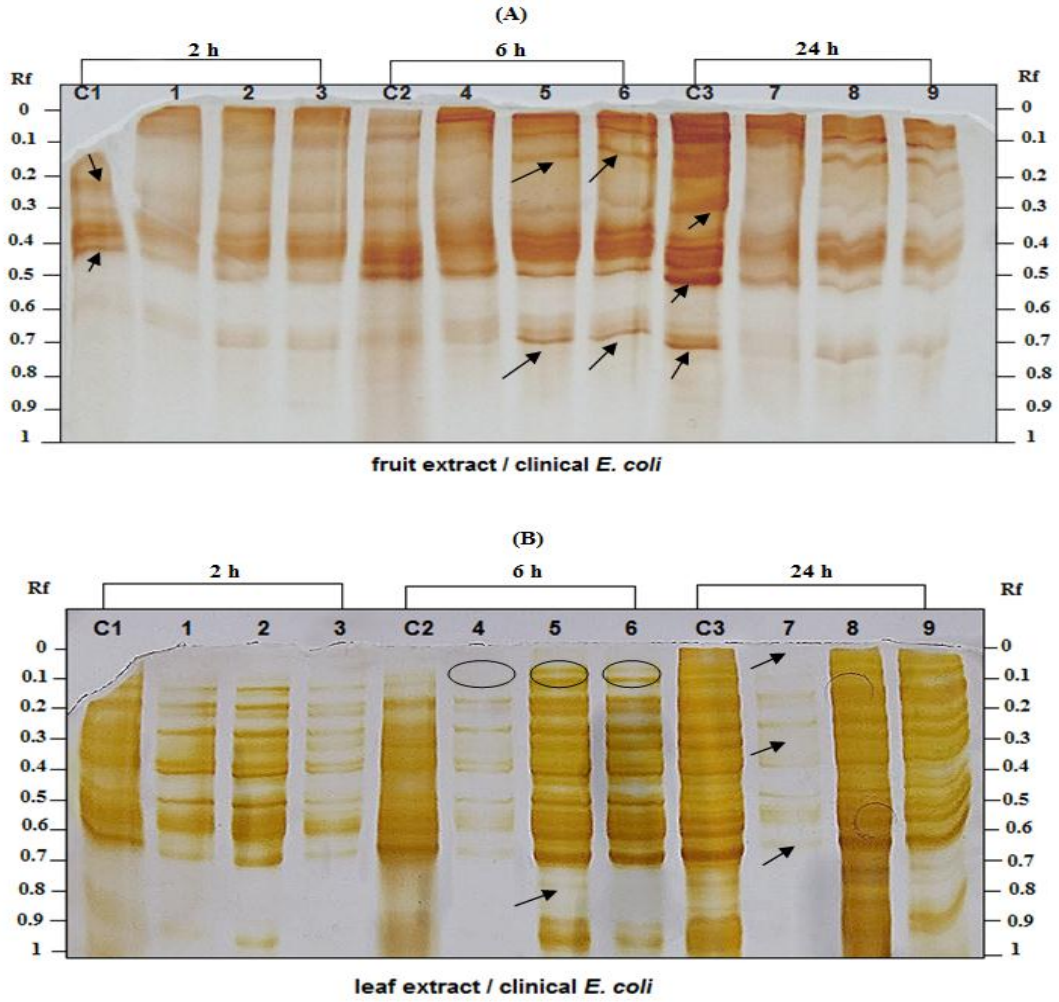

Figure 3.4: Protein profile of clinical *E. coli* strain treated and untreated with different concentrations of fruit (A) and leaf (B) ethanolic extracts of *E. elaterium* at different time intervals. Lanes C1, C2 and C3 are untreated (negative controls); Lanes 1,4 and 7 are treated with 25 mg/ml; Lanes 2, 5 and 8 are treated with 10 mg/ml; Lanes 3, 6 and 9 are treated with 6 mg/ml. Arrows and ovals indicate some of the treatment-affected bands mentioned in the text.

Chapter Four
Discussion and Conclusion

Ecballium elaterium is commonly used as a folkloric remedy in different countries. It is utilized traditionally for the treatment of various ailments, including jaundice, rhinosinusitis, sinusitis, fever, cancer, liver cirrhosis, constipation, hypertension as well as others (Salhab, 2013). Moreover, *E. elaterium* juice is rich with cucurbitacins that were reported to have broad-spectrum pharmacological bioactivities (Alghasham, 2013). Cytotoxic, hepatoprotective, antioxidant, antimicrobial, anti-inflammatory, and anti-tumor activities are all reported in the literature (Bernard and Olayinka, 2010; Alghasham, 2013). With these activities, cucurbitacins may be a source of potential drug formulations for curing many illnesses.

In fact, drugs derived from natural products represent 70 % of these approved since 1981 (Jafargholizadeh *et al*., 2016). Moreover, the traditional use of medicinal plants in treating diseases is increasing in many countries (Basaran *et al*., 1996). In spite of this, increasing evidence has shown that many of medicinal plant products may have toxic, mutagenic, and even carcinogenic effects (Celik, 2012). Thus, investigating the genotoxicity of plants is of great importance for safe traditional use and safe use in drug formulations.

In the present study, the potential genotoxic effect of the ethanolic leaf and fruit extracts of *E. elaterium* against two different strains of *E. coli* was examined using ERIC-PCR, and SDS-PAGE analytical methods. Initially,

the minimal inhibitory concentration (MIC) of the ethanolic fruit and leaf extracts of *E. elaterium* was determined using the microbroth dilution method. Aligiannis, Kalpoutzakis, Mitaku, and Chinou (2001) and Duarte et al. (2007), proposed a classification of the antimicrobial activity of plants as either strong (MIC < 500 μg mL−1), moderate (MIC of 600–1500 μg mL−1) or weak (MIC > 1600 μg mL−1). According to this classification, the antimicrobial activity of *E. elatruim* extracts against both tested *E. coli* strains were found to be weak (MIC of 25mg/ml). The detected weak antimicrobial activity against *E. coli* is in general agreement with the findings of Abbassi *et al*., (2014), Oskay *et al*., (2009). Differences in cell wall composition is the most logical reason behind this decreased sensitivity of Gram-negative bacteria as reported by other authors (Marasini *et al*., 2015; Abbassi *et al*., 2014; Oskay and Sari, 2007). Gram-negative bacteria possess a unique outer membrane that interferes with the entrance of antibacterial agents due to its hydrophilic character (Abbassi *et al*., 2014). The presence of efflux pump system of Gram-negative bacteria also may attribute to the decreased sensitivity (Marasini *et al*., 2015).

In literature, many plants were tested by different genotoxicity methods showed genotoxicity potential. Examples include *Curcuma longa*, *Melia azedarach*, *Rhazya stricta*, *Urtica dioica*, *Salvia triloba*, *Arctium minus*, *Plantago major*, *Momordica charantia*, *Thermopsis turcica*, *Moringa peregrina* (Lalrotluanga *et al*., 2011; El-tarras *et al*., 2013; Basaran *et al*., 1996; Hajar and Gumgumjee, 2014; Cigerci *et al*., 2016).

To the best of our knowledge, this is the first study of an analysis of genomic alteration that tested on *E. coli* strains treated with different concentrations of ethanolic leaf and fruit extracts of *E. elaterium* using ERIC-PCR assay. ERIC-PCR banding pattern showed significant differences between the treated and untreated *E. coli* strains used in this study. The main changes in the treated *E. coli* included the disappearing or appearing of certain bands in comparison with untreated control, as well as differences in band intensity. The loss of bands in treated *E. coli* strains in comparison with that found in control samples may be due to the effect of the genotoxins present in the plant extracts. These genotoxins can induce DNA damage such as single and/or double strand breaks, point mutations and/or chromosomal rearrangements. These damages in the DNA may have a potential effect on the primer annealing sites and or inter-priming distances (Cigerci *et al*., 2016; Hajar and Gumgumjee, 2014; Lalrotluanga *et al*., 2011). Point mutations, large deletions, and/or homologous recombination are considered as mechanisms that can produce new primer annealing sites, thus resulting in the appearance of extra new bands or change the amplicon size (Cigerci *et al*., 2016). However, understanding and determining the specific mechanisms that lead to differences in ERIC-PCR profile is difficult. Other techniques can support and assist in understanding the proposed mechanisms such as analysis of amplicons using DNA sequencing or probing (Lalrotluanga *et al*., 2011).

Results of the current study support the evidences reported previously that extracts of *E. elaterium* have genotoxic and mutagenic potential (Celik and Aslanturk, 2009; Rencuzogullari *et al*., 2006; Basaran *et al*., 1996).

The observed genotoxicity of *E. elaterium* possibly were mediated by cucurbitacins. Different types of cucurbitacins such as B, D, E, I, L and R have been identified in the juice of *E. elaterium* (Celik and Aslanturk, 2009). The fruits juice of *E. elaterium* is especially rich with cucurbitacins that represent between 20-30% of the juice (Jaradat *et al*., 2012). It was reported that cucurbitacins B, D, E, and I possess strong anticancer activities (Lee *et al*., 2010). In addition, the genotoxic potential of cucurbitacin B was reported, as it induced DNA damage in A594 and MCF-7 cells using the comet assay (Guo *et al*., 2014; Ren *et al*., 2015). The damage mediated by cucurbitacin B is due to induction of reactive oxygen species (ROS) formation, which results in double-stranded breakage and subsequently G2/M phase arrest in A594 cells.

The effect of fruit and leaf ethanolic extracts of *E. elaterium* on total protein profile of both *E. coli* strains was assessed using SDS-PAGE gels, where several alterations in the protein profile of *E. elaterium* treated *E. coli* compared to the untreated control was observed. These changes included increase or decrease in band intensity, absence or appearance of bands. The absence of protein bands indicates the interruption of protein synthesis pathways possibly stimulated by *E. elaterium* extract bioactive constituents (Othman and Ahmed, 2017). The appearance of new bands

and increasing the intensity of some bands can be explained by the ability of *E. elaterium* extract to apply a stress on treated *E. coli*. The stressed *E. coli* may respond under these conditions by up-regulation (increasing the expression) of some proteins and stimulating the expression of others (El-tarras *et al*., 2013). Another explanation for this proposes the happening of frame shift mutations due to stress (El-tarras *et al*., 2013).

In conclusion, our results showed that *E. elaterium* fruit and leaf ethanolic extracts possess genotoxic and mutagenic potential. The results also point out the capability of using *E. elaterium* to treat *E. coli* infections. More studies are required to find out the exact mechanism responsible for the observed genotoxicity.

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جامعة النجاح الوطنية
كلية الدراسات العليا

تقييم التأثيرات البيوكيميائية والجزيئية لمستخلص نبات القثاء البري (*Ecballium elaterium*) على بكتيريا الإشريكية القولونية

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قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

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الملخص

تستخدم نبتة القثاء البري (*Ecballium elaterium*) في العديد من الدول حول العالم كدواء شعبي لمعالجة العديد من الأمراض مثل اليرقان والتهاب الجيوب الأنفية. تم اجراء هذه الدراسة بهدف فحص فعالية مستخلصات الكحول الايثيلي لثمار وأوراق نبات القثاء البري ضد نوعين من البكتيريا الاشريكية القولونية احداهما معزولة من شخص مصاب بالتهاب في الجهاز البولي والأخرى هي عينة مرجعية. أظهرت نتائج طريقة Microbroth dilution أن المستخلصين لديهما نفس الفعالية ضد كلا النوعين الذين تم فحصهما من البكتيريا الاشريكية القولونية وبقيمة MIC مساوية ل 25 mg/ml. أيضا تم دراسة تأثير كلا المستخلصين على المستوى الجزيئي بواسطة تقنية ERIC-PCR ودراسة تاثيرهما على محتوى الخلية الكلي من البروتينات بواسطة تقنية SDS-PAGE. أظهرت نتائج تقنيتي ERIC-PCR و SDS-PAGE حدوث تغييرات على ال DNA والبروتين المستخلصين من كلا النوعين من البكتيريا الاشريكية القولونية اللذين تمت معالجتهما بواسطة مستخلصات ثمار وأوراق نبتة القثاء البري مقارنة بتلك غير المعالجة. تراوحت التغييرات بين زيادة أو نقصان في شدة سطوع بعض قطع ال DNA أو البروتين أو اختفاء أو ظهور قطع جديدة. كان من بين التغييرات التي لوحظت بواسطة تقنية ERIC-PCR هو اختفاء قطعة DNA بحجم مساوي تقريبا ل 450–bp بعد مضي 6 و 24 ساعة على معالجة البكتيريا المرجعية بواسطة 6 mg/ml من مستخلص ثمار القثاء البري مقارنة بتلك غير المعالجة. أيضا لوحظ نقصان سطوع قطعة DNA بحجم مساوي تقريبا ل 550–bp بعد ساعتين من

معالجة البكتيريا المعزولة من مريض بواسطة 25 mg/ml من مستخلص أوراق القثاء البري مقارنة بتلك التي لم تخضع للمعالجة. من بين التغييرات التي لوحظت في ال SDS-PAGE هو ظهور قطعتي بروتين جديدتين عند Rf تقريبا 0.47 و 0.45 بعد 6 ساعات من معالجة البكتيريا المرجعية بواسطة 25 mg/ml من مستخلص الأوراق لنبات القثاء البري. بالاضافة الى ذلك لوحظ زيادة في شدة سطوع قطعتي بروتين عند Rf مساوية تقريبا ل 0.15 و 0.7 بعد 6 ساعات من معالجة البكتيريا المعزولة من مريض بواسطة 10 و6 mg/ml من مستخلص الثمار لنبات القثاء البري مقارنة بالبكتيريا التي لم تخضع للمعالجة. بالاضافة الى ذلك لوحظ أن زيادة تركيز مستخلصات القثاء البري وزيادة وقت المعالجة أدى الى زيادة ملحوظة في تركيز البروتين الكلي في كلا نوعي البكتيريا اللذين تم فحصهما. هذه النتائج تشير بقوة الى قدرة مستخلصات نبات القثاء البري على التاثير على جينات الاشريكية القولونية وتعد مشيرا أوليا على أن استخدام القثاء البري في العلاج الشعبي هو غير أمن. وتشير النتائج أيضا الى امكانية استخدام مستخلصات القثاء البري في علاج الأمراض التي تسببها الاشريكية القولونية. ننصح بأن بتم عمل دراسات أخرى لمعرفة الاليات المسؤولة عن هذه التأثيرات الملحوظة لمستخلصات القثاء البري على جينات وبروتينات البكتيريا الاشريكية القولونية.