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Original Article

In vitro Screening of Glutathione- S -Transferase Inhibition by Different Extracts of *Verbascum sinuatum*

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Abstract

Background and Aim: The concern toward using herbs as enzyme inhibitors such as glutathione-s-transferases (GSTs) may result in the improvement of proliferative diseases. Moreover, it may overcome the problem of multidrug resistance tumors. Based on that, this experiment was conducted to detect the impact of using aqueous, ethanol and methanol extracts from *Verbascum sinuatum* L. (Scrophulariaceae) that grow wild in Palestine as enzymatic inhibitors.

Materials and Methods: The impacts of these three extracts at four studied concentrations (250, 500, 750 and 1000 µg/mL) on the activity of purified hepatic glutathione-s-transferases were estimated spectrophotometrically by using-chloro-2,4-dinitro-benzene (CDNB) as substrate.

Results: The results of the present research indicated that all the three prepared extracts of *V. sinuatum* could inhibit the activity of GSTs at all examined concentrations. Moreover, according to the results, the alcoholic extracts from the studied plant species were more influential in the activity of GSTs than the aqueous ones . However, minor inhibitory variations were observed between methanol and ethanol extracts. It is notable that all the studied extracts manifested inhibitory effects in a dose-dependent manner.

Conclusion: The results of this study emphasized the possibility of utilizing *V. sinuatum* extracts in pharmaceutical industry of new medications to fight drug resistant tumors in general and GST-induced tumors in particular.

Keywords: *Verbascum sinuatum*, Glutathione-S-transferases, Plant extracts

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Introduction

Medicinal plants are among the valuable sources of bioactive antimicrobial materials. These materials are considered as secondary metabolites generated by herbs to fight microbes (1). *Verbascum sinuatum* L, from the Scrophulariaceae family, is one of these medicinal herbs (2). The leaves and flowers of various *Verbascum* species are helpful in the treatment of respiratory disorders, including irritating coughs with bronchial congestion (3). Moreover, it has been reported that *Verbascum* species have diuretic, assuasive and anti-inflammatory impacts on the urinary tract (4). Additionally, the leaves, roots and flowers from different *Verbascum* species are also used as sedative, analgesic, astringent, emollient, nervine, antiseptic, antispasmodic, antihistaminic, antioxidant and anticancer agents (5). The presence of several secondary metabolites is indicative of the wide application of *Verbascum* species. The phytochemical

investigation of *Verbascum* species was indicative of the existence of diverse biologically active compounds like flavonoids, saponins, iridoids, phenylethanoid, neolignan and mucilage (6). In this respect, there are various experiments that have highlighted the antioxidant and antimicrobial potentials of *V. sinuatum* (7, 8).

Glutathione-S-transferases (GSTs) isoenzymes are widely found in the natural world as they can be found in mammals, plants, fish, birds, insects and microbes (9). Moreover, the presence of glutathione-S-transferases enzymes in biological systems is of high significance for the detoxification of lipid peroxidation byproducts or DNA hydroperoxides (9, 10). Otherwise, mammalian GSTs significantly act in the detoxification and metabolism of several xenobiotic and endogenous compounds. Moreover, these isoenzymes are able to have interactions with several exogenous compounds, including herbal remedies (11). Glutathione-S-transferases is divided into two main superfamilies (12). The first characterized family is cytosolic GSTs that has at least 16 members in human beings. Whereas, the second family is known as a protein which is associated with membrane in eicosanoid and glutathione metabolism (MAPEG) (12, 13, 14). Cytosolic enzymes belong to the major phase II detoxification enzymes which are responsible for the catalyzation of the conjugation of glutathione (GSH) to different exogenous and endogenous compounds. Furthermore, these isoenzymes contribute to drug resistance as they hinder the mitogen-activated protein (MAP) kinase pathway (15).

As it can be understood from the previous background, the present study was carried out to determine the inhibitory effect of *Verbascum sinuatum* aqueous, ethanol and methanol extracts on the activity of the purified hepatic glutathione-S-transferases.

Materials and Methods

Collection and Preparation of the Plant Material

Verbascum sinuatum was obtained from West Bank, Palestine, and classified by Ghadeer Omar, from the Biology & Biotechnology Department, An-Najah National University, Palestine. A herbal specimen was placed on herbarium sheet and given a voucher number. Then, this sheet was deposited at An-Najah National University herbarium. The collected plant material for the enzymatic study was washed. Subsequently, it was dried, ground into powder and kept dry at room temperature until extract preparation.

Preparation of Plant Extracts

Preparation of Aqueous Extract

Five grams of *V. sinuatum* leaves powder were soaked in 100 mL of distilled water and shacked for three days at room temperature using a rotary shaker. Subsequently, the soaked plant was sonicated for 15 minutes at room temperature. Then, the mixture was centrifuged for 10 minutes at 4500 rpm. The supernatant that had been obtained was filtrated and evaporated by freeze-drying. The obtained powder of the herbal species was dissolved in distilled water making a stock working solution that was equal to 1000 µg/mL.

Preparation of Alcoholic Extracts

Five grams of *V. sinuatum* leaves powder were soaked in 100 mL of each solvent (70% methanol and 70% ethanol) and shacked for three days at room temperature using a rotary shaker. After that, the soaked plant species were sonicated for 15 minutes at room temperature. Then, the centrifugation of the mixture occurred in 10 minutes at 4500 rpm. The resultant extract supernatants were filtrated and evaporated using a rotary evaporator. The extracted powder of plant species was dissolved in 10% dimethyl sulfoxide (DMSO) forming a stock working solution equal to 1000 µg/mL.

Extraction and Purification of Glutathione-S-Transferases

Glutathione-S-transferase enzyme was obtained from protein purification laboratory, affiliated to the Biology and Biotechnology Department, Faculty of Science, An-Najah National University. The enzyme solution was prepared by homogenization and centrifugation of fresh liver.The sheep was the source of the fresh liver in this experiment, which directly obtained from a slaughterhouse in Nablus city. At which all animals were under the ethics of the Ministry of Health in Palestine. Then, the cytosolic fraction was purified in three steps: ammonium sulfate precipitation (30-70) %, gel filtration column chromatography (Ultrogel ACA 44 column, Sigma), and affinity column chromatography (GSH-agarose, Sigma). The protein

levels for all fractions and GSTs activity for protein containing fractions were determined (16, 17). 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.

Effects of Different Concentrations of *Verbascum sinuatum* Extracts on Glutathione-S-Transferases Activity

The activity of Glutathione-S-transferase was evaluated spectrophotometrically by the use of 1-chloro- 2, 4- dinitrobenzene (CDNB) as a substrate (17). The enzyme reaction mixture contained 0.2 M sodium phosphate buffer (pH 7), 50 mM GSH, 30 mM of CDNB and 50 µL of diluted enzyme (1 µg/mL) in a final volume of 1.0 mL. The alteration in absorbance at 340 nm was measured against a blank with all the reactants except CDNB. Expression of the GSTs activity was as µmol conjugate formed/min/mL by the use of a molar extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$. The impacts of all the three *V. sinuatum* extracts prepared at various four concentrations (250, 500, 750 and 1000µg/mL) were evaluated spectrophotometrically at 340 nm by the addition of 50 µL from each extract concentration to the previous enzyme reaction mixture. Subsequently, the results were expressed as inhibition percentage using the following formula

$$\text{Inhibition \%} = (\text{GSTs activity without treatment} - \text{GSTs activity under treatment})/\text{GSTs activity without treatment} \times 100\ \%.$$

Data Statistical Analysis

All the experiments were carried out in five replicates. The results were indicated as mean ± standard deviation (SD). The IC_{50} (concentration giving 50% inhibition) was measured using Microsoft Excel using the calculated non-linear regression after plotting the percentage inhibition versus concentration to obtain the dose–response curve. The significance of the acquired results was conducted via applying mean values and standard deviations using T test to determine whether there was an important distinction among the various concentrations of every examined plant extract type relative to the control. Moreover, one-way ANOVA test was applied to identify if a remarkable distinction could be found among the various studied extract types. P value < 0.05 was considered to be significant.

Results and Discussion

Glutathione-s-transferases isoenzymes are involved in xenobiotic and endogenous compounds detoxification and are over-expressed in some cancer cells. Hence, they play a role in the detoxification of anticancer drugs, resulting in the emergence of tumors characterized by resistance to drugs. Consequently, it is

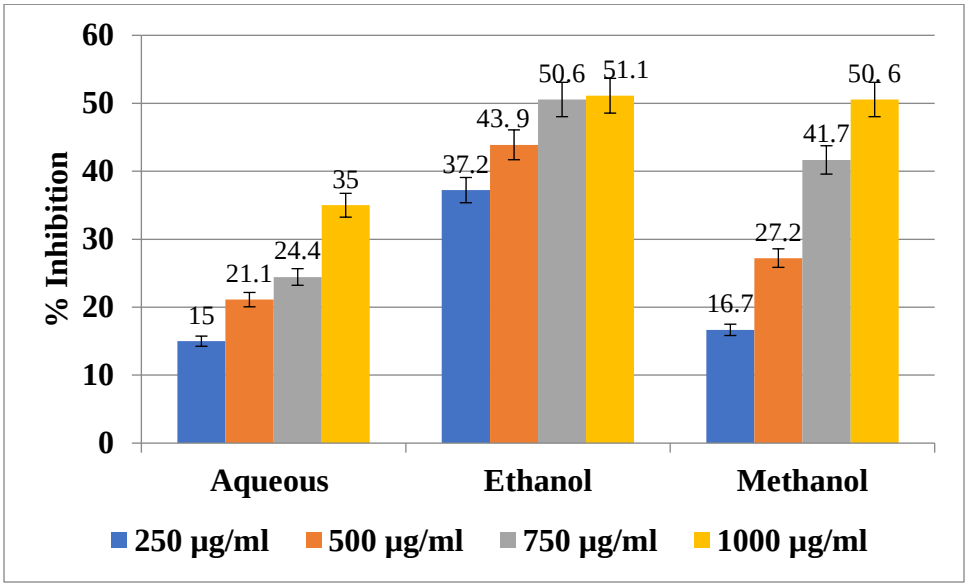

Figure 1. The percentage of the inhibitory effect of different extract types at the studied concentrations of *Verbascum sinuatum* on GSTs activity.

suggested that the inhibition of GSTs is a strategy to fight GST-induced resistance tumors (18). Moreover, many compounds that are derived from plants possess anticancer activity, including their ability to inhibit GSTs. Since this feature provided additional antitumor potency to these plant-derived phytochemicals, they could prevent drug resistance (11). The obtained results revealed that the aqueous, ethanol and methanol extracts of *V. sinuatum* reduced GSTs activity at all examined concentrations. The recorded GSTs activity results after the exposure to the examined extract concentrations of *V. sinuatum* have been summarized in Table 1. The results demonstrated that both of alcoholic *V. sinuatum* extracts were more efficient than the aqueous extract in the alleviation of GSTs activity at all studied concentrations. The inhibition percentage data for all the prepared *V. sinuatum* extracts concentrations is presented in Figure 1. The inhibition percentage of the aqueous extract was lower than that of alcoholic ones. Moreover, based on the obtained *in vitro* IC50 results, the alcoholic extraction was also more influential in hindering GSTs than the aqueous extraction (Table 2). Regarding the half maximum inhibitory concentration values (IC50), the most significant difference was between the alcoholic extracts and the aqueous ones. The ethanol and methanol extracts of this herbal species demonstrated GST inhibitory activity with IC50 values of 862.6 µg/mL and 978.3 µg/mL respectively, while the IC50 value for the aqueous extract was 1677.8 µg/mL. Moreover, according to the obtained P values (Table 1). The ethanol extract had significant effects at

Table 1: Glutathione-s-transferases activity (µmole/min/mL) with the statistical P values under the studied three extract types of *Verbascum sinuatum*.

Extract Type	Extract Concentration µg/mL	GSTs Activity µmole/min/mL	P value
Aqueous	250	6.426 ± 0.192	0.6268
	500	5.964 ± 0.042	0.3489
	750	5.712 ± 0.128	0.2093
	1000	4.914 ± 0.135	0.1801
Ethanol	250	4.746 ± 0.084	0.1834
	500	4.242 ± 0.083	0.0495
	750	3.738 ± 0.087	0.0387
	1000	3.696 ± 0.064	0.0283
Methanol	250	6.3 ± 0.133	0.4243
	500	5.502 ± 0.192	0.3907
	750	4.41 ± 0.169	0.186
	1000	3.738 ± 0.111	0.0499
Control		7.56 ± 0.120	

*P value ≤ 0.05 was significant among the different studied *V. sinuatum* extract concentrations relative to the control (GSTs without *V. sinuatum* extract).

500, 750 and 1000 µg/mL concentrations with P values ≤ 0.05. Also, the methanol extract exhibited a significant inhibitory effect at 1000 µg/mL concentration with P values ≤ 0.05, while there was no significant effect of the aqueous extract at the examined concentrations with P values >0.05. Furthermore, the results indicated that there was a remarkable GSTs inhibitory distinction between the alcoholic extracts and the aqueous ones (Table 3). The reported P values between them were less than 0.05. However, there was no remarkable distinction between the methanol and ethanol extract with P values >0.05. The recorded data suggested that the alcoholic extracts of *V. sinuatum* may contain some biologically active ingredients that are responsible for the *in vitro* inhibition of GSTs, and these ingredients are not found in the aqueous extract. It is worth mentioning that all the screened extract types exhibited a dose-dependent manner in their effect on GST inhibition.

The results of the present research approve the medicinal significance of *V. sinuatum*. All the three examined extracts from this plant species have the potency to inhibit GSTs. In this aspect, many researchers have reported that several isolated plant compounds have been confirmed to be *in vitro* inhibitors of GSTs enzymes. For example, tannic acid, thonningianin A, cibacron blue, hematin, ethacrynic acid, ellagic acid, ferulic acid, caffeic acid, stilbene, quercetin, chlorogenic acid and curcumin have been frequently stated by several researchers (19, 20, 21, 22). With regard to that, the phytochemical analysis of *Verbascum* species showed the presence of some of those biologically active compounds (6). From the previous literature, *Verbascum* species contain iridoid glycosides, saponins, flavonoids, phenylethanoids, and neolignanglycosides (23). It was indicated that plant total saponins could strongly inhibit the glutathione-S-transferase activity (24). Moreover, some falvonoids exhibited inhibitory potency toward GSTs activity. Their inhibitory effect is fundamentally related to the pattern of hydroxylation and number of hydroxyl groups (25). The observed *in vitro* activity of plant extracts might be different from their observed *in vivo* activity (26). In this context, the contradictions between *in vitro* and *in vivo* results have been explained previously for antioxidants such as ellagic acid and curcumin. These antioxidants are *in vitro* inhibitors for GSTs, even so they are *in vivo* inducers of the same enzyme (27). The proposed explanation of this contradiction is the presence of some compounds in the plant extract that might reduce the expression of GSTs *in vivo*, and this action does not take place in *vitro* (28). Furthermore, it has been shown that the *in vivo* effect may vary according to the extraction solvent (11). Thus, it is recommended to examine the effects of different types of *V. sinuatum* extract using animal models.

Table 2: The half maximum inhibitory concentration values (IC50) of different extract types studied concentrations of *Verbascum sinuatum* on GSTs activity.

Plant Extract	IC50 (µg/mL)
Aqueous	1677.8
Ethanol	862.6
Methanol	978.3

Table 3: The statistical P values among the different extract types of *Verbascum sinuatum*.

Extract Type	* P value
Aqueous	0.000
Ethanol	
Aqueous	0.001
Methanol	
Ethanol	0.334
Methanol	

*P value ≤ 0.05 was significant among the different extract types

Conclusion

The results of the present research pointed up the ability of different extracts of *V. sinuatum* as sources of numerous biological compounds that had inhibitory impacts on some drug metabolizing enzymes such as GSTs. Consequently, additional research is needed to explain the mechanism of action of those valuable compounds. Moreover, it is recommended to conduct more *in vivo* studies on the impacts of various extracts of the investigated herbal species on the activity of GSTs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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