Assessment of the Cyto-Toxicity of *Teucrium Capitatum* to the Hepatic Cell Line, HepG2

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ABSTRACT

Teucrium capitatum (TC) is used in the Palestinian folk medicine to treat colic and intestinal infections. Our previous investigation revealed antimicrobial virtues of TC (Ghareeb and Weld Ali, 2015; Hijjawi Prize 2013). However, TC is suspected to cause hepatitis. This research intends, therefore, to assess the effect of TC on human hepatic cells (HepG2 cell line) using the cellular viability test, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. TC is extracted in the Palestinian folk medicine by the juice or the infusion in boiled water and these two methods are assessed in this research.

For infusion extraction method and at a cellular confluence of 10,000 cells/well (in the 96-well plate), cytotoxicity of TC starts at 1.6 mg of the plant extract/ ml of HepG2 culture medium, toxicity increases in a directly-proportional manner as the plant dose increases (up to 35 mg/ml).

At the same confluence (10,000cells/well) but using juice extraction method, cytotoxicity starts at 6.4 mg/ml, toxicity increases also in a directly proportional manner as the plant dose increases (up to 35 mg/ml). Assays are also conducted using a different confluence of 5,000 cells/ well (in the 96-well plate). Using infusion method, toxicity starts at 0.2 mg/ml, and increased as the concentration increases to 35 mg/ml. Using juice method, toxicity starts at a higher dose (6.4 mg/ml).

In both cases, the HepG2 viability decreases dramatically when applying high doses of TC (either as a juice or as infusion). Juice extraction method appears, however, to decrease viability of HepG2 cell line to a less extent compared with the infusion method. This result conforms to the Palestinian traditional extraction of TC. After more validation on more cells then on humans, we could be able to recommend using juice rather than infusion extraction method to treat colic without side effects on hepatic cells. Palestinian traditions reveal to be efficient in both the choice of medicinal plants and the extraction method!

Keywords-*Teucrium capitatum*, hepatitis, HepG2, toxicity, medicinal plants, MTT assay, Arab Palestinian folk medicine.

1. INTRODUCTION

For a very long time, plants were used in the treatment of many diseases. Palestinian historical heritage as a part of the Arabic culture harbor a reservoir of traditions of use of medicinal plants as folk medicines. The whole above ground parts of *Teucrium capitatum* (Ja'da) is used in traditional Palestinian folk medicine especially in Palestinian Bank areas and Al-Nakab desert, as well as Persian medicine to fight against colic and diarrhea. *Teucrium capitatum* seems to possess a positive effect against the bacteria that cause colic and Stomach problems like *Escherichia coli* and *Pseudomonas aeruginosa*. This research has to be conducted in order to complement the 2013 Hijjawi Prize winner research entitled: Assessment of Antibacterial Virtues of *Teucrium capitatum* (Ja'da) on Pathogenic Bacteria (From Folk to Complementary Medicine). Our previous work demonstrated antibacterial virtues of *Teucrium capitatum* (Ghareeb and Weld Ali, 2015; Hijjawi Prize 2013). The present research assays for the appropriateness of using this plant especially for the human liver because hepatitis was reported to be caused by *Teucrium* (Dourakis et al., 2002).

Teucrium capitatum (TC) is used as herbal tea also in some Mediterranean cultures (Shebaro, 1997). These traditions should not be underestimated as they emerge from thousands of years of empirical experience. Its flowers are small and range from pink to white (see fig 1). Leaves grow in September- November; the flowers appear in April-August (Ishtayeh, 2008).

TC is a perennial, pubescent, aromatic plant, 20–50 cm high, with green to greyish leaves and white flowers. It grows wild in southern Europe, central and south-west Asia and North Africa. This plant is scarcely found in continental France. In the world, there are more than 200 type of *Teucrium*, for example *Teucrium ajugaceum*, *Teucrium botrys*, *Teucrium chamaedrys*, *Teucrium polium*, *Teucrium capitatum* (under assessment in this research) and others (Mostefa-Kara et al., 1992; Dao et al., 1993).

Figure 1: Leaves and flowers of (*Teucrium capitatum*). Leaves appear opposite, entire, dentate, and color of the flower is cream and white. Specimens collected and images captured from Qabatia Mountains (Jenin District-Palestine) on 13/11/2013.

Teucrium capitatum is reported to contain many important alkaloids known as cetkaderan as well as saastron, volatile oil, arbohydrate (glucose, fructose, sucrose, ramnoz, and raffinose), sterolat and turbines, flavone, gelokozydat, flavonoids, and volatile oil. More than 45 compounds are described in TC, including the compound responsible for the well-known bitter taste of this plant (pkrobolin). (Al-Qahtani, 2005).

Studies report that the oil isolated from *Teucrium capitatum* grown in Portugal was characterized by a high content of oxygenated monoterpenes (33.0%), isomenthone (7.7%) being the major constituent. Another oil from a population collected from the same region was dominated by monoterpenes and sesquiterpene hydrocarbons (43.9% and 23.2%, respectively). The oils from other three populations were characterized by a high content of both sesquiterpene hydrocarbons (23.0%, 32.2% and 33.2%) and oxygenated sesquiterpene (39.7%, 23.4% and 20.4%). T-cadinol (24.1%) and α-cadinol (9.8%) were the major compounds in the oil (José et al., 2004; Calzada et al., 2006; Tutin and Wood, 1972; Maranta, 1981)

Such components were demonstrated to act as antimicrobial agents though in another plant species (oregano) (Lambert et al., 2001).

HepG2 is a perpetual human hepatic cell line, these cells are epithelial in morphology, the cells secrete a variety of major plasma proteins, for example albumin, transferrin, and the acute-phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin, and plasminogen. HepG2 cells used as a model system for studies of liver metabolism and toxicity of xenobiotics, the detection of environmental and dietary cytotoxic and genotoxic (and thus cytoprotective, anti-genotoxic, and cogenotoxic) agents (Mersch-Sundermann et al., 2004).

Poisons undergo metabolic processes where drugs are chemically altered by cells to more water soluble metabolites to allow elimination in urine or bile or by increasing access to drug excretory transporters. A feature of hepatic metabolism pathways is the never-ending array of drug substrates that are successfully metabolized by the liver. The liver is perfectly designed as a drug removal organ. (Anonymous, 2007). The liver provides an efficient barrier that prevents poisonous compounds like xenobiotics from entering the body's circulation (Wilkening et al., 2003).

Previous studies report that TC causes hepatitis. A 62-year-old Caucasian man was admitted to hospital due to an acute icteric hepatitis-like illness. The patient reported having hypercholesterolaemia and hyperglycaemia, for which he started daily consumption of a tea containing the medicinal plant TC. Anorexia, nausea, and malaise appeared 4 months later. The patient also noticed hyperpigmentation of his urine for 5 days. He stopped drinking the TC tea but became jaundiced the next day. He denied taking any drugs orally or intravenously, and denied alcohol abuse (Larrey, 1997; Mostefa-Kara et al., 1992). However, this is only one case of hepatitis claim to be caused by this species of *Teucrium*. Importantly, there are many hepatitis cases claimed to be caused by other species of *Teucrium*, like *Teucrium chamaedrys* where an outbreak of hepatotoxicity occurred in 27 cases with one death from acute, non-viral hepatitis (Alberti et al., 1992; Dourakis et al., 2002). Consequently, it is of utmost importance to assess the effect of TC on the hepatic cells which is the main objective of this research. In this work, TC is assayed in perspective of using it as an anticolic and antidiarrheal folk medicine (Hijjawi Prize 2012-2013) without detrimental effects on the liver. TC extracts are therefore assayed for their effects on the human hepatic cell line, HepG2.

2. MATERIALS AND METHODS

2.1 Cell lines

The human hepatic cell line HepG2 cell line was kindly provided by Dr. Hilal Zaid; from Al Qasemi Center Research center in 15/11/2013 and kept in the freezer at -80 C°. It was culture and subculture in order to assay for

the TC effect on the viability of hepG2 cell line using the MTT viability test.

The cells are grown in DMEM (Dulbecco's Modified Eagle Medium) which is a widely used basal medium for supporting the growth of many different mammalian cells, with a high glucose content(4.5g/L) supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acid, 1% glutamine, 100 U penicillin /ml and 10 mg streptomycin/ml. Cells are maintained in humidified atmosphere with 5% CO_2 at 37C. The medium of cells is changed twice a week. At 70-80 % confluence, cells are trypsinized and seeded in 96-well plates in cell density of 1.0×10^4 and 5.0×10^3 HepG2 cells in each well (for assessment of cytotoxic and cytostatic effects of TC respectively). 24 hours after cell seeding, cells exposed to various concentrations of the plant extracts in fresh serum-free medium. Hepatic cell are known to represent the detoxification center of animal (Celton-Morizur et al., 2011). Therefore, any measured plant toxicity on HepG2 can be expected to appear in the whole organism.

***Teucrium capitatum*:** Harvested from Qabatia Mountains on 13/11/2013, and then kept in the freezer at -20 C° after weighting.

2.2 Plant Extractions

Teucrium capitatum was extracted using two methods both of them are in NaCl solution (0.9%):

(1) Making juice by crushing or maceration with gloved hands for 3 minutes (in Arabic mars), applied on the above ground parts (leaves and stems). This was accomplished after washing of the plant and letting it to dry for 1 hr. The extracts was collected and filtered using sterile micro filters (0.2 µm) and kept in freezer until application on HepG2.

(2) Infusion method by putting the above-ground parts of the plant in boiled 0.9% NaCl solution and letting for infusion until the extract attains room temperature (5 hrs.), and then filtering the infusion extract using 0.2 µm and keeping it under freezing until application on cells.

2.3 Controls

Two negative controls are used in this research

1- HepG2 Cell line (100 µl of an overnight culture) in medium without any addition of TC.

2- HepG2 Cell Line (100 µl of an overnight culture) in medium with lettuce, but without TC. These negative controls used in parallel with HepG2 cells treated with TC aim to objectively assess the effect of TC on cells.

2.4 MTT assay

The MTT assay is performed to assess the effect of the plant extracts on the viability and proliferation as cell. MTT (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) standard colorimetric assay, is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolim rings of the pale yellow MMT and from a dark blue or purple Formosan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells solubilization of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formosan products created. The color can then be quantified using a simple colorimetric assay. The results can be read on a multi-well scanning spectrophotometer (ELISA reader) at 570 nm (Berridge and Tan, 1993).

2.5 Cytotoxicity assay

At 70-80% confluence, cells were detached from the cultured flask by treatment with 0.05% trypsin- EDTA and a suspension of 1.0×10^4 cell/ml viable cells was seed in a 96-well micro-titer plate and incubated for 24 h. At this cell density, cells were in confluent monolayer. When cells reached >80% confluence, the medium was replaced and cells were incubated with stock solutions of crude extracts serially diluted to reach concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 and 35 mg/ml. After 24 h of incubation, add 70 ml of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution (0.5mg/ml) were added to each well and incubated at 37°C for 4 h. The medium then aspirated, and the formazan product was solubilized with 70 ml acidified isopropanol (0.4N HCl).

2.6 Cytostatic Assay

For the determination of cytostatic effects, cells were seeded at lower cell density at which cells were at about 50-70% confluence. Therefore, 5.0×10^3 were plated in 96-well-plate and were treated with the plant extract in different concentrations as mentioned in the step previous, then incubated for 24 hours at 37°C. Following the removal of plant extracts from each well, the cells were incubated in which MTT (0.5 mg/ ml) was added to each well (70 ml), and then cells were incubated for 4h at 37°C. The medium was removed and 70 ml of isopropyl alcohol was added to dissolve formazan crystals. The plate then was covered with tinfoil and agitated on orbital shaker for 5 min.

3. RESULTS AND DISCUSSION

3.1 Cytotoxic vs Cytostatic Properties

The agents that cause death of cells through controlling cell division and interrupting DNA synthesis is called cytostatic agents. In contrast, cytotoxic agents cause the death of existing cells in culture. Such agents may also be toxic to normal cells such as bone marrow, hair, and mucosa and this depends on the intensity of the dose from these agents (Blakeley and Grossman, 2012). In case of cytotoxicity, cells may undergo cell lysis and decrease in cell viability, or apoptosis. In the cytotoxicity assays of this research, the number of cell is high 10,000 cell/well, as contrasted with 5000 cell/well, which are used in cytostatic assays.

The following table depicts the optical density (OD) readings with different concentrations of *Teucrium capitatum* after applying of different doses of *TC* compared to appropriate controls (cells without TC, cells with lettuce). In addition, the two extraction procedures are contrasted here (the juice method vs the infusion method). Assays done thrice and the average are shown.

Table 1: Different concentration of the plant (*Teucrium capitatum)* applied to (1.0×10^4 cell/well) HepG2 cells. The applied concentration is expressed in terms of mg of plant extract /ml of HepG2 medium. The effect is measured using MTT viability assay through optical density (OD) readings and ELISA reader. 8-replicate average using plant extract is referenced to 8-replicate average without plant extract.

Concentration (mg/ml)	juice	Infusion	fresh lettuce
0	1.287375	1.080875	1.04125
0.1	1.23175	0.943375	1.17725
0.2	1.223875	0.94525	1.396625
0.4	1.168625	1.04975	1.347125
0.8	1.179625	0.972	1.326625
1.6	1.22475	0.653	1.266375
3.2	1.055875	0.615	1.310125
6.4	0.844375	0.551	1.5015
12.8	0.35875	0.406875	1.38025
25.6	0.0795	0.141625	1.35675

Table 2: Different concentrations of the plant (*Teucrium capitatum)* applied to (5.0×10^3 cell/well) HepG2 cells. The applied concentration is expressed in terms of mg of plant extract /ml of HepG2 medium. The effect is measured using MTT viability assay though the optical density (OD) readings and ELISA reader. 8-replicate average using plant extract is referenced to 8-replicate average without plant extract

Concentration(mg/ml)	juice	Infusion	fresh lettuce
0	0.1445	0.14875	0.178125
0.1	0.144625	0.155125	0.20525
0.2	0.14	0.11825	0.20275
0.4	0.141375	0.122	0.20175
0.8	0.138625	0.119625	0.1665
1.6	0.1535	0.114125	0.17975
3.2	0.1505	0.110125	0.182875
6.4	0.130625	0.06925	0.156875
12.8	0.114625	0.049125	0.145125
25.6	0.064625	0.047	0.132375
35	0.05225	0.05075	0.053625

The following figure demonstrates response of HepG2 cells under the effect of *Teucrium capitatum* extracted by the juice and infusion methods contrasted with appropriate controls (e.g. lettuce) through the MTT viability assay. We referenced HepG2 viability values with plant extract to the average of HepG2 viability without plant extract combined for both extraction methods. For example, at 6.4 mg of plant extract/ml of medium (Figure 2 below), the MTT readings were averaged for 8 replicates and the average was divided by the MTT readings averaged also for HepG2 cells without plant extract.

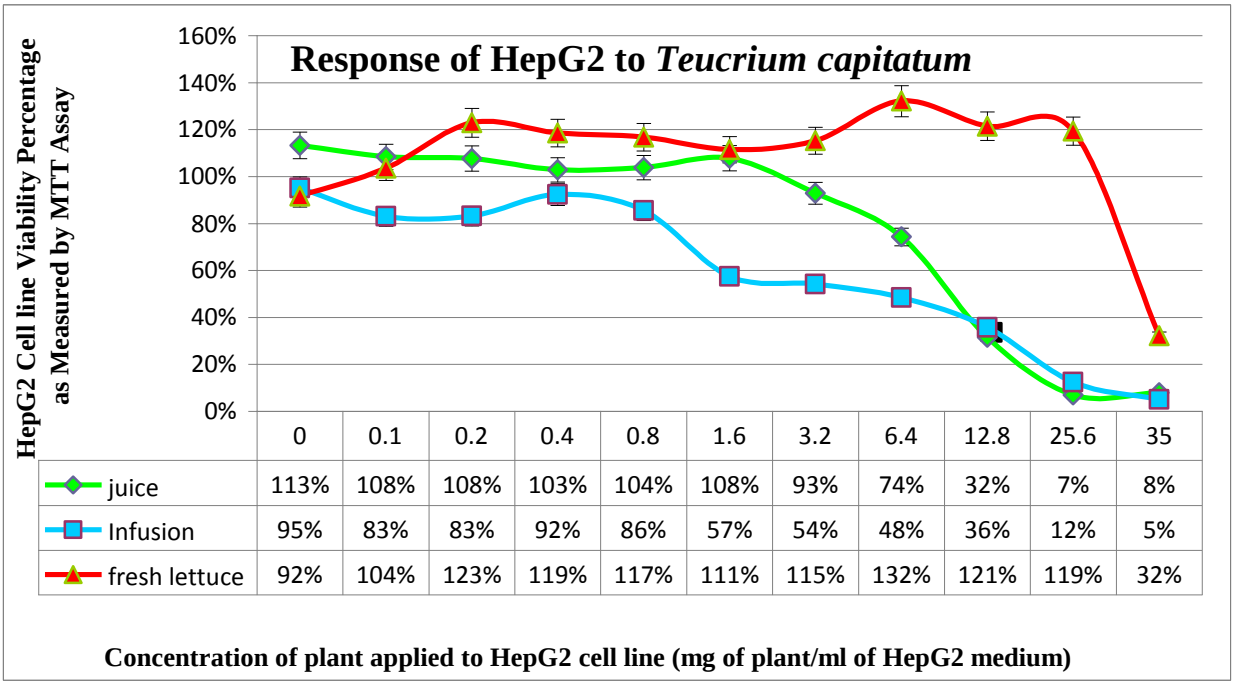

Figure 2: Different concentrations of the plant (*Teucrium capitatum)* are applied to HepG2 (10,000 cell/ml). The applied concentrations are expressed in terms of mg of plant extract /ml of HepG2 medium. The effect is measured in terms of MTT viability assay. Values are means ±SD, N=8. Error bars Percentage ≥5 for each point. 8-replicate average using plant extract is referenced to 8-replicate average without plant extract.

At the used doses, *Teucrium capitatum* extracted by infusion demonstrate a clear toxicity to HepG2 cell line (cytotoxicity at 10000 cell/ml) more than the juice method (figure 2). Interestingly, lettuce does not show toxicity at most concentrations (up to 25.6 mg/ml). When using infusion extraction method, 1.6 mg/ml leads to HepG2 viability of 57%. Nevertheless, when using the juice extraction method and at the same plant dose (1.6 mg/ml), the viability of HepG2 is 108%. This result can be meaningful for the better plant extraction method (i.e. that should not be toxic for hepatic cells). However, at higher concentrations (e.g. 6.4 mg/ml), both methods lead to HepG2 toxicity even though to a less extent in case of juice method (viability 74%) as compared to the infusion method where viability is about 50%.

The above results demonstrate the cytotoxic effects of *Teucrium capitatum* on HepG2 cells. To complement results, we attempted to demonstrate also the cytostatic effect of the plant on the same cells by applying different concentrations of the plant on 5000 cells/well. Results are shown in figure 2 (below)

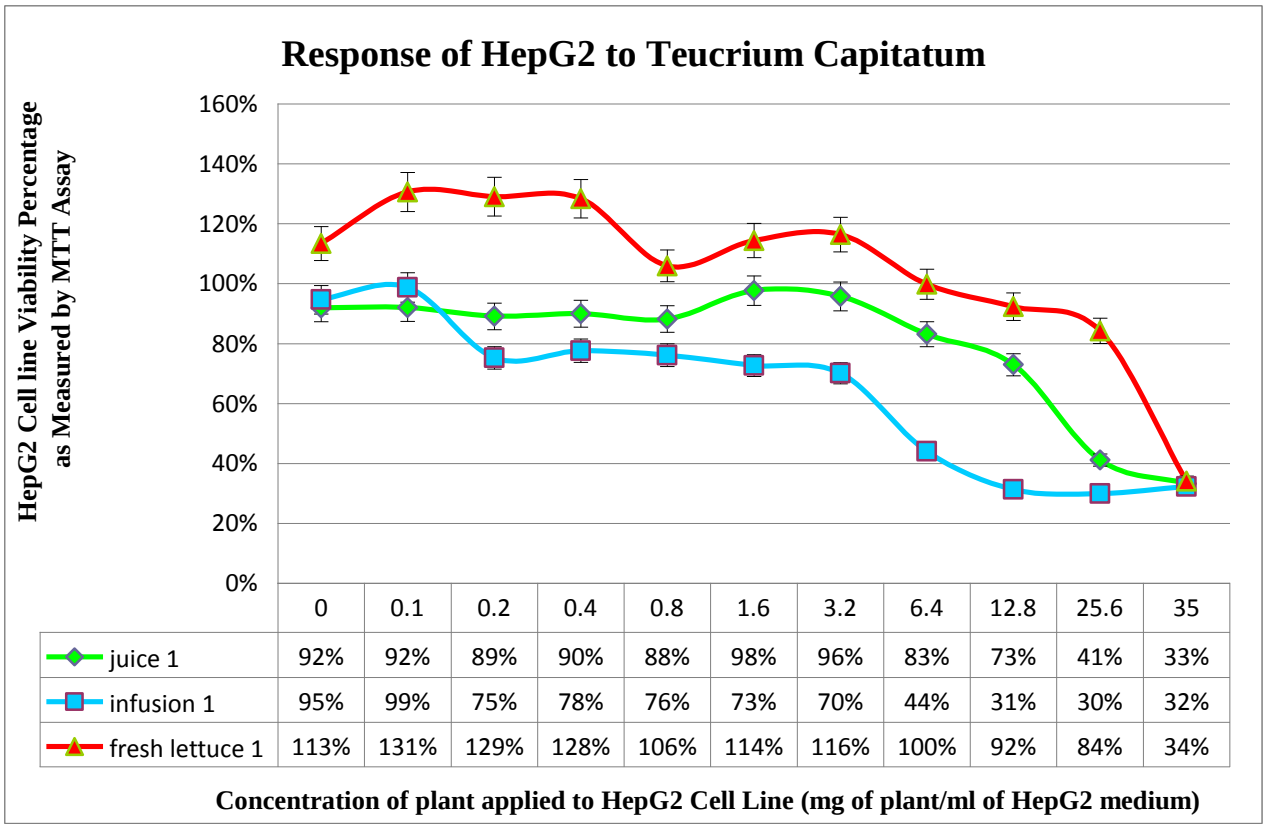

Figure 3: Different concentrations of *Teucrium capitatum* applied to HepG2 (5000 cell/well). The applied concentrations are expressed in terms of mg of plant extract /ml of HepG2 medium. The effect is measured in terms of MTT viability assay. Values are means ±SD, N=8. Error bars Percentage ≥5 for each point. 8-replicate average using plant extract is referenced to 8-replicate average without plant extract.

Similarly to the results demonstrated in figure 1 (Cytotoxic effect at 10000 cell/well), results here (Cytostatic effect at 5,000 cells/well) show a cytostatic effect of *Teucrium capitatum* on HepG2 cell line. Once again, the infusion extraction method is more severe than the juice method on HepG2 cell line. This confirms the promising results (in figure 2) about the better extraction method! Lettuce has been very useful as a control in the above experiments. It shows no detrimental effect on the growth of HepG2 cell line especially at concentrations up to 6.4 mg/ml.

These results are relieving regarding the Palestinian old traditions in extraction of medicinal plants. Juice method (practiced by our ancestors) proved to be both efficient against bacteria (2013 Hijjawi Prize) and to be less cytotoxic and less cytostatic than the infusion extraction method. The reported hepatitis cases in France after using *Teucrium capiatum* could be the result of an inappropriate plant extraction method. (Dourakis et al., 2012). Our research demonstrates original scientific and cultural results!

4. CONCLUSIONS

Our previous study was the first to assess the antimicrobial virtues of *Teucrium capitatum* on pathogenic bacteria (Hijjawi Prize 2013). Those results fit with Palestinian use of this herb since long time against colic. The present study aims to assess the safety of this folk traditional knowledge more precisely to the liver in the light of some reports about hepatitis caused by this plant. Results here show that *Teucrium capitatum* is neither cytotoxic nor cytostatic to HepG2 cell line at plant doses up to 3.2 mg/ml of cells medium. This is promising for perspective use of this plant against colic without harming the liver. In addition, the Palestinian-traditionally and originally employed juice method proves to be less toxic to HepG2 cell line than the commonly used infusion extraction method (encountered frequently in literature). In fact, extraction of plant juice accompanied by maceration and squeezing under pressure is expected to extract both organic and inorganic soluble substances.

4.1 Perspectives and Recommendations

Further investigations can be needed to determine the cytotoxic and cytostatic compounds found in *Teucrium capitatum*. Such compounds can be saponins, glycosides, flavonoids, several furan-containing neoclerodane penoids (NCD) and Teuchamaedryn A compounds. Teucrine A is another toxic furano neoclerodene diterpenoid substance, which is found in flowers belonging to the *Teucrium* genus

and in in Wood Sage. It is a hepatotoxic agent that can damage the liver. (Ulubelen et al., 1994; Piozzi et al.)

Further investigations can idealize the results obtained on *Teucrium capitatum* regarding the antimicrobial virtue of this medicinal plant (Hijjawi Prize 2013) and also regarding the cytotoxic and cytostatic effect of this plant on the human hepatic cells (Research presently submitted to Hijjawi Prize 2014). More plant doses and more cell lines can refine the obtained results. Experiments on animals are essential for validation and should be conducted with medical control. We hope that *Teucrium capiatum* will be validated as an efficient folk medicinal plant against colic without detrimental effects on the human liver especially when using the Palestinian traditional extraction method (i.e. the juice method described above rather than the commonly used infusion method). This is an original finding at both the scientific and rich Palestinian cultural levels!

CONFLICT OF INTEREST

The authors declare no conflict of interest

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