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The curative effect of *Cymbopogon citrates* volatile oil against chlorambucil drug toxicity

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Abstract

Chlorambucil (CLB) is a bifunctional alkylating drug widely used as an anticancer agent and immunosuppressant. CLB mutagenicity, teratogenicity and carcinogenicity are indicated based on their structure and clinical history. This study aims to evaluate the antigenotoxic effect of *Cymbopogon citratus* essential oil, CC, (75 mg/kg) against CLB (7.5 mg/kg) genotoxicity in rats. GC/MS for essential oil has identified 19 compounds representing approximately 99.7% Geranial was the most abundant **(**53.5%) followed by Neral (35%) and Myrcene (5.3%). The lowest was α-Muurolene (0. 1%). The marked damage was observed in total genomic DNA and total protein profile of CLB-intoxicated rat's spleen tissues. Lymphocytes single strand breaks of treated rats were examined by comet assay after CC had ameliorated these effects in a time dependent manner (5, 10 and 15 days) for spleen and after 48 hours for lymphocytes. In conclusion, this study suggests that *Cymbopogon citrates* oil possesses antigenotoxic potential in CLB-intoxicated rats. It can constitute natural, new and safe co-therapeutics.

Keywords: Chlorambucil; *Cymbopogon citratus*; DNA damage; Comet assay; GC/MS and essential oil.

1. Introduction

Chlorambucil (CLB) is a bifunctional aromatic alkylator. It is used clinically as an anticancer drug and immunosuppressant. CLB kill cells, non-selectively (Thomas et al., 2000). It was found to inhibit transcription and translation. DNA is a critical molecule in the mode of action of mustards (Masta et al., 1995). Besides, chlorambucil was found to be carcinogenic in $^{BALB}/_{c}$ *mice* (Cavaliere et al., 1990). It was reported that the drug-induced DNA alkylation/damage lesions were single-stranded (DNA mono-adducts) or double-stranded (DNA inter-strand cross links) (Wang et al., 2003).

Chlorambucil induces both crosslink DNA in the absence of metabolism (McLean et al., 1980) and monoalkylate DNA following metabolic removal of one of its chloroethyl groups (Adams et al., 1996). It induces ^{32}P-labeled DNA adducts in calf thymus DNA used as a target for the direct detection of adducts (Yourtee et al., 1992). It induced inter-chromosomal mitotic recombination in the w/w^{+} assay in *Drosophila* (Vogel and Nivard, 1993). CLB also caused chromosomal aberrations and micronucleus test after 48 h of spleen cells treatment (Moore et al., 1995), and *In vitro* chromosomal aberrations induction and sister-chromatid exchanges in a dose-dependent manner in V79 cells (Speit et al., 1992).

Natural compounds are considered chemoprotective agents against anticancer therapy-induced cytotoxicity in normal tissues (Guerriero et al., 2014; Sobhy et al., 2017; El-Garawani et al., 2017). They possess anticancer potential against different types of cancers (El-Nabi et al., 2018; Elkhateeb et al., 2018). The carcinogenic, mutagenic and toxic effects caused by some medications could be diminished by natural compounds such as essential oils due to its accessibility and nontoxic effect on human cells and the environment (Huang et al., 2000; Lee et al., 2001; Sakr et al., 2016). *Cymbopogon citratus* essential oil is one of these chemopreventive agents (Melo et al., 2001). It has no toxic properties when daily administrated, for two months, to male and female rats and in offspring exposed "in utero" (Souza-Formigoni et al., 1986).

The CC oil is characterized by high percentages of citral (70–85%) according to the geographical area (Ferreira and Fonteles, 1989) as determined by GC-Mass method. It was found that citral (20 mg/kg) had significantly decreased the frequency of micronucleus induced by the known mutagens in bone marrow and peripheral blood erythrocytes (Rabbani et al., 2005). A good superoxide scavenging activity was observed in citral treated groups, too (Rabbani et al., 2006).

Kauderer et al. (1991) concluded that β- myrcene was negative for the induction of point mutations *in vitro* and showed no induction of any chromosomal aberrations in cultured human peripheral blood lymphocytes. No carcinogenicity data on β-myrcene were located. It was generally negative for genotoxic activity in mammalian *in vitro* and *in vivo* (Roscheisen et al., 1991; Kauderer et al., 1991; Zamith et al., 1993).

Finally, the current study was conducted to evaluate the curative and antigenotoxic effect of *Cymbopogon citratus* against genetic disorders induced by CLB in spleen as a target organ, because of its importance for human immunity.

2. Materials and Methods

2.1. Materials

2.1.1. Chlorambucil (LEUKERAN)

Chlorambucil is water-soluble aromatic nitrogen mustard, 4-[bis (2-chlorethyl) amino] benzenebutanoic acid. It was purchased from a local pharmacy. Tablets of Leukeran 2 mg (GlaxoWellcome, UK) were dissociated and suspended in tap water. A concentration of 7.5 mg/kg/day was administered daily to rats by a gastric tube (Tomenendalova et al., 2008).

2.1.2. Volatile oil of Cymbopogon citratus (CC)

The plant was obtained from National Organization for Drug Control and Research (medicinal plant field) at Kafr El- Gabal, Giza,

Egypt. Extraction of total volatile oils from fresh lemon grass leaves was done by hydro distillation method according to (Egyptian Pharmacopoeia, 1984). The oil was mixed with corn oil (1:5 v/v) and a concentration of 75 mg/kg/day was administered daily to rats using a gastric tube (Fandohan et al., 2008).

2.1.3. Experimental animals

The present study was carried out using adult male albino rats (*Rattus norvegicus*) of an average body weight about 170 ±10 g. Animals were obtained from the National Organization for Drug Control and Research at Kafr El-Gabal, Giza, Egypt. Animals were acclimatized for a period of two-weeks in the animal house as established by Institutional Animal Ethical Committee (IAEC) at Zoology Department, Faculty of Science, Menoufia University.

2.1.4. Experimental design

Initially, 15 animals were treated with 7.5 mg/kg/day of Chlorambucil as preliminary study to investigate the effect of the drug as a toxicant, while other 15 untreated animals served as a control group (five animals at each dissection at fixed intervals, 5^{th}, 10^{th} and 15^{th} days).

The other experimental animals were divided into five groups, five animals each, as the following for DNA and protein analysis:

Control included corn oil (which received 200 µl of the commercial corn oil daily), CLB, CC oil and CC oil as a curative agent against CLB toxicity (they received the oil dose after an hour of CLB dose. All groups were sacrificed by the 15^{th} day of treatment).

2.2. Methods

2.2.1. Gas Chromatography/Mass Spectrometry (GC/MS)

Identification of the oil constituents was performed on the Agilent 5989B, VL MS Detector system operating in EI mode (equipped with a HP5MS 30m x 0.25mm x 0.25µm film thickness capillary column), using He (1 ml/min) as the carrier gas. 100 µl of the EODM was diluted with 400 µl of dichloromethane and was injected into the GC-MS system in the split mode (split ratio 1: 33). Helium was used as the carrier gas with a flow rate of 0.7 ml/min. The column temperature was maintained at 50ºC for 2 min. Then, it was programmed to 140ºC at a rate of 3 /min, then it was increased up to 160ºC at a rate of 0.5 /min and the final temperature, 260ºC at a rate of 3 /min, was held for 2 min. Injector and detector temperatures were optimized at 230ºC and 260ºC, respectively. The MS operating parameters were as follows: ionization energy, 70 eV; ion source temperature, 200ºC ; quadruple, 100ºC; solvent delay, 8.0 min; scan speed 2000/us and scan range 30–600 u, EV voltage 3000 volts. Determination of the components was based on direct comparison of the retention times and MS data with those for standard compounds, and matching with the combined Wiley 229, Nist 107 and Nist 21 libraries (Version 1998) (Adams, 2007).

2.2.2. Total genomic DNA extraction and apoptosis detection

DNA extraction and detection of apoptosis (DNA fragmentation assay) were done according to "salting out extraction method" of Aljanabi and Martinez (1997) and modification by Hassab El-Nabi (2004). Protein was precipitated by a solution of NaCl (4M). Apoptotic bands of DNA fragmentation appeared and were located at 180 bp and its multiples 360, 540 and 720 bp against 100bp DNA ladder (Thermo Scientific™ O'gene ruler™). The intensity of released DNA fragments was analyzed using (Biogene software, France) as maximum optical density values where the height is a maximum intensity at 256 grey levels.

Agarose gel electrophoresis

Gel was prepared using 1.8% electrophoretic grade agarose (BRL) obtained from (Hispangar D-1 LE, Spain) (Surzycki, 2000a).

Determination of DNA purity

According to Surzycki (2000b), DNA purity was estimated spectrophotometrically at optical density

(A_{260}/A_{280}), using (Nicolet Evolution 100, Thermo, England) spectrophotometer.

2.2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of spleen tissues:

SDS-PAGE was done according to Laemmli (1970) and the separation was done using 12% continuous gel method.

2.2.4. Alkaline single cell gel electrophoresis (SCGE)/comet assay

Leukocytes isolation

Heparinized venous blood samples were drawn from treated and non-treated rats then blood was incubated twice by erythrocyte lysing buffer (0.015 M NH_4Cl, 1mM $NaHCO_3$, 0.1mM EDTA). Cells then were resuspended in serum-free RPMI medium and forwarded to further investigations (El-Garawani, 2015).

Alkaline single cell gel electrophoresis (comet assay) was performed in this study (Singh et al., 1988). Briefly, cells were embedded into low melting point agarose gel between two layers of ultra-pure normal melting agarose on microscopic glass slides and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0) with freshly added 1% Triton X-100 (Sigma-Aldrich, Germany) and 10% dimethyl sulfoxide (DMSO) for 1 h at 4°C. Subsequently, the slides were incubated for 20 min in cold and freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13). The slides were applied in an electric current of 25 V and 300 mA for 10 min. The slides were then neutralized for 3 min by 0.4 M Tris-HCl buffer, pH 7.5. Next, they were stained with ethidium bromide (Sigma-Aldrich, Germany). Visualization of cells was performed using fluorescence microscope (Olympus BX 41, Japan), and representative images were photographed. For DNA damage assessment, about 200 randomly selected cells were examined per one field of total examined five fields. The results were divided as normal with no tail, damage with migrated tail not more than the diameter of the nucleus and strong damage with no distinct nucleus.

2.3. *Statistical analysis*

Data were presented as Mean ± Standard Deviation (M ± SD) for DNA and protein bands intensities. Comparison were made between control (untreated) and treated individuals. Data statistically analyzed by using Student's *t*-test, (Mc Clave and Dietrich, 1991), ($P<0.05$) were considered significant.

3. Results

3.1. Cymbopogon citratus oil composition by GC/MS

Cymbopogon citratus essential oil (i.e. > 1.5% (V/W) is a volatile oil on a fresh weight basis). The gas chromatographic analysis results, coupled with the mass spectrometry of essential oil, identified 19 compounds. This represents approximately (99.7%) as shown in Table (1) and reveals the presence of the following compounds: α -Thujene**,** α -Pinene, Myrcene, Limonene, (E) -β-Ocimene, Linalool, Citronellal, α –Terpineol, Citronellol, Neral, Geraniol. Geranial, Geranyl acetate, β –Caryophyllene, α -Humulene, α -Muurolene, γ-Cadinene and α –Cadinol**.** Geranial presented the abundant **(**53.5%) followed by Neral (35%) and Myrcene (5.3%) while the lowest was α -Muurolene (0. 1%).

Table 1. Essential oil (%) identified in the oils of *Cymbopogon citratus* by GC/MS.

No.	Retention indices (Literature)	Name	%
1	916	α Thujene	0.2
2	929	α Pinene	0.5
3	988	Myrcene	5.3
4	1028	Limonene	0.6
5	1045	(E) -β-Ocimene	0.3
6	1095	Linalool	0.5
7	1150	Citronellal	0.7
8	1180	α Terpineol	0.3
9	1230	Citronellol	0.2
10	1242	Neral	35
11	1256	Geraniol	0.9
13	1273	Geranial	53.5
14	1380	Geranyl acetate	0.5
15	1416	β -Caryophyllene	0.4
16	1447	α Humulene	0.2
17	1495	α Muurolene	0.1
18	1520	γ Cadinene	0.2
19	1650	α Cadinol	0.3
Total			99.7

*Retention indices on HP-5MS

3.2. Total genomic DNA damage in CLB-treated rats

CLB induced severe damage in DNA of spleen after fifteen days of treatment while a non-significant damage was observed after five and ten days. There was an increase of the height value at (1000 bp) which recorded 20±2.9, 24±2.2, 27±1.9 and 93±1.8 for control, 5, 10, and 15 days, respectively. The third band (360 bp) has only a value for 15 days which recorded a height value of 30±2.3. These results indicated that the highest DNA damage detected was for the treatment period of 15 days (Figure 1, 2).

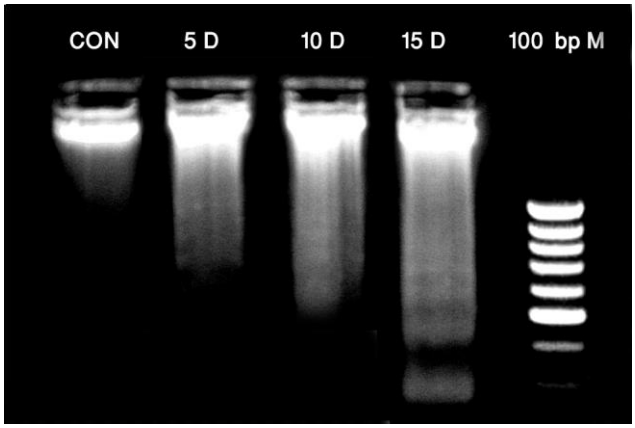

Figure (1): Photomicrograph of DNA electrophoresis of spleen tissues shows the effect of (CLB) (7.5 mg/kg/day) after 5, 10, and 15 days of treatments, CON resembles control spleen; 5D: after 5 days; 10D: after 10 days; 15D: after 15days and M: 100 bp ladder.

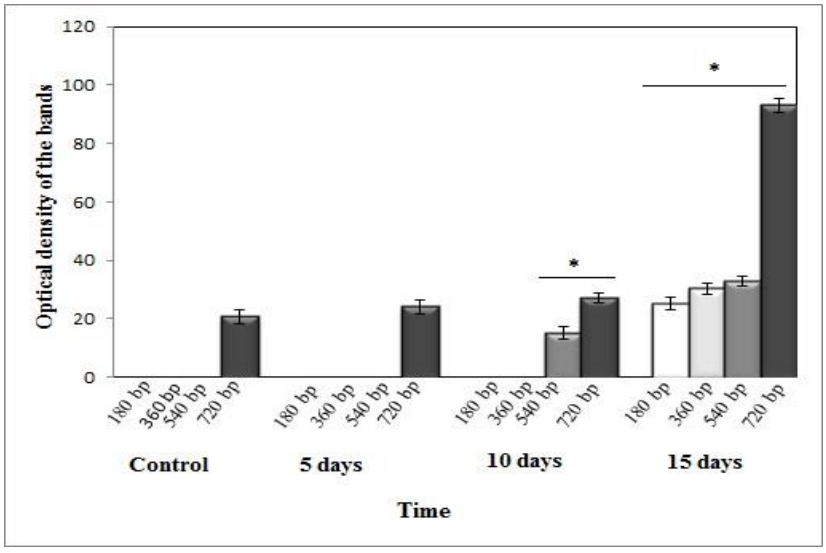

Figure (2): The average maximum intensity (height) of fragmented DNA in spleen treated with CLB after 5, 10 and 15 days.

3.3. The curative role of Cymbopogon citratus oil against DNA damage induced by CLB after fifteen days

The curative effect was observed in all lanes when compared with control and corn oil lanes at the level of DNA fragmentation in spleen tissues. Otherwise, CLB-treated animals countered the apoptotic DNA fragmentation at (180, 360, 540 and 720bp) when compared with *Cymbopogon citratus* oil-treated groups (Figure 3,4).

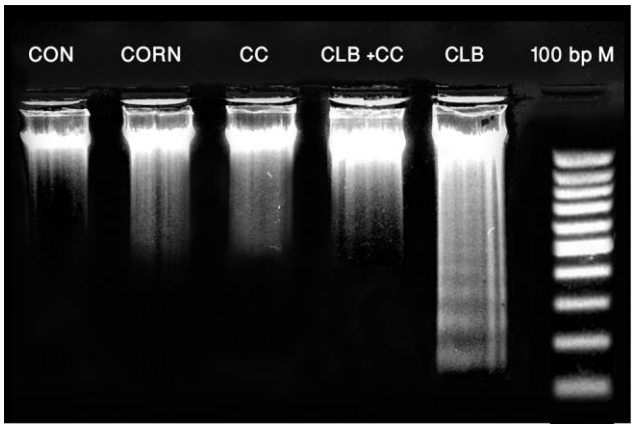

Figure (3): Photomicrograph of DNA electrophoresis of spleen tissues shows the effect of CLB (7.5 mg/kg/day) and the curative effect of *Cymbopogon citratus* against CLB after fifteen days of treatment. CON resembles control; CORN: corn oil; CC: *Cymbopogon citratus* oil; CLB+CC: *Cymbopogon citratus* oil after one hour of (CLB) dosing and M: 100 bp ladder**.**

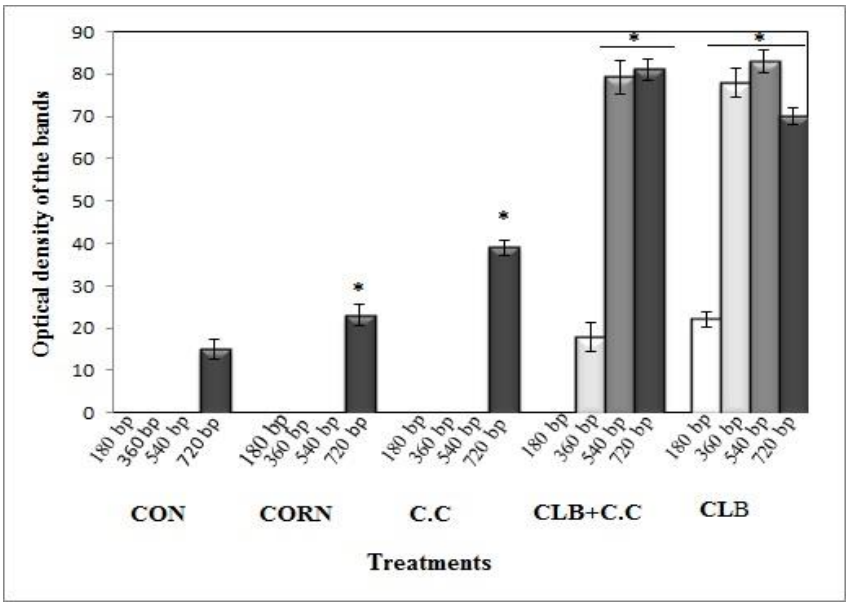

Figure (4): Maximum intensity (M±SD) of fragmented DNA in spleen of animals treated with *Cymbopogon citratus* (CC) against CLB toxicity after 15 days.

3.4. *DNA single strand breaks (Comet assay)*

DNA single strand breaks were detected as a length DNA migration tail. The length and intensity were classified as undamaged (normal), damaged (+) and severely damaged cells (++) with fan-like tail. In this study, animals treated with CLB only showed severe damaged cells with percentile value 51±2.1% and damaged cells 39±2.9%. On the contrary, CC-treated animals exhibited a significant (P< 0.0005) genotoxic elevated effects when compared with CLB treated group. Data showed only about 6±1.1% severe damaged cells and 23±2.4% damaged cells (Figure 5&6).

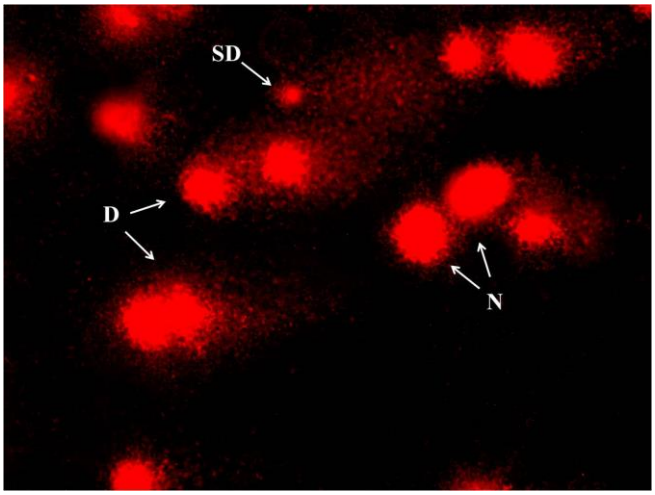

Figure (5): Photomicrograph showed single strand breaks (comet assay or Single Cell Gel Electrophoresis assay) of DNA of rat leukocytes. (N) Normal DNA spots (no migration), (D) Damaged DNA spots (migration towards the anode), (SD) Strong damaged DNA spots (more migration towards the anode).

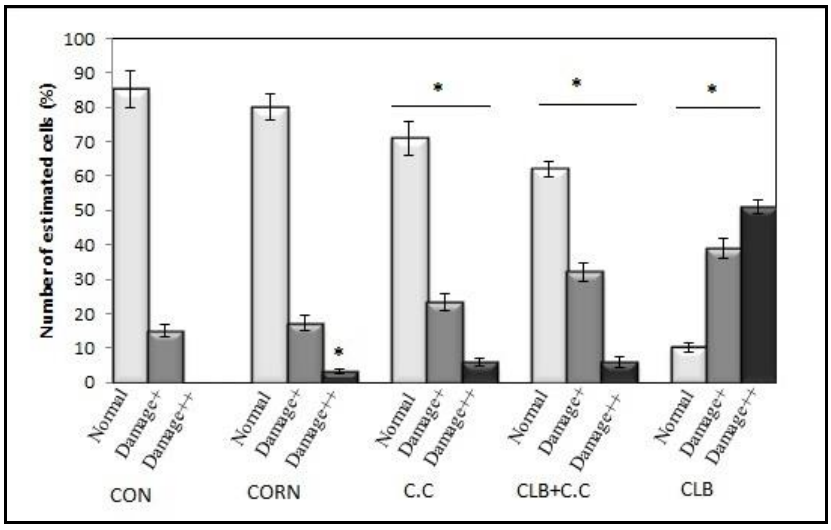

Figure (6): The effect on lymphocytes DNA damage of rat detected by comet assay after treatment with *Cymbopogon citratus* (CC) against CLB toxicity for 48 hours**.**

3.5. *The curative role of CC volatile oil against CLB effect on total proteins of spleen tissues*

Protein banding patterns of spleen tissues on 12% of SDS-PAGE after 15 days of treatments revealed that spleen tissues of CC-treated animals showed improved protein pattern when compared with CLB damaging effects (Figure 7).

Samples for control, corn oil and CC showed no significant differences between protein bands among the electrophoretic migration; ranging between 26.6 and 180 KDa. On the contrary, there was an absence of three bands in CLB-treatments lanes. This was improved to only one absent lane in CC-treated lane (Figure 7).

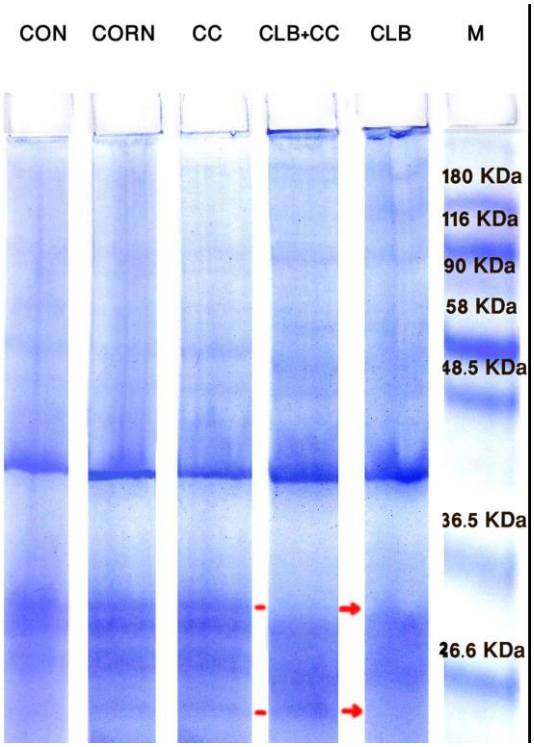

Figure (7): Photomicrograph of the spleen proteins profile by SDS-PAGE (12%) showed the effect of CLB (7.5 mg/kg/day) and the curative effect of *Cymbopogon citratus* (CC) against CLB after fifteen days of treatment. CON resembles control liver; CORN: corn oil; CC: *Cymbopogon citratus* oil; CLB+CC: *Cymbopogon citratus* oil after one hour of CLB dosing and M: (26.6 – 180 KDa).

4. Discussion

Mutations and DNA damage could be induced by drugs or irradiation commonly used in cancer treatment strategies. It not only kills cancer cells but also normal cells. One of these strategies is chemotherapy which is commonly used in cancer treatment.

Pre-carcinogenic lesions were found to be formed by covalent binding of alkylating agents (mutagens) to DNA (Miller and Miller, 1976). The DNA adducts are formed as a result of the reaction of metabolically generated electrophilic ions with nucleophilic sites in DNA (Magee and Barnes, 1967). Chlorambucil is considered as one of the alkylating anticancer drugs. They stop tumor growth by cross-linking guanine bases in DNA double-helix strands preventing DNA replication and cell division (Rai et al., 2000).

Chlorambucil also lead to DNA damage via the formation of cross-links or mispairing of the

nucleotides leading to mutations (Wishart et al., 2008). The most frequent location of adducts in the DNA is at guanines (Sanderson and Shield, 1996). Bank (1992) revealed that the majority of adducts induced by CLB are guanyl adducts. The reactivity of alkylating agents with DNA, RNA and proteins can cause cell death. It has been shown to be potentially carcinogenic, mutagenic and teratogenic, and to promote secondary malignancies (Saffhill et al., 1985; Sanderson and Shield, 1996).

The present results showed that CLB induced mutagenic effects including DNA damage after fifteen days of treatment, while a non-significant damage in spleen was observed after five and ten days. Apoptosis as a cell death mode could be shifted towards necrosis by stimulation of genotoxic stimuli that are responsible for damaging the proteins or genes that make these proteins and other cellular macromolecules which may be required for apoptosis Singh (2000).

These results are in agreement with the Bacsia et al., (2005) who mentioned that the induction of DNA double-strand breaks in CLB-exposed cells. In addition, the present findings are in agreement with the results of Wang et al., (2003) which reported that the drug-induced DNA alkylation / damage lesions were single-stranded (DNA mono-adducts) or double-stranded (DNA inter-strand crosslinks).

Mughal et al., (2010) concluded that alkaline single cell gel electrophoresis (SCGE)/comet assay can successfully detect the genetic damage of peripheral blood. The results revealed that CLB induced severe damage in peripheral blood leukocytes cells when compared with normal cells. These results were in parallel with (Mughal et al., 2010), who reported the DNA damage occurrence in the peripheral blood lymphocytes evaluated by comet assay between 36 and 48 h of CLB treatment in rats.

For thousands of years, physicians had believed that certain foods and plants had curative or restorative powers, and much of early medicine involved the prescription of certain foods or plant extracts to cure medical conditions (Stainmetz and Potter, 1991). The prevention of medication side effects is a worldwide interest. There have been many studies on the functionality of natural chemicals in foods and drinks.

The results of phytochemical investigation of lemongrass oil constituents by Gas Chromatographic-Mass Spectrometry (GC-MS) showed the presence of one major compound, citral, which is a mixture of the *cis* and *trans* non-cyclic monoterpene aldehyde isomers, neral and geranial. Myrcene was also detected. These results agree with most authors in this point of investigation with some geographical variations (Lewinsohn et al., 1998; Muhammad et al., 2003; Lewinsohn et al., 2005; Tchoumbougnang et al., 2005). These findings were found to be the reason of considering that lemon grass oil is one of the natural plants with antigenotoxic prosperities and protect cells from cell death and apoptosis.

In this study, results showed that *C. citratus* can exert preventive effects on the development of CLB mutagenic potential. Results of comet assay revealed that administration of *C. citratus* showed significant improvement by reduction of tail length and less DNA damaged cells percentage when compared with CLB treatment groups.

The curative effects of lemon grass may be due to the antioxidant activity or its ability to inhibit beta-glucuronidase activity (Suaeyun et al., 1997). In addition, the chemoprevention associated with lemongrass volatile oil treatment is characterized for monoterpenes compounds and citral (Onawunmi et al., 1984; Rabbani et al., 2004). It is the major component (65-85%) and consists of small quantities of monoterpene olefins, such as myrcene (Silva et al., 2008). Myrcene exerts its antimutagenic activity by inhibiting certain forms of the cytochrome P-450 isoenzymes required for activation of premutagens. Precarcinogenes is discussed too (Kauderer et al., 1991; Kim et al., 1992).

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