

In vitro inhibition of sheep liver glutathione-s-transferases activity by different extracts from *Ephedra aphylla* and *Ephedra foeminea*[†]

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ABSTRACT

Glutathione-S-transferases (GSTs) play an important role in the detoxification of chemicals that may lead to mutagenic or cytotoxic effects. In this study, we aimed to find the effect of aqueous, methanol and ethanol extracts of both *Ephedra aphylla* and *Ephedra foeminea* on the activity of the purified sheep liver GST at the following different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) spectrophotometrically using 1-chloro- 2, 4- dinitrobenzene (CDNB) as substrate. The obtained results revealed that all examined extracts types of *Ephedra* species under study manifested inhibitory effect on GSTs activity at all screened concentrations. The inhibitory effect on GST activity by the alcoholic extracts of the two species under investigation at all tested concentrations was more than the extracts when compared to controls. Nevertheless, this was more pronounced in *E. aphylla* rather than in *E. foeminea*. It is worth mentioning that all the GSTs inhibitory effects displayed dose dependent manner under all studied extracts. On that account, the obtained results in this research confirms the medicinal importance of both *Ephedra* species under study, which provides the possibility of their use in whatever benefit human health in general and combat GST-induced resistance in drug resistant tumors in particular.

Keywords: Glutathione-S-Transferases, *Ephedra aphylla*, *Ephedra foeminea*, Plant Extracts.

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INTRODUCTION

Glutathione-S-transferase isoenzymes (GSTs) are widely distributed in nature and present in different organisms such as microbes, plant, fish, insect, birds and mammals [1]. Glutathione-S-transferases contains two super families of enzymes that possess transferase activity [2]. The first enzyme family type is cytosolic [3]. Eight families of cytosolic mammalian enzymes are designated as Alpha, Mu, Pi, sigma, theta, Zeta, Omega and Kappa [4, 5, 6, 7]. However, there are four additional classes of this super family, called Beta, Delta, Phi and Tau which are represented in bacteria, insect and plants [8]. The second enzyme family type is membrane-associated protein in eicosanoid and glutathione metabolism (MAPEG) [9]. Cytosolic GSTs are mostly involved in the metabolism of foreign chemicals, such as carcinogens, environmental pollutants and cancer

chemotherapeutic drugs as well as the detoxification of potentially harmful endogenously derived reactive compounds [1]. By contrast, MAPEG members are not principally involved in detoxification reaction, but instead involved in the biosynthesis of leukotrienes and other things [9]. Never the less, their activity is motivated by the action of the cytochrome P450 (CYP) enzymes. As they catalyze the introduction of a functional group, such as an epoxide, into inactive xenobiotic (toxic substance). The electrophilic center of the functional group attacked by the reduced glutathione (GSH), which is catalyzed by GSTs through the conjugation reaction [3]. The addition of GSH to the toxic molecules gives it a molecular flag, which allow the xenobiotic-conjugate to be removed from the cell [8]. Those transferases are able to detoxify broad spectrum of noxious chemicals that may lead to mutagenic event or cytotoxicity [1]. Specific isoenzyme of GSTs are over

expressed in a wide variety of tumors and may play a role in the etiology of other diseases, so it has emerged as a promising therapeutic target.

Throughout history, the Plant Kingdom has represented a significant source for the discovery of new drugs with important therapeutic effects in different areas of medicine. Various types of plant extracts include a large range of phytochemicals, which can be on their own or through a synergistic mechanism useful for different therapeutic activities. At the moment, many of the currently used drugs in well-established therapeutic protocols are directly obtained from, or are chemical derivatives of phytochemicals [10].

Glutathione-S-transferases activities have previously been shown to be modulated by plant products [11]. As a result, the effect of different plant extracts on the GST activity was investigated. In this aspect, some of the studied plant species increased GST activity such as *Orthosiphon stamineus* [12]. Furthermore, other plant species like *Areca catechu* showed activity inhibition for GST [13]. Moreover, the effects of plant species on the activity of GST depend on the extract type used in the experiment. Also *in vivo* and *in vitro* studies for the same extract type may showed variable effect on GST activity [14].

Palestine has a rich and prestigious heritage of herbal medicines. More than 700 species of medicinal plants are known to exist, and approximately 63 of these are actively used for the preparation of traditional medicines [15]. Therefore, many herbal remedies are still used by herbalists in Palestine for treatment of several diseases, some of them have been approved scientifically while others are not [16].

One of those medicinal plants in Palestine is *Ephedra* different species. Therefore, the clinical interest in the folk use of *Ephedra* increased during the 20th century. *Ephedra* is a Chinese shrub belong to the family of *Ephedraceae* that is phylogenetically very old plant belonging to the group of gymnosperms (literally ‘naked seeds’), which also includes pines, firs and larches [17]. There are some 45 species of *Ephedra* distributed all over the world, particularly in coastal and subalpine areas [18]. Furthermore, *Ephedra*

species are used in the folk medicine as decoction as a stimulant, a deobstruent, to treat kidney, bronchi, circular system, digestive system disorders and to relief asthma attack as well as used for treatment of cancer also the plant stems are chewed for treatment of bacterial and fungal infections [19]. For example, *E. foeminea* recently became one of the most commonly utilized plants by the Palestinian population primarily due to local media reporting of its decoctions as an effective herbal remedy for cancer patients [20]. Several investigations considering the bioactivity of different species of *Ephedra* in Palestine were carried out considered different aspects such antioxidant activity, antibacterial or anticancer ones [16, 21].

So keeping in view all this background and from this point of view, the present study was designed to investigate the impact of the prepared aqueous, ethanol and methanol extracts from *Ephedra aphylla* and *Ephedra foeminea* on the activity of the purified hepatic GSTs. As this study was not previously studied in Palestine considering that issue which provides its novelty.

MATERIALS AND METHODS

Plant Collection

Plant species *Ephedra aphylla* and *Ephedra foeminae* were collected from Tulkarim, West Bank, Palestine. They were identified by Ghadeer Omar, Department of Biology & Biotechnology, An-Najah National University; Palestine. Representative plant specimens were pressed till drying, treated chemically, mounted on herbarium sheets and provided with voucher numbers 1812 and 1895, respectively and then they were deposited at An-Najah National University herbarium. Then, whole parts of two plants species were washed with water, air dried for approximately 2 weeks in shade away from sunlight at room temperature. After that, the dried parts were placed in a grinder and crushed in order to get a fine powder. The powder was kept in plastic bags in dark place at room temperature until their use in preparation of plant extracts.

Plant Extracts Preparation

Ten grams of plants powder of the two species were soaked in 100 ml of distilled

water which were shaken on rotary shaker at 30 °C for 72 hours. After that, the soaked plant species were macerated by a probe sonicator (3 seconds sonication and 5 seconds rest) for 20 minutes at 40°C. Then, the mixtures were centrifuged for 10 minutes at 4000 rpm. The obtained extract supernatants were filtrated and evaporated by freeze-drying. The extracted powder of each plant species was dissolved in distilled water forming a stock working solution to a final concentration equal to 1000µg/ml. For alcoholic extraction, ten grams of each plant powder were soaked in 100 mL of (70%) ethanol or methanol for one week with interval shaking. Then the same steps of the aqueous extraction procedure were repeated, except that (10%) dimethyl sulfoxide (DMSO) was used as a solvent as ethanol may decrease the activity of GSTs and evaporation was performed by a rotary evaporator [22, 23].

Glutathione-S-Transferase Preparation

Sheep liver Glutathione-S-transferase enzyme was obtained from Protein Purification Laboratory, Biology and Biotechnology Department, Faculty of Science, An-Najah National University. The enzyme solution was prepared as the following, fresh sheep liver was washed, homogenized and centrifuged. Then the resulting supernatant that contains cytosolic glutathione-s-transferases was precipitated by ammonium sulfate at (30-70) %. After that, the obtained ammonium sulfate fraction was purified by gel filtration column chromatography using (Ultrogel ACA 44 column, Sigma). The protein levels for all fractions and GSTs activity for protein containing fractions were determined [24, 25]. Then, all fractions with GSTs were pooled and applied to affinity column (GSH-agarose, Sigma). After elution, all fractions were tested to determine GST activity and protein level [24, 25]. The fractions with GSTs were pooled, dialyzed, concentrated by freeze-drying to a concentration equal to 200 µg/ml and subsequently used for the activity studies.

Glutathione-S-Transferase Assay under Different Concentrations of the Plant Extracts

Glutathione-S-transferase activity was carried out on 1-chloro- 2, 4- dinitrobenzene

(CDNB) as a substrate, spectrophotometrically using (Seacomam spectrophotometer) as described by Habig et al., 1974 [25] with slight modifications as the used substrate concentration in the current assay was 1.5 mM instead of 1 mM. The cuvettes contained 0.2 M phosphate buffer (pH=7), 1.5 mM GSH, 1.5 mM of CDNB and 50 µl of diluted enzyme (1 µg/ml) in a final volume of 1.0 ml. Change in absorbance at 340 nm was followed against a blank containing all reactants except CDNB. The GSTs activity was expressed as µmol conjugate formed/min/ml using a molar extinction coefficient of 9.6 mM⁻¹.cm⁻¹. To determine the effect of all prepared extracts on the activity of GST enzyme different concentrations of the two plant species under study aqueous, methanol and ethanol extracts were prepared using distilled water for aqueous extracts and 10% DMSO for alcoholic extracts (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml). Then 50 µl from each of the extract types concentration was added to the enzyme reaction mixture. Distilled water and 10 % DMSO were used as negative controls for the aqueous extracts and the alcoholic extracts, respectively. Then the effect of each extract concentration on the activity of GSTs was measured by Seacomam spectrophotometer at 340nm and expressed as Inhibition percentage " $Inhibition \% = (GSTs \text{ activity without treatment} - GSTs \text{ activity under treatment of specific plant extract concentration}) / GSTs \text{ activity without treatment} \times 100 \%$ ".

Data Analysis

All samples were analyzed in triplicate. The results were expressed as mean ± standard deviation (SD). The concentration giving 50% inhibition (IC₅₀) was calculated by non-linear regression with the use of Microsoft Excel. The dose-response curve was obtained by plotting the percentage inhibition versus concentration.

RESULTS

The obtained results revealed that all examined different extract types (aqueous, methanol and ethanol) of *Ephedra* species under study manifested inhibitory effect on GSTs activity at all screened concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml). The recorded data of the GSTs activity after the ex-

posure to the different extract types concentrations of *E. aphylla* and *E. foeminea* are presented in Table 1. Results showed that the

effect of alcoholic extracts from the two species on the activity of GST at all studied concentrations is more than the aqueous ones.

Table (1): Effect of different extract types studied concentrations of *Ephedra aphylla* and *Ephedra foeminea* on GSTs activity.

Plant species Studied Concentrations (mg/ml)	<i>Ephedra aphylla</i>			<i>Ephedra foeminea</i>		
	Aqueous Extract	Methanol Extract	Ethanol Extract	Aqueous Extract	Methanol Extract	Ethanol Extract
0.1	6.8 ± 0.57	4.3 ± 0.31	4.6 ± 0.30	5.5 ± 0.11	3.7 ± 0.13	4.1 ± 0.30
0.2	6.2 ± 0.37	4.1 ± 0.53	4.2 ± 0.38	5.3 ± 0.73	2.5 ± 0.45	3.7 ± 0.48
0.4	5.8 ± 0.21	3 ± 0.22	3.3 ± 0.22	5.2 ± 0.35	2.3 ± 0.32	3.4 ± 0.29
0.6	5.7 ± 0.37	2.8 ± 0.14	2.5 ± 0.06	4.7 ± 0.28	2.1 ± 0.25	2.4 ± 0.52
0.8	5.5 ± 0.44	2.7 ± 0.32	2.2 ± 0.15	4.6 ± 0.31	1.9 ± 0.14	1.8 ± 0.38
1	5.3 ± 0.48	1.6 ± 0.09	1.4 ± 0.27	4.6 ± 0.47	1.6 ± 0.29	1.2 ± 0.17
Control	7.1 ± 0.63	7.6 ± 0.33	7.6 ± 0.33	5.8 ± 0.18	5.4 ± 0.49	5.4 ± 0.49

The IC₅₀ values for all plant extracts in the current research were determined (Table 2). It was observed that the IC₅₀ values of both the methanol and ethanol extracts of both plant species exhibited higher inhibitory effect on GSTs activity than the aqueous

ones. As the IC₅₀ values for methanol and ethanol extracts of *E. aphylla* and *E. foeminea* were 0.261, 0.294, 0.33 and 0.539 mg/ml respectively. While, the aqueous extracts of the two species had IC₅₀ values equal to 2.17 and 2.4 mg/ml, respectively.

Table (2): The IC₅₀ values (mg/ml) for *Ephedra aphylla* and *Ephedra foeminea* different extracts types.

Plant Extract	IC ₅₀ (mg/ml)
<i>E. aphylla</i> Aqueous Extract	2.170
<i>E. aphylla</i> Methanol Extract	0.261
<i>E. aphylla</i> Ethanol Extract	0.294
<i>E. foeminea</i> Aqueous Extract	2.400
<i>E. foeminea</i> Methanol Extract	0.330
<i>E. foeminea</i> Ethanol Extract	0.539

The inhibition percentage for all prepared extracts concentrations from the two studied plant species were shown in figure 1 and 2. All inhibition percentage values for the alcoholic plant extracts were higher compared to those of aqueous ones. *Ephedra aphylla* ethanol and methanol extracts were more potent inhibitor of the hepatic GSTs than the other alcoholic extracts from *E. foeminea* as observed by their inhibition percentage values. On the other hand, both methanol and ethanol extracts showed minor variations between them in both *E. aphylla* and *E. foeminea*. Contrary to that, the aqueous extract of *E. aphylla* was of higher recognized inhibitory effect than *E. foeminea* compared to the control. It is worth mention-

ing that all the GSTs inhibitory effects were observed in dose dependent manner in all studied extracts.

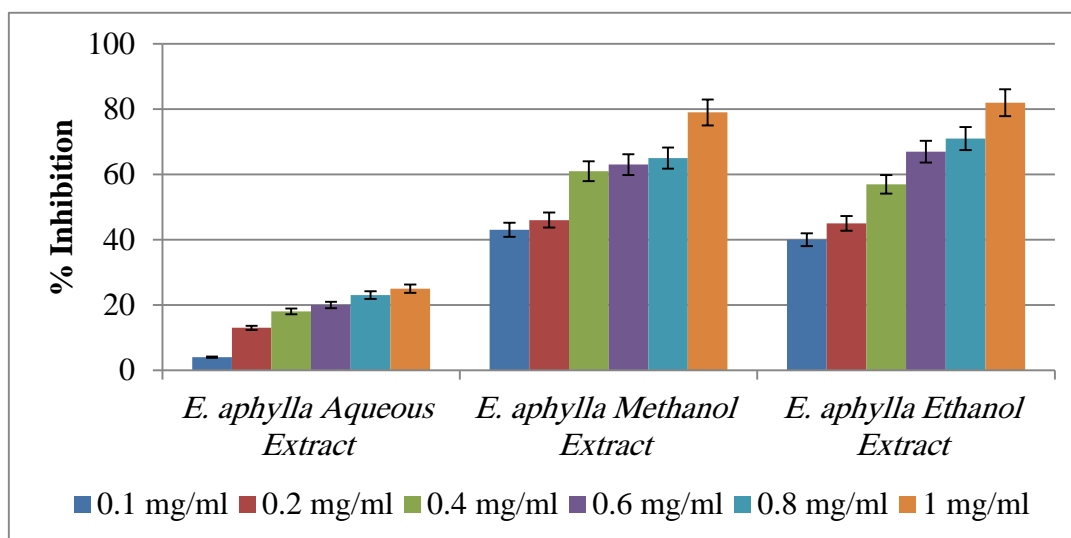


Figure (1): Effect of different extract types studied concentrations of *Ephedra aphylla* on GSTs activity.

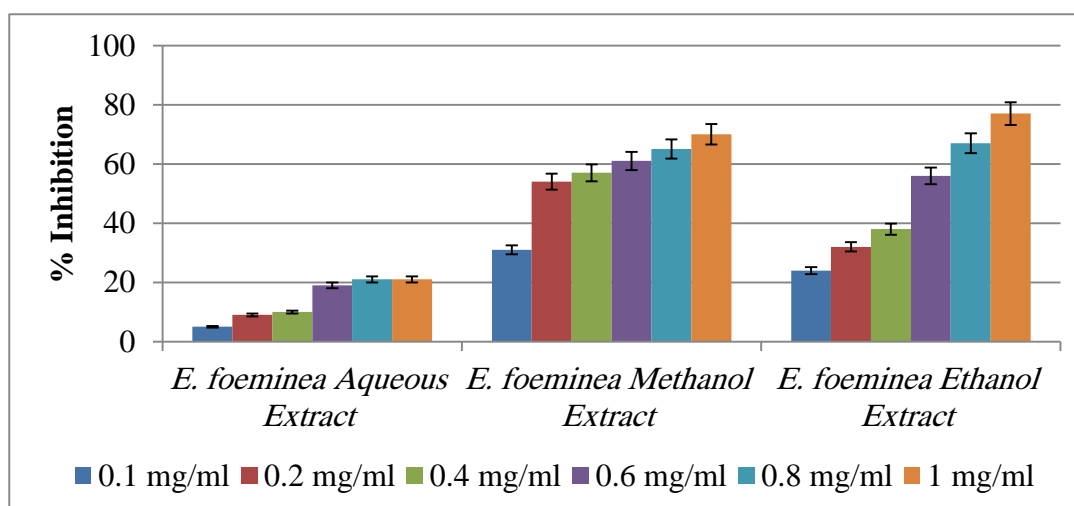


Figure (2): Effect of different extract types studied concentrations of *Ephedra foeminea* on GSTs activity.

DISCUSSION

Inhibition of the enzymes related to the family of GSTs is important from several points of view. These involve applications in studies of the catalytic mechanism, such as studying the topology and binding characteristics of the active site. Also, from a therapeutic standpoint, inhibition of GSTs steadily becomes more interesting. Since these enzymes appear to be involved in drug resistance, and in the biosynthesis of a number of important arachidonic acid metabolites such as prostaglandins and leukotrienes [26].

The main role of GSTs is the detoxification and metabolism of many xenobiotic and

endogenous compounds. Also they are over-expressed in some cancer cells. Therefore, GSTs contribute to the detoxification of anti-cancer drugs, leading to drug resistant tumors. For that reason, their inhibition has been suggested as an approach to fight GST-induced resistance [27]. Metabolism of the drug by GST renders the drug more soluble and enhances its excretion from the body. This would in turn reduce the therapeutic effects of the drug or even cause therapeutic failure as the residence time of the drug in the body is reduced. This in certain cases could be disadvantage while in others is an advantage [28].

Therefore, this means that the use of inhibitors to modulate the activity of GSTs during chemotherapy are a promising strategy in the battle against multi-drug resistance that could result in enhanced therapeutic efficiency of anticancer compounds. The inhibitors of GSTs may increase the sensitivity of cancer cells to antitumor drugs and then they may be used for several therapeutic applications [29].

According to that, the inhibition of GSTs has been extensively studied *in vitro* by several approaches. One of those approaches is the studying of plant compounds effect on the activity of GSTs. Different compounds from plants have been found to be inhibitors of GSTs enzymes as reported by many researchers. These compounds include: tannic acid, thoningianin A, cibacron blue, hematin, ethacrynic acid, ellagic acid, ferulic acid, caffeic acid, stilbene, quercetin, chlorogenic acid and curcumin [30].

Since GSTs are responsible for the synthesis of prostaglandins, the inhibition of GSTs by *Ephedra* examined species extracts may support their uses in the treatment of fever, pain and inflammation in ethnopharmacology [14]. As these enzymes are involved in endogenous activities that produce certain physiological products including pain mediators. Consequently, there is need to carry out further studies to isolate the bioactive compounds responsible for inhibiting GSTs and identify their structures.

On the other hand, their consumption should be under professional's supervision as their GSTs inhibition effect would in turn reduce the therapeutic effects of drugs or even cause therapeutic failure as the residence time of the drug in the body is reduced. Therefore, *in vivo* studies concerning the effect of the examined plant species different extracts on the activity of GSTs is recommended. Moreover, further experimentation is needed in order to study the precise mechanism of action of the effective examined plant extracts on the GSTs activity.

CONCLUSION

The obtained results in this research confirms the medicinal importance of both *Ephedra* species under study. This out-

finding provides the possibility of their use in whatever benefit human health in general and combat GST-induced resistance in drug resistant tumors in particular. So they increase the sensitivity of cancer cells to antitumor drugs.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interests.

REFERENCES

- 1) Hayes JD, Pulford DJ. The glutathione s-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol.* 1995; 30(6): 445-600.
- 2) Hayes JD, Strange RC. Glutathione-S-transferase polymorphisms and their biological consequences. *Pharmacology.* 2000; 61(3):154-166.
- 3) Mannervik B. The isoenzymes of glutathione transferase. *Advances in Enzymology and Related Areas of Molecular Biology.* 1985; 57: 357-417.
- 4) Meyer DJ, Coles B, Pemble SF, Gilmore KS, Fraser GM, Ketterer B. Theta, a new class of glutathione transferases purified from rat and man. *Biochem J.* 1991; 1(274): 409-414.
- 5) Meyer DJ, Thomas M. Characterization of rat spleen prostaglandin- H Disomerase as a sigma class GSH transferase. *Biochem J.* 1995; 1(311): 739-742.
- 6) Pemble SE, Wardle AF, Taylor JG. Glutathione-S-transferase Kappa: characterization by the cloning of rat mitochondrial GST and identification of a human homologue. *Biochem J.* 1996; 1(319): 749-754.
- 7) Board PG, Baker TR, Chelvanayagam G, Jermin LS. Zeta, a novel class of glutathione transferases in a range of spe-

- cies from plants to human. *Biochem J.* 1997; 328(3): 929-935.
- 8) Hayes JD, McLellan LI. Glutathione and Glutathione-dependent enzymes represent a co-ordinately regulated defense against oxidative stress. *Free Radic Res.* 1999; 31(4): 273-300.
 - 9) Jakobsson PJ, Morgensten R, Mancini J, Ford-Hutchinson A, Persson B. Common structural features of MAPEG- a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci.* 1999; 8(3): 689-692.
 - 10) Appiah-Oppong R, Commandeu JNM, Istyastono E, Bogaards JJ, Vermeulen NPE. Inhibition of glutathione S-transferases by curcumin and analogues. *Xenobiotica.* 2009; 39(4): 302-11.
 - 11) Hayeshi R, Mukanganyama S, Hazra B, Abegaz B, Hasler J. The interaction of selected natural products with human recombinant glutathione transferase. *Phytother Res.* 2004; 18(11): 877-883.
 - 12) Chin JH, Ismail S, Hussin AH. Induction of hepatic glutathione-s-transferase activity by *Orthosiphon stamineus*, benth in stz-induced diabetic rats. *Malays J Pharm Sci.* 2008; 6(1): 59-68.
 - 13) Hamsar MN, Ismail S, Mordi MN, Ramanathan S, Mansor SM. Antioxidant activity and the effect of different parts of areca catechu extracts on Glutathione-S-Transferase activity *in vitro*. *Free Rad Antiox.* 2011; 1(1): 28-33.
 - 14) Gweshelo D, Muswe R, Mukanganyama S. *In vivo* and *in vitro* inhibition of rat liver glutathione transferases activity by extracts from *Combretum zeyheri* (*Combretaceae*) and *Parinaricurate llifolia* (*Chrysobalanaceae*). *BMC Complement Altern Med.* 2016; 16: 238.
 - 15) Ali-Shtayeh MS, Jamous RM. Complementary and alternative medicine use amongst Palestinian diabetic patients. *Complement Ther Clin Pract.* 2012; 18(1): 16-21.
 - 16) Jaradat NA, Shawahna R, Eid AM, Al-Ramahi R, Asma MK, Zaid AN. Herbal remedies use by breast cancer patients in the West Bank of Palestine. *J Ethnopharmacol.* 2015; 3(178): 1-8.
 - 17) Hyam R, Pankhurst R J. *Plants and their names: a concise dictionary.* Oxford; New York: Oxford University Press; London: Royal Botanic Gardens, 1995.
 - 18) Lee M. The history of Ephedra (ma-huang). *Journal of the Royal College of Physicians of Edinburgh.* 2011; 41(1): 78-84.
 - 19) Freitag H, Maier-Stolte M. The Genus Ephedra in NE Tropical Africa. *Kew Bull.* 2003; 58(2): 415-426.
 - 20) Ali-Shtayeh MS, Jamous RM, Salameh NM, Jamous RM, Hamadeh AM. Complementary and alternative medicine use among cancer patients in Palestine with special reference to safety-related concerns. *J Ethnopharmacol.* 2016; 187: 104-122.
 - 21) Sawalha MKM, Abu-Lafi S, Rimawi F. *Phytochemical screening of wild Ephedra foeminea and Smilax aspera fruits and their in-vitro pharmacological activities* (M.Sc. dissertation, Al-Quds University). 2018. Retrieved from: https://dspace.alquds.edu/bitstream/handle/20.500.12213/2077/MT_2018_21512_712_8081.pdf?sequence=1&isAllowed=y.
 - 22) Gholivand M, Yamini Y, Dayeni M. Optimization and Comparison of Ultrasound-Assisted Extraction of Estragole from Tarragon Leaves with Hydro-Distillation Method. *Anal Bioanal Chem Res.* 2014;1(2): 99-107.
 - 23) Omar G, Abdallah L, Rahim A, Othman R, Barakat, A. Selected wild plants ethanol extracts bioactivity on the coagulation cascade. *J Sci Res Rep.* 2017;13(6): 1-10.
 - 24) Warburg O, Christian W. Isolation and crystallization of enolase. *Biochem Z.* 1942; 310: 384-421.
 - 25) Habig W, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymat-

- ic step in mercapturic acid formation. *J. Biol. Chem.* 1974; 294(22): 7130-7139.
- 26) Bladeren PJV, Ommen BV. The inhibition of glutathione S-transferases: Mechanisms, toxic consequences and therapeutic benefits. *Pharmacol Therapeut.* 1991;51(1): 35-46.
- 27) Schipper DL, Wagenmans MJH, Wagener DJT, Peters WHM. Glutathione S-transferases and cancer (review). *Int J Oncol.* 1997; 10(6): 1261-1264.
- 28) Testa B, Krämer SD. The Biochemistry of Drug Metabolism – An Introduction Part 4. Reactions of Conjugation and Their Enzymes. *Chem Biodivers.* 2008; 5(11): 2171-336
- 29) Mahajan S, Atkins WM. The chemistry and biology of inhibitors and pro-drugs targeted to glutathione S-transferases. *Cell Mol Life Sci.* 2005; 62(11): 1221-1233.
- 30) Weber JE, Oakley AJ, Christ AN, Clark AG, Hayes JD, Hall R, Hume DA, Board PG, Smythe ML, Flanagan JU. Identification and characterisation of new inhibitors for the human hematopoietic prostaglandin D₂ synthase. *Eur J Med Chem.* 2010; 45(2): 447-454.