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RESEARCH ARTICLE

Inhibition of glutathione S-transferases by some Malvaceae flowers

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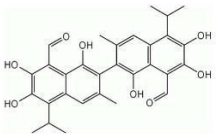
Abstract

Glutathione S-transferase (GST) enzymes have important roles in detoxification and have been implicated in resistance of tumor cells against chemotherapeutic agents. Exhibiting inhibitory potential on GST activity by plant extracts will be crucial in increasing the efficiency of cancer chemotherapeutics.

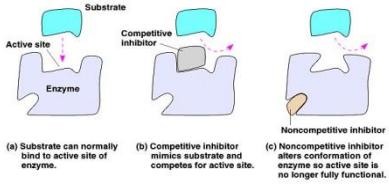
Eight Malvaceae plant extracts were evaluated for their inhibitory effects on different mammalian GST isoenzymes along with their polyphenol, flavonoid, anthocyanin contents and antioxidant efficiencies.

Hibiscus subdriffa extracts exhibited the highest phenolic contents (60.74 ± 7.1 mg gallic acid/g dry tissue). The highest flavonoid content was obtained with Hibiscus cannabinus (17.38±2.12 mg rutin/g dry tissue). H. subdriffa and H. cannabinus extracts showed high antioxidant activities (IC_{50} equal 0.79 ± 0.09 and 0.820 ±0.04 mg /ml, respectively), also had high phenolic, flavonoide and anthoyanin contents. Among the plant extracts studied for GST inhibition activity, Gossypium hirsutum (cotton) was the most efficient inhibitor for all studied GSTs (rat liver & kidney and human erythrocytes & placenta). Erythrocyte hGSTP1-1 only inhibited by G. hirsutum extracts with IC_{50} value equal 0.35 mg/g dry tissue, correlating with its relatively high phenolic contents.

Inhibition of hGSTP1-1 by gossypol (major constituent of G. hirsutum) in vitro indicates the potential use of these compounds both as an in vivo inhibitor for GST P1-1.



Gossypol



G. hirsutum extracts inhibited the purified erythrocyte hGSTP1-1 competitively, while, non-competitive inhibition was observed when gossypol (the major constituent of cotton flower) was used as inhibitor for both GST substrates, GSH and CDNB.

Introduction

Glutathione S-transferase (GST, E.C.2.5.1.18) is a phase II enzyme that accounts for multifunctional role in the cell defense system against electrophilic compounds. GSTs also exhibit antioxidant properties due to their selenium-independent glutathione peroxidase activity. Alternatively, GSTs may act as ligandins by binding and sequestering a variety of small or large toxic compounds and peptides. An example of this ligandin role is the specific binding of GSTP1-1 to Jun-kinase, a pro-apoptotic enzyme that becomes inactive when bound to GST. [1] Based on amino acid sequence, seven mammalian classes of cytosolic GSTs have been defined namely α, μ, π, σ, θ, ώ and ζ. [2] The members of the Alpha, Mu, and Pi classes are the most abundant GSTs and are the enzymes most likely to be involved in the metabolism of xenobiotics. Multidrug resistance (MDR), the principal mechanism by which many cancers develop resistance to chemotherapy drugs, is a major factor in the failure of many forms of chemotherapy. Resistance of tumor cell to electophilic xenobiotic such as alkylating agent after cancer chemotherapy treatment has been linked with over-expression of certain GST isoenzyme. Consequently, the effort to detoxify the drug during continuous chemotherapy treatment leads to the increase in the GST level. Thus, exhibiting inhibitory potential on the GST activity will be crucial in increasing the efficiency of cancer chemotherapeutics agent. [2]Polyphenolic compounds are products of secondary metabolism, which protect plants against oxidative and other types of stress, and against pathogenic organisms. Polyphenols are a large group of substances that include such classes as phenolic acids, flavonoids, stilbens, and lignans. [3] Plant extracts that are high in polyphenols are known to have important inhibitory effect on GST. Today, there is compelling evidence that strongly suggests that the mechanisms by which plant polyphenols exert their protective actions against cardiovascular and neurodegenerative diseases, as well as cancer and diabetes, are not simply due to their redox properties, but rather to their ability to directly bind to target proteins (or peptides). Such a mode of action would induce the inhibition of key enzymes, the modulation of cell receptors or transcription factors, as well as the perturbation of protein (or peptide) aggregates, which can regulate cell functions related to, for example, growth and proliferation, inflammation, apoptosis, angiogenesis, metastasis, and immune responses, in various ways by affecting signal transduction pathways. [4] Malvaceae is a family of flowering plants containing over 200 genera with close to 2,300 species. In Egypt Malvaceae are represented by 26 species belonging to 10-11 genera. [5] Ethnobotanical investigations have shown that Malvaceae species are frequently used in traditional medicine to treat various diseases such as malaria, fever, pain, variola, and also have anti-bacterial, anti-inflammatory, anti-viral activities and hepatoprotective properties. [6] In this study, eight Malvaceae plant extracts were used to investigate their polyphenol, flavonoid, anthocyanin contents, antioxidant capacities and there in vitro inhibitory effects on different mammalian GSTs in order to develop inhibitors with optimized isozyme specificity, antioxidant capacity and fewer side effects.

2. Results and Discussion:

Total phenolic contents (TPC)

Total concentrations of phenolic compounds in extracts of eight plant flowers (Malvaceae) were determined using gallic acid as standard. The results were expressed as mg of total phenolics in g of extracts as gallic acid equivalents (Table 1). Among the studied plants, H. sabdriffa (karkadey) extracts exhibited the highest phenolic compounds (60.74 ± 7.1 mg gallic acid/g dry tissue). While extracts of A. esculentus (okra) have the lowest content of phenolic compounds (3.91 ± 0.5 mg gallic acid / g dry tissue). G. hirsutum (cotton) and H. cannabis (kenaf) extracts exhibited relatively high amounts of phenolic compounds (48.17 ± 4.4 and 47.02 ± 3.83 mg gallic acid/g dry tissue respectively) compared to the flower extracts of the other studied plants.

Total flavonoid contents (TFC)

Total flavonoids of plant extracts are represented by the following decreasing order: H. cannabinus (kenaf) > H. rosa-sinensis > G. hirsutum (cotton) > H. sylvestris > H. syriacus > H. vitifolius > H. sabdriffa > A. esculentus (okra). The lowest content was found in okra, with 1.85±0.24 mg rutin / g dry tissue. TFC varied from 1.85 ± 0.24 mg rutin / g dry tissue to 17.38 ± 2.12, where the highest flavonoid content was obtained with H. cannabinus. These results suggested that flavonoids were principal constituent of kenaf extract (Table 1).

Total anthocyanin contents

Hibiscus anthocyanins are water soluble and among the most important groups of plant pigents which significantly reduced oxidative stress. Total anthocyanin content of the extracts was ranging from 7.7±1.9 to 0.195±0.03 mg anthocyanin / g dry tissue (Table 1). The highest values of total anthocyanin were in the following decreasing order: H. sabdriffa > H. cannabinus (kenaf) > H. rosa-sinensis > A. esculentus (okra) > H. vitifolius. Anthocyanins could not be detected in both G. hirsutum (cotton) and H. sylvestris extracts. These results indicated that H. sabdriffa extract contained the highest percentage of anthocyanin yield (12.98%) relative to total phenolics.

The antioxidant activities using DPPH free radical scavenging activity method

Antioxidant activities of the present studied plant extracts were investigated by (DPPH) scavenging inhibition method. The IC_{50} values for DPPH % scavenging activity were determined from the % inhibition versus log plant extract concentration curve using vitamin C as a standard. Our results indicate that both H. cannabinus (kenaf) and H. sabdriffa extracts have approximately the same effect on scavenging free radicals (IC_{50} were 0.79 ± 0.09 and 0.820 ±0 .04 mg /ml, respectively). On the other hand, the lowest antioxidant capacity was given by okra extract (4.17±0.46 mg /ml). The scavenging ability of the extracts of the eight studied plants and the standard vitamin C on DPPH radical decreased in the following order: vitamin C > H. cannabinus (kenaf) > H. sabdriffa > H. rosa-sinensis > H. sylvestris > H. vitifolius > G. hirsutum > H. syriacus. The calyxes with high phenolics as H. sabdriffa, also has low IC_{50} DPPH radical scavenging activity (0.8202 mg /ml) which indicate high antioxidant capacity. Extracts of A. esculentus (okra) have the highest IC_{50} DPPH radical scavenging activity which indicates low antioxidant capacities (Table 1).

Effect of some natural compounds of Malvaceae **flower extracts on the mammalian GST activities**

The inhibitory effect of some natural occurring compounds of Malvaceae flowers on the affinity purified rat liver, kidney, human erythrocyte and placenta GST activities were investigated (Table 2). The percent inhibition of GST activity of both ascorbic acid and α-tocopherol was evaluated in the presence of varied concentration (2-40 µM). Cibacron blue (2µM) the strong synthetic GST inhibitor was used as a control for inhibitory effects. Not all the compounds have shown considerable inhibition on the GST activities. Comparing the used compounds for their IC_{50} values, gossypol was the most effective inhibitor of both human erythrocytes and placenta GST activity (0.035 and 0.092 mM, respectively). The strong inhibitor for erythrocytes was obtained with protocateuic acid and ascorbic acid while quercetin, tannic acid and delphinidin-HCl have no inhibitory effect on erythrocytes and placenta affinity purified GST activity. Tannic acid has a strong inhibitory effect on the GST affinity purified of rat liver and kidney (0.0017 mM and 0.0027 mM, respectively). Gossypol and quercetin are strong inhibitors with almost the same efficiency for rat liver and kidney enzymes. GST inhibitory effects of the tested compounds showed the following increasing order, in human erythrocyte and placenta GST: gossypol > protocateuic acid > ascorbic acid > α-tochopherol. However rat liver and kidney were inhibited by all the tested compounds and their IC_{50} values were in the following decreasing order: tannic acid > gossypol > quercetin > α- tochopherol > protocateuic acid and ascorbic acid.

Effects of plant extracts on the mammalian GST activities

All the studied plant extracts have shown considerable inhibition on the GST activity and the percent inhibition of GST enzymes was evaluated in the presence of varied extract concentrations (0.2–150 mg/g dry tissue). Comparing all the plant species for their IC_{50} values, cotton and kenaf extracts were the most effective inhibitors of rat liver GST activity toward CDNB, with almost comparable values. While cotton extracts was the most effective inhibitor of rat kidney GST activity, with a value of 0.19 mg/g dry tissue, followed by okra extracts (0.41 mg/g dry tissue) and kenaf (0.52 mg/g dry tissue). Of all the plant extracts tested in our study, the results showed that extract of cotton plant was the most effective inhibitor of all the affinity purified GST enzymes. Specifically, cotton extracts inhibit both human erythrocytes and placenta with IC_{50} values of 0.35 and 0.83 mg/g dry tissue, respectively. Kenaf and okra extracts inhibit erythrocyte GST activity with a higher IC_{50} values (2.87 and 4.52 mg/g dry tissue, respectively). GST affinity purified from human placenta inhibited by okra extracts with IC_{50} value of 7.1 mg/g dry tissue, however, extracts of H. rosa-sinensis, H. vsyriacus, H. vitifolius and H. sylvestris showed negligible or no inhibition on the four tested GST isoenzymes (Table 3).

Effect of cotton extracts and gossypol (IC_{50} concentration) on variable substrate concentration of the purified erythrocyte GST

G. hirsutum (cotton) extracts and gossypol inhibited GST activity dose-dependently with 50% inhibitory concentration of 0.308ug. GST activity was measured with variable concentrations of either CDNB or GSH. Figs. 1a and b showed the Lineweaver–double reciprocal plots with varying concentrations of CDNB and GSH. The Km value was increased from 0.42 to 1.33 mM with a Vmax equal 107.8 and 113.8 µmoles / min / mg protein by cotton extracts, respectively. While by using gossypol as an inhibitor, the Vmax value was decreased to 66.2and 71.07µmoles / min / mg protein with a Km equal 0.34, and 0.58 mM, respectively, for both GSH and CDNB (Table 4).

Time dependent inactivation of erythrocyte GST

G. hirsutum (cotton) extracts and ethacrynic acid inactivated GST in a time-dependent manner at concentrations of 0.308 µg and 20 nM, respectively, while gossypol inactivated GST in a time-independent manner. Ethacrynic acid was used as a positive control since it is known to inactivate GST P1-1 (Townsend and Tew, 2003). It was noted that at time 0 min, cotton extracts, gossypol and ethacrynic acid had a direct inhibitory effect on the enzyme and this resulted in a loss of almost 50% of enzyme activity. By increasing the incubation time to more than 30 min, almost 80% of enzyme activity was lost when cotton extracts and ethacrynic acid were used as inhibitors.

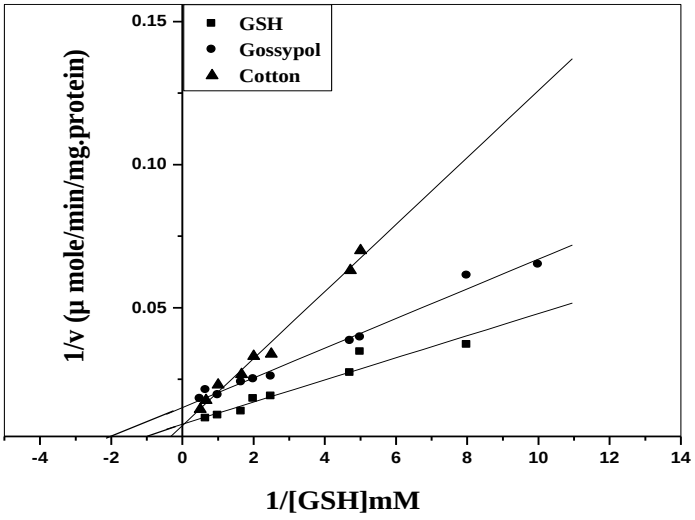
Effect of GSH on the inactivation of erythrocyte GST by cotton extracts and gossypol

Glutathione at 5 mM was shown to protect the erythrocyte purified GST enzyme from inactivation by cotton extracts and gossypol as shown in Fig 2a. However, GSH alone enhanced the activity of the enzyme in the absence of an inhibitor. This trend was also observed when ethacrynic acid was used as the inactivation agent (Fig.2b).

Figures

Fig. 1a, b

(a)



(b)

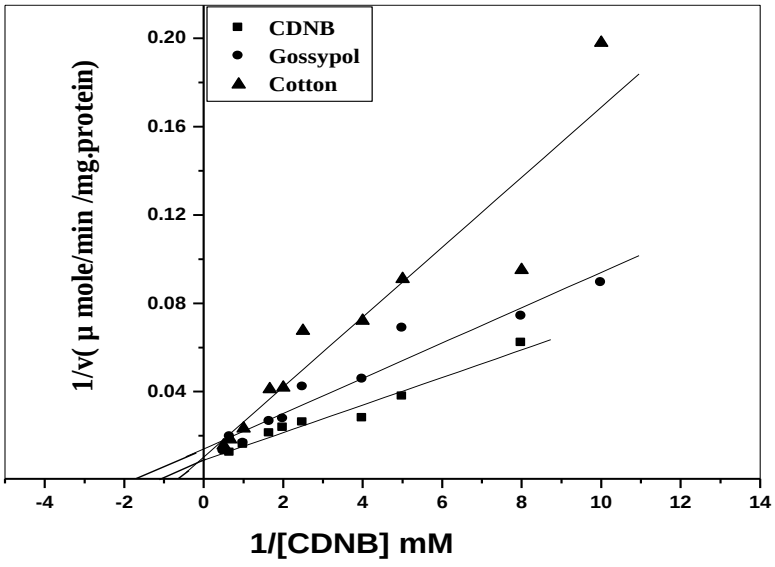
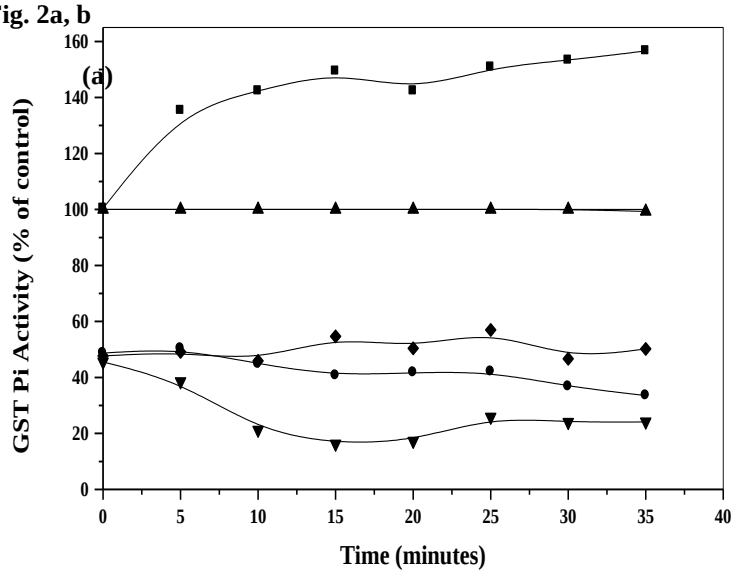
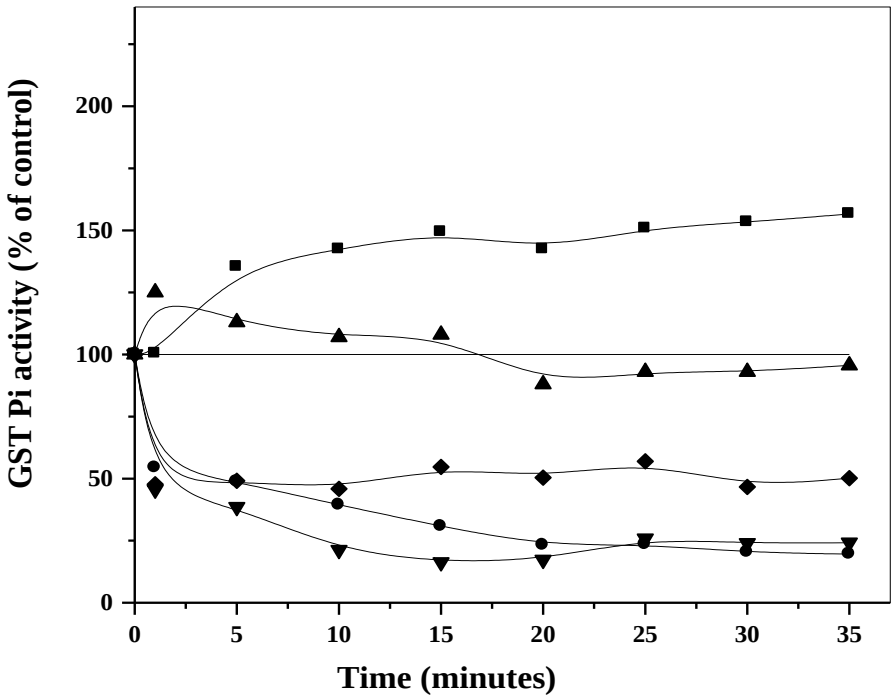


Fig. 2a, b



——GSTP1-1,—■—GSTP1-1 +GSH (5mM), —●—GSTP1-1 + gossypol (40mM), —▲— GSTP1-1 + gossypol (40mM) + GSH (5mM), —▼— GSTP1-1 + ethacrynic acid (20 nM), —♦— GSTP1-1 + ethacrynic acid (20 nM) + GSH (5mM)

(b)



—— GSTP1-1, —■— GSTP1-1 + GSH (5mM), —●— GSTP1-1 + cotton, —▲— GSTP1-1+ cotton + GSH (5mM),—▼— GSTP1-1 + ethacrynic acid (20 nM),—♦— GSTP1-1 + ethacrynic acid (20 nM) + GSH (5mM)

Tables

Table 1: Total phenolics, flavonoids, anthocyanins of Malvaceae plant extracts and their DPPH free radical scavenging activity

Plant sample	Total phenolics (mg gallic acid/ g dry tissue)	Total flavonoids (mg rutin/ g dry tissue)	Total anthocyanine (mg / g dry tissue)	DPPH IC_{50} (mg /ml)
Gossypium hirsutum	**48.17 ± 4.4**	**8.90 ± 0.59**	**ND**	**1.37 ± 0.12**
Abelmoschus esculentus	**3.91 ± 0.50**	**1.85 ± 0.24**	**0.21 ± 0.04**	**4.17 ± 0.46**
Hibiscus cannabinus	**47.02 ± 3.83**	**17.38 ± 1.2**	**0.73 ± 0.15**	**0.79 ± 0.09**
Hibiscus rosa-sinensis	**11.84 ± 1.09**	**9.26 ±0.62**	**0.23 ± 0.03**	**1.03 ±0.11**
Hibiscus syriacus	**10.58 ± 0.31**	**6.30 ± 0.27**	**ND**	**1.52 ± 0.10**
Hibiscus vitifolius	**7.88 ± 0.63**	**5.70 ± 0.47**	**0.20 ± 0.03**	**1.37 ± 0.06**
Hibiscus sylvestris	**9.72 ± 0.77**	**7.03 ± 1.0**	**ND**	**1.32 ± 0.15**
Hibiscus sabdriffa	**60.70 ± 7.10**	**1.91 ± 0.2**	**7.70 ± 1.9**	**0.82 ± 0.04**

- Values are expressed as mean ± SE of triplicate experiments

- ND: could not be detected under our experiment conditions

Table 2: Effect of some natural compounds of Malvaceae flower extracts on the mammalian GST activities affinity purified from different mammalian organs, human erythrocytes and placenta

IC_{50} of GST inhibition (mM)				
compound concentration (mM)	**Rat liver**	**Rat kidney**	**Human erythrocytes**	**Human placenta**
Ascorbic acid	**27.96**	**12.12**	**2**	**15**
α-Tochopherol	**1.34**	**0.52**	**20**	**NI**
Protocateuic acid	**2.56**	**1.27**	**1.36**	**3.98**
Quercetin	**0.005**	**0.016**	**NI**	**NI**
Tannic acid	**0.0017**	**0.0003**	**NI**	**NI**
Delphinidin HCl	**NI**	**0.19**	**NI**	**NI**
Gossypol	**0.004**	**0.0048**	**0.035**	**0.092**
Cipacron blue	**0.0004**	**0.0002**	**0.0002**	**0.00016**

IC50 (amount of extract which cause 50% inhibition)

NI: no inhibition (inhibition of GST activity could not be detected under our experiment conditions)

Table 3: Effects of plant extracts on the activities of the affinity purified GST from different mammalian organs, human erythrocytes and placenta

IC50 (amount of extract which cause 50% inhibition)

NI: no inhibition (inhibition of GST activity could not be detected under our experiment conditions)

Tissue / Plant sample	IC_{50} (mg/g dry tissue)			
	Rat liver	**Rat kidney**	**Human erythrocyte**	**Human placenta**
Gossypium hirsutum	**0.43**	**0.19**	**0.35**	**0.83**
Abelmoschus esculentus	**4.13**	**0.41**	**4.52**	**7.1**
Hibiscus cannabinus	**0.42**	**0.52**	**2.87**	**NI**
Hibiscus rosa-sinensis	**NI**	**NI**	**30.7**	**50.9**
Hibiscus syriacus	**18.7**	**14.3**	**NI**	**NI**
Hibiscus vitifolius	**NI**	**8.4**	**NI**	**33**
Hibiscus sylvestris	**NI**	**15.4**	**19.7**	**13.2**
Hibiscus sabdriffa	**13.6**	**10.7**	**19.8**	**41.1**

Table (4): kinetic parameters of the purified erythrocyte **h**GSTP1-1

Substance concentration	Inhibitor concentration	Erythrocyte GST	
		Km **(** mM**)**	**Vmax** **(**μmoles / min / mg protein**)**
GSH (0.1-2mM)	**-**	**0.42**	**107.8**
	Cotton (0.308 µg)	1.33	113.8
	Gossypol (40 µM)	0.34	66.2
CDNB(0.1-2mM)	**-**	**0.70**	**111.9**
	Cotton (0.308µg)	1.52	96.15
	Gossypol (40µM)	0.58	71.07

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Table 3: Effects of plant extracts on the activities of the affinity purified GST from different mammalian organs, human erythrocytes and placenta
Table 4: kinetic parameters of the purified erythrocyte hGSTP1-1

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Fig. 1: Lineweaver-Burk plot relating GST activity purified from human erythrocytes to GSH (a) and CDNB (b).
Fig. 2: Time-dependent effect of cotton extract, gossypol and ethacrynic acid on the erythrocyte purified GST activity presented as percentage of erythrocyte GST alone where,
(a) preincubation with cotton extract, cotton extract and glutathione, and erythrocyte GST alone (horizontal line).
(b) preincubation with gossypol, gossypol and glutathione, ethacrynic acid was used as a positive control and the preincubation period was 30 min in all cases.

3-Discussion

Our results clearly indicate that there is a relation between the content of phenolic compounds in the plants extracts and their antioxidant capacity. This could be an electron donating capability effect of the plant extracts, thus forming a stable product and consequently terminate free radical chain reaction. Flavonoids are large compounds occur as glycosides and contain several phenolic hydroxyl groups on their ring structure. Many flavonoids are found to be strong antioxidants capable of effectively scavenging the reactive oxygen species (ROS) because of their phenolic hydroxyl groups. [7]

In this study, H. sabdriffa and H. cannabinus extracts showed high antioxidant activities also had high phenolic, flavonoid and anthoyanin contents. The antioxidant effect of H. sabdriffa extracts could be related to the presence of anthocyanins, which could not be detected in the G. hirsutum (cotton) extracts. Hibiscus anthocyanins are water soluble and among the most important groups of plant pigents which significantly reduced oxidative stress. On the other hand, anthocyanin contents in the other Hibiscus plant extracts represent relatively low amounts with no relation to the color of the plant flower studied. Although anthocyanins are thought to be a pigent responsible for the intense red color of H. sabdriffa petals, in the other studied plants having red color the anthocyanins content was very low compared to H. sabdriffa, indicating that their colors are due to other pigents rather than anthocyanins. The calyx and flowers of H. sabdriffa have been known to contain many chemical constituents. The main constituent of H. anthocyanins (2.5% dry weight) was identified as delphinidin, delphinidin-3-glucoxyloside (also known as hibiscin), polyphenolic acids (1.7% dry weight), flavonoids (1.43% dry weight) and anthocyanins. [8] Although there was no difference between TPC of both cotton and kenaf extracts, cotton extracts did not exhibit the same antioxidant capacities.

In the present study we choose liver, kidney, erythrocyte and placenta having different classes of GST responsible for detoxification in the body to examine the effect of natural phenolic compounds (protocateuic acid, quercetin, tannic acid, delphinidin HCl and gossypol) and known antioxidant compounds (ascorbic acid and α-tochopherol) on the affinity purified GSTs. GST subunits are differentially expressed in mammalian tissues. Liver has mainly Alpha class GSTs and GSTM1. Kidney contains the four main classes, alpha, mu, pi, and theta of cytosolic GSTs, plus the trimeric microsomal form, but at a low level relative to the liver. [9] GSTP1-1 is the most abundant form of intra-erythrocyte transferase representing 95% of entire GST pool. [1]The differential expression of the GSTs may therefore have profound consequences for tissue-selective metabolism and organ-specific toxicity caused by drugs and other xenobiotics. [9] Our results revealed that gossypol (the major phenolic compound of cotton flower) is a potent inhibitor of the conjugation of CDNB by affinity purified GSTs from erythrocyte and placenta. No inhibition of the human enzymes was observed when quercetin, tannic acid and delphinidin-HCl were used as inhibitors. Rat liver and kidney GST enzymes were inhibited by gossypol with lower IC_{50} values than the human and placenta purified enzyme. Tannic acid was the most effective inhibitor of rat liver and kidney GSTs. Also gossypol and quercetin are strong inhibitors with almost the same efficiency for rat liver and kidney enzymes. This result is in agreement with the observation that rat liver isoenzymes of GST are more sensitive to inhibition by plant phenols than the human isoenzymes. [10] While in our study human GSTs are more sensitive to ascorbic acid inhibition than rat GST enzymes. The IC_{50} values of all the tested compounds are higher than those of cibacron blue, a known potent inhibitor of GSTs. The antioxidant capacity of most of the studied plant extracts was weaker than its GST inhibitory potency revealing that these extracts might employ different mechanisms in each process. 1- GSTs are able to interact covalently and non-covalently with various compounds that are not substrates for enzymatic activity. 2- GST activity has been shown to be modulated by natural plant products.

Flavonoids have been shown to inhibit GSTs in human blood platelets as well as in cancer cell lines. Some compounds were able to activate GST M isoforms whilst inhibiting GST A and P. The observed distribution of GST subunits suggests potential differences in the ways by which individual human tissues can detoxify, or otherwise

handle, certain chemical compounds or drugs. Multiple different GSTs with different catalytic specificities suggest that there should be dissimilar tissue-specific responses to the types of compounds acted upon by the proteins. [11] Regarding the importance of the role played by GST enzymes in multidrug resistance to chemotherapy, the interested plant extracts were screened for their effects on GST activity besides their antioxidant efficiencies. This is the first study concerned with the evaluation of the effect of the eight Malvaceae plants on the GST activity affinity purified from rat liver, kidney, human erythrocytes and placenta. Our results have suggested that high GST inhibitory activity of plant extracts could be attributed to the high polyphenolic content, as indicated in the literature for the GST inhibitory effects of naturally occurring plant polyphenols. [11]These results are in good agreement with the total phenolic and flavonoid contents of both cotton and kenaf extracts. Inhibitory effects of naturally occurring plant polyphenols such as tannins, ellagic acid, ferulic acid, caffeic acid, stilbene, quercetin, curcumin and chlorogenic acid against GST have been demonstrated. Quinines are well-known examples of covalent inhibitors of GST enzymes. Although H. sabdriffa extracts contain the highest TPC and TAC, their inhibitory effect on rat liver, kidney, human erythrocytes and placenta GST was found to be weak compared to cotton and kenaf inhibitory effects. Similarly okra extracts have a good inhibitory effect on rat kidney GST, while TPC, TFC and TAC of these extracts were low compared to the other tested plants. There are several reasons to explain the ambiguous relationship between the inhibitory potency and the phenolics and flavonoids. The total phenolics content did not include all the possible inhibitors; the synergism among the inhibitors in the mixture accounted for the inhibition but was not only dependent on the concentration of individual inhibitors but also on the structure and interaction among them. On the other hand, the method used to quantify the flavonoids was limited to flavones and flavonol. [12] A detailed examination of phenolic of different plant extracts is necessary for a comprehensive assessment of the individual compounds enzyme inhibitory ability. Our results clearly indicates a high amount of phenolic compounds does not necessary translated into a high inhibitory effect on GST enzymes, although the type and structure of phenolics of the plant extracts contributes more to their effects and role. In the present study, a simple reproducible procedure for the purification of GST P1-1 from human erythrocytes was established. The GSTP1-1 is the most abundant form of intra-erythrocyte transferase representing 95% of entire GST pool. [1] Our results indicate that G. hirsutum (cotton) extracts inhibits competitively erythrocyte hGSTP1-1 enzyme with respect to GSH and CDNB. Lineweaver–Burk plots (Fig. 2a, b) showed that the competitive inhibition lines intersect on the y-axis, illustrating that, such inhibitors (cotton extracts) do not affect Vmax while increased the km. In competitive inhibition, the substrate and inhibitor cannot bind to the enzyme at the same time. Competitive inhibitors are often similar in structure to the real substrate. [13] Gossypol, $C_{30}H_{30}O_8$, is a polyphenolic aldehyde compound derived from the cotton plant (genus Gossypium, family Malvaceae) that has been reported to have potent anticancer activities in many types of cancer. [14] It exhibits pro- and antioxidant behavior. Its role, as antioxidant, may be dependent on the dose level or the tissue type. [15] It inhibits GST, impairing the liver's ability to metabolize xenobiotic compounds. [14] It's a toxic compound that occurs as a mixture of enantiomers in cotton plant tissues including seed and flower petals. [16] In the present study, kinetic studies indicated that gossypol lowered the Vmax but did not affect the Km for GSH and CDNB suggesting a non-competitive inhibition towards both substrates. Non-competitive inhibition is a form of mixed inhibition where the binding of the inhibitor to the enzyme reduces its activity but does not affect the binding of substrate. [13] Our results suggesting that the inhibitory binding site of gossypol as a non-competitive inhibitor is remote from the catalytic sites leading to conformational changes and hence enzyme inactivation. Gossypol toxicity is related to its ability to bind macromolecules before and after absorption. It may affect certain enzymes in two ways, first: by reacting with the substrate thereby blocking the action of the enzyme or, second: through binding with the enzyme, changing the ionic character of the active site or creating steric hindrance. [15] Our kinetic studies indicated that G. hirsutum (cotton) extracts inhibited erythrocyte GST (hGSTP1-1) competitively, while gossypol non-competitively with respect to GSH and CDNB. The mechanism of inhibition of GSTs by plant polyphenols has not been fully elucidated however it has been suggested that the presence of polyhydroxylations in plant polyphenols is important for GST inhibition. In the present study, it was revealed that exposure of human erythrocyte purified enzyme to both cotton extracts and gossypol decreased the ability of GSTP1-1 to conjugate a variety of electrophilic substrates especially CDNB. Although the different classes of GST show different active site structures at the GSH binding site, it is known that all GSTs share similar catalytic mechanism where the thiol group of glutathione is activated to thiolate. Kaempferol, genistein and quercetin were specific for one class of GST isoforms in that they significantly inhibited only M1-1 and M2-2. The type of inhibition in most cases was mixed or uncompetitive with respect to both GSH and CDNB probably due to binding of the compounds to the ligandin-binding site of the GSTs. [10] Moreover, it has been reported that the specific sensitivity of the pi-class GST to inactivation is linked to the oxidation of a reactive thiol, at position 47 in the amino acid sequence with a concomitant formation of a disulfide. Modification of amino acid residues such as cysteine, tryptophan and methionine have also been found to be sensitive to oxidation besides tyrosine and hence inactivation of enzymes

such as GST. [10] Inhibition of human GSTP1-1 involves conformational changes as suggested by the antioxidants, tocopherols and tocotrienols. Furthermore tannic compound has the potential to bind proteins including the GST enzyme through hydrogen bond formation causing steric hindrance and hence enzyme inactivation. Previous studies with tannins suggested that the $_{O}$-dihydroxy groups in the natural tannin molecule were sites of complex formation. The diuretic drug ethacrynic acid is a potent reversible inhibitor of GST isoenzymes has been used as an inhibitor of GST in vivo. [10] Ethacrynic acid interferes with GST mediated GSH conjugation by two related mechanisms: (a) ethacrynic acid acts as a direct inhibitor of GST activity; (b) ethacrynic acid is also a substrate for GST-mediated GSH conjugation. As a consequence, cellular GSH levels are reduced by treatment with ethacrynic acid, which may result in a further impairment of GST-mediated GSH conjugation. [17] Our results indicate that addition of reduced GSH could reverse the inactivation inhibitory effects of both G. hirsutum (cotton) extracts and gossypol on the erythrocyte hGSTP1-1 enzyme. GSTs M1-1, M2-2 and P1-1 were inactivated by the two polyphenolic compounds ellagic acid and curcumin. The time-dependent inactivation observed indicates irreversible inhibition of these isoforms due to covalent binding. Glutathione and dithiothreitol are capable of protecting GST P1-1 from ethacrynic acid-induced inactivation due to their thiol groups. [11] Under normal physiological conditions (glutathione concentration 1-10 mM) , glutathione may be expected to reverse any covalent binding of ethacrynic acid to GST P1-1, and the inhibition of GST would occur only reversibly, through the glutathione conjugate of ethacrynic acid and of ethacrynic acid itself. However, in those cells with high levels of GSTP1-1 and/or low levels of glutathione, covalent inhibition of GST P1-1 might be predominant. [17] These findings may have several implications. Under normal physiological conditions the inhibition of GST P1-1 by covalent binding of ethacrynic acid would be reversed by glutathione, however, when glutathione levels are low the covalent inhibition might be predominant, resulting in a completely different time course for the inhibition.

Conclusion

Regarding the importance of GST enzymes in cellular defense mechanisms and in multidrug resistance, selected plant extracts belong to the Malvaceae were evaluated for their effects on different mammalian GST activities along with their antioxidant efficiencies. When their antioxidant activities are compared, H. sabdriffa and H. cannabinus extracts which showed high phenolic, flavonoid and anthoyanin contents were by far a better antioxidant than the others. Among the plant extracts studied for GST inhibition activity, G.hirsutum (cotton) was the most efficient inhibitor for the erythrocyte purified hGSTP1-1, correlating with its relatively high total phenolics content. G. hirsutum was less effective in antioxidant capacity than H. sabdriffa**,** however, it can still be considered as potential GST inhibitor. In conclusion, this study has shown that cotton flowers extracts inhibit the major human erythrocyte GST enzyme in vitro (hGSTP1-1). These observations may be of importance in view of the potential use of these compounds both as an in vivo inhibitor for GST P1-1 in drug resistance as well as chemopreventive agents.

6. Experimental Section:

Chemicals

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH), Folin-Ciocalteau-phenol reagent (FC), ascorbic acid, bovine serum albumin, 2, 4-dithiotheritol (DTT), reduced glutathione (GSH), and 1-chloro-2, 4-dinitrobenzene (CDNB) were purchased from Merck Company. SDS-molecular weight standard protein kit, ascorbic acid, α-tochopherol, protocateuic acid, quercetin, tannic acid, delphinidin-HCl, gossypol and cipacron blue were purchased from Sigma Company. Epoxy activated Sepharose 6B and molecular weight standard proteins were purchased from Pharmacia Company. All other chemicals were of the highest purity commercially available.

Plant materials

Collection of plants

Plant materials constituted of eight different flowers belong to the family Malvaceae, G. hirsutum (cotton), Abelmoschus esculentus (okra), H. cannabinus (kenaf), Hibiscus rosa-sinensis, Hibiscus syriacus, Hibiscus vitifolius, Hibiscus sylvestris and H. sabdriffa (Karkadey) were collected from different localities and markets in Egypt, at various periods in 2011-2012. The plants were botanically identified by botany department, National Research Center in Cairo.

Preparation of plant extracts

Tissue samples of each plant (flowers) were dried at room temperature and ground to a fine powder, using a mortar and pestle. The extraction was processed using 2 g of each sample in 100 ml of 70% ethanol at room temperature for 24 h. The extracted material was centrifuged at 1000 g, filtered through Whatman No. 1 filter paper and saved at -4°C for further analyses.

Biological materials (Preparation of the cytosolic fraction from different mammalian sources)

Preparation of human erythrocyte hemolysate

Venous blood samples were purchased from National Center for blood transfusion in Cairo and centrifuged within 4 h of sampling at 1030 g for 10 min. The plasma and buffy layer were then removed; the erythrocytes were washed three times with a 9.0 g/L NaCl solution, and hemolyzed by the addition of an equal volume of ice cold 25 mM Tris-HCl buffer, pH 8.0 containing 1 mM DTT, 5 mM EDTA to yield a 50% hemolysate. The erythrocytes hemolysate was then centrifuged at 10,000 g for 25 min and saved at -4°C for further analyses.

Preparation of human placenta homogenates

Twenty-five villous placental tissue samples were collected by blunt dissection within 30 min of delivery from women (age range 20 years) at El-galaa hospital, Cairo, Egypt (participants gave informed consent). Placental tissue was dissected, placed in ice-cold 25 mM Tris-HCl buffer, pH 8.0 containing 1 mM DTT, 5 mM EDTA, homogenized immediately and centrifuged at 10,000 g for 25 min and saved at -4°C for further analyses. [18]

Preparation of rat liver and kidney homogenates

A 4% homogenates of rat liver and kidney (removed from each rat immediately after the sacrifice) were prepared in 25 mM Tris-HCl buffer, pH 8.0 containing 5 mM β-mercaptoethanol using a glass homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 10,000 g for 30 min to obtain the cytosolic fractions and stored at -4°C for further analyses.

Methods

Determination of total phenolic content (TPC):

Total concentration of phenolic compounds in the extracts was determined using a series of gallic acid standard solutions (2.5 - 20 µg / ml) as described by, [18] but with some modifications. Each extract solution (0.1 ml) was mixed with 2 ml of a 2% (w/v) sodium carbonate solution and vortexed vigorously. The same procedure was also applied to the standard solutions of gallic acid. After 3 min, 0.1 ml of Folin Ciocalteau's phenol reagent was added and each mixture was vortexed again. The absorbance at 750 nm of each mixture was measured, after incubation for 30 min at room temperature.

Determination of total flavonoid content (TFC):

Total concentration of flavonoid compounds in extracts was determined using a series of standard rutin solutions (2.5 - 50 µg / ml) as described in the aluminum chloride colorimetric method. [20]A known volume of each extract solution was mixed with 5% sodium nitrite solution, vortexed vigorously, then 10% aluminum chloride solution was added and vortexed again. After 6 min, 4.3% of sodium hydroxide solution was added, followed by addition of water and each mixture was vortex again. At the end of incubation for 2 h at room temperature, absorbance of each mixture was measured at 510 nm.

Determination of total anthocyanin content (TAC):

Total anthocyanins were measured according to the pH differential method. [21]

Two dilutions of the plant samples, one with 0.025 M potassium chloride buffer, pH 1.0, and the other with 0.4 M sodium acetate buffer, pH 4.5 were prepared. Absorbance of each dilution at 520 nm and 700 nm were measured using distilled water as a blank. The difference in absorbance values at pH 1.0 and 4.5 was directly proportional to the total anthocyanin concentration, which was calculated based on cyanidin-3-glucoside, with a molecular weight of 449.2 g/mol and molar absorption coefficient equal 26,900 mol^{-1} $cm^{-1.}$

Determination of the antioxidant capacity using the free radical scavenging activity DPPH method

The free radical scavenging activities were determined by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method with some modifications of the method proposed by, [22.] A 0.05 mg/ml (0.1 mM) of DPPH ethanol solution which absorbs at 517 nm produces approximately 1.3 U of absorbance. A series of extract solutions with varying concentrations were prepared, 0.1 ml of solutions from each extract was added to 1.4 ml of DPPH solution. The absorbance at 517 nm was recorded after 30 min of incubation at room temperature. IC_{50} concentrations were calculated after constructing the percent inhibition versus log extract concentrations curve.

Preparation of GST enzyme using glutathione-Sepharose affinity chromatography

The glutathione affinity column was prepared, GST activity was determined by conjugation activity towards CDNB and absorbance at 280 nm was recorded. [23]

GST activity determination

Glutathione S-transferase activity was determined by measuring the increase in the concentration of the conjugation product of GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm over 3 min at 25°C. [24]

Unless otherwise stated, the assay mixture contained in a total volume of 1 ml, 0.1 M potassium phosphate buffer, pH 6.5, 1mM CDNB in ethanol (final concentration of ethanol less than 4%), 1mM GSH, and the enzyme solution. One unit is equivalent to the amount of enzyme conjugating 1 µmole of CDNB in 1min at 25°C. The extinction coefficient of the product was taken to be 9.6 $mM^{-1}cm^{-1}$. Protein was estimated using bovine serum albumin as standard. [25]

Screening for GST inhibition

Plant extracts were screened for inhibition of the major cytosolic GSTs affinity purified from rat, liver, kidney, human erythrocytes and placenta. Inhibitory effect of some known polyphenolic compounds (protocateuic acid, quercetin, tannic acid, delphinidin-HCl and gossypol) and powerful antioxidants (ascorbic acid and α-tochopherol) were examined on the isolated GST enzymes. The concentration of inhibitor required to bring about 50% inhibition of GST activity, the IC_{50} value, was determined by plotting sigoidal dose response curves of enzyme activity versus log plant extract concentrations.

Purification of human erythrocyte GST

Erythrocyte GSTP1-1 was purified to homogeneity using procedure included ammonium sulphate precipitation, affinity chromatography on glutathione-Sepharose followed by chromatography on DEAE- Sepharose.

Electrophoresis

Purity of the enzyme purified fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), carried out on 12% slab gels, 7.5% Native PAGE was performed and stained for GST activity. [26-28] Blue insoluble formazan appeared on the gel surface in about 3–5 min, except in the GST.

Kinetic studies

Kinetic and inhibition studies of the purified human erythrocyte GST

All kinetic and inhibition studies were carried out using the purified preparations of the enzyme in the presence and absence of the cotton plant extract and gossypol at a concentration which cause 50% inhibition of enzyme activity (IC_{50}). The apparent Km and Vmax values for GSH were determined at pH 6.5 using a GSH range from 0.1 to 2mM and a fixed CDNB concentration of 1.0 mM. The apparent Km and Vmax values for CDNB were determined using a CDNB range from 0.1 to 2 mM. Data were plotted as double reciprocal Lineweaver–Burk plots to determine the apparent Km values.

Inactivation of the purified human erythrocyte GST

Time-dependent inactivation

Incubation mixtures contained erythrocyte purified GST enzyme with 40 µM of gossypol and 0.308 µg cotton extracts, the incubations were carried out at room temperature. At fixed time intervals, an aliquot of the incubation mixture were withdrawn and assayed for GST activity. These incubations were run in parallel with positive and negative controls. The negative control contained GST enzyme only. The positive control contained GST enzyme and 0.02 µM ethacrynic acid. The inactivation parameter was obtained by plotting graphs of the percentage remaining enzyme activity with time.

Effect of reduced glutathione (GSH) on the inactivation of the purified GST by gossypol, cotton extracts and ethacrynic acid

The possible role of the thiol group of GSH in the protection of erythrocyte GST from inactivation by gossypol, cotton extracts was investigated by incubating the enzyme with gossypol and cotton extracts using the same concentrations as described for time-dependent inactivation, but also including 5 mM GSH in the incubation mixture. An incubation mixture containing GSH and enzyme alone was run to determine the effect of the presence

of these reducing gents on the activity of the enzyme. Incubations were also carried out with the enzyme in buffer alone. A positive control containing ethacrynic acid (0.02 μM) was also run under similar conditions.

7-Conflict of interest: The authors declare no conflict of interest.

8-Ethical statement

All experiments were carried out in accordance with the Egyptian laws and national research center guide lines for the care of experimental animals.

9-References

[1] Noce, A, Ferrannini, M, Fabrini, R, Bocedi, A, Dessı, M, Galli, F, Federici, G, Palumbo, R, Daniele, N D, Ricci, G. (2012). Erythrocyte glutathione transferase: a new biomarker for hemodialysis adequacy, overcoming the Kt/V urea doga? Cell Death and Disease, 3 (377), 112-118.

[2] Zabri Tan, M S M, Abhalim M R, Ismail S, Mustaffa F. (2011). Inhibitory effect of selected Malysian herbal plants on glutathione S-transferase activity. Int J pharmacol, 7(3): 349-355.

[3] Zinoveva, V N, Spasov, A A. (2011). Mechanisms of the anticancer effects of plant polyphenols, I blockade of initiation of carcinogenesis. Biomed Chem, 5(2), 113-123.

[4] Quideau, S, Deffieux, D, Casassus, C D, Laurent, P. (2011). Plant polyphenols: chemical properties, biological activities, and synthesis. Angewandte Chemie, 50 (3), 586 - 621.

[5] El-Naggar, S M. (2004). Pollen morphology of Egyptian Malvaceae: An assessment of taxonomic value. Turk J Bot, 28, 227-240.

[6] Kiessoun, K, Souza. A, Meda, N T R, Coulibaly, A Y, Kiendrebeogo, M, Lamien-Meda, A, Lamidi, M, Millogo-Rasolodimby, J, Nacoulma, O G (2010). Polyphenol contents, antioxidant and anti-inflammatory activities of six Malvaceae species traditionally used to treat hepatitis B in Burkina Faso. **Eur. J. Sci. Res,** 44 (4), 570-580.

[7] Maganha, E G, Halmenschlager, R C, Rosa, R M, Henriques, J A P, Ramos, A L L P, Saffi, J. (2010). Pharmacological evidences for the extracts and secondary metabolites from plants of the genus Hibiscus. Food Chem, 118, 1–10.

[8] Kuo, C Y, Kao, E S, Chan, K C, Lee H J, Huang, T F, Wang, C J. (2012). Hibiscus sabdariffa L. extracts reduce serum uric acid levels in oxonate-induced rats. J Funct Foods, 4375-4381.

[9] Eklund, B I, Moberg, M, Bergquist, J, Mannervik, B. (2006). Divergent activities of human glutathione transferases in the bioactivation of azathioprine. Mol Pharmacol, 70, 2, 747-754.

[10] Gyamfi, A M, Ohtani, I I, Shinno, E, Aniya, Y. (2004). Inhibition of glutathione S-transferases by thonningianin A, isolated from the African medicinal herb, Thonningiasanguinea, in vitro. Food Chem Toxicol, 42, 1401-1408.

[11] Hayeshi, R, Mutingwende, I, Mavengere, W, Masiyanise, V, Mukanganyam, S. (2007)**.** The inhibition of human glutathione S-transferases activity by plant polyphenolic compounds ellagic acid and curcumin. Food Chem Toxicol, 45(2), 286-295.

[12] Bangou, M J, Kiendrebeogo M, Compaore M, Coulibaly A Y, Meda N T R, Abarca, N A, Zeba, B, Millogo-Rasolodimby, J, Nacoulma, O G. (2011). Enzyme inhibition effect and polyphenolic content of medicinal plant extracts from Burkina Faso. J Biol Sci, 11(1), 31-38.

[13] Segel, I H (1993). Enzyme kinetics: behavior and analysis of rapid equilibrium and steady state enzyme systems (new ed.). John Wiley & Son Inc, New York.

[14] Gunassekaran, G R, Gayathri, R, Priya, D K D, Murugan, S, Sakithsekaran, D. (2010). Protective role of gossypol against N-methyl-N-nitro-N-nitrosoguanidine (MNNG) induced Gastric carcinogenesis in experimental rats. Int J Med Sci, 2 (4), 121-127.

[15] El-Sharaky, A S, Wahby, M M, Bader El-Dein, M M, Fawzy R A, El-Shahawy I N. (2009). Mutual anti-oxidative effect of gossypol acetic acid and gossypol-iron complex on hepatic lipid peroxidation in male rats. Food Chem Toxicol, 47, 2735-2741.

[16] Vshivkov, S, Pshenichnov, E, Golubenko, Z, Akhunov, A, Namazov, S, Stipanovic, R D. (2012). Capillary electrophoresis to quantitate gossypol enantiomers in cotton flower Petals and seed. J Chromatogr, 908, 94 - 97.

[17] Ploemen, J H T M, Van Schanke, Z A, Van Ommen B, Van Bladeren P J. (1994). Reversible conjugation of ethacrynic acid with glutathione and human glutathione S-transferase P1-1. Cancer Res, 54, 915-919.
[18] Collier, A C, Tingle, M D, James, W P, Murray, D M, Keelan, J A. (2002). Metabolizing enzyme localization and activities in the first trimester human placenta: The effect of maternal and gestational age, smoking and alcohol consumption. Hum Reprod, 17 (10), 2564-2572.
[19] Singleton, V L, Rossi, J A. (1965). Colorimetry of total phenolic substances. ACS Symposium series, 26, 47-70.
[20] Zhu, H, Wang, Y, Liu, Y, Xia, Y, Tian, T. (2010). Analysis of flavonoids in Portulacaoleracea L. by UV- Vis spectrophotometry with comparative study on different extraction technologies. Food Anal. Methods, 3, 90–97.
[21] Fuleki, T, Francis, F J. (1968). Determination of total anthocyanin and degradation index for cranberry juice. Food Sci., 33(1), 78-83.
[22] Blois, M S. (1958). Antioxidant determinations by the use of a stable free radical. Nature. 26, 1199-1200.
[23] Simons, P C, Vander-Jagt, D L. (1974). Purification of glutathione S-transferases from human liver by glutathione affinity chromatography. Anal Biochem. 82, 334-341.

[24] Habig, W H, Pabst, M J, Jakoby, W B. (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem, 249, 7130-7139.
[25] Bradford, M M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72, 248-254.
[26] Laemmli, U K (1970). Cleavage of structural proteins during assembly of head bacteriophage T4. Nature, 227, 680-685.
[27] Davis, B Y. (1964). Disc electrophoresis. Method and application to human serum protein. Ann Ny Acad Sci, 121, 404-427.

[28] Ricci, G, Bello, M L, Caccuri, A M, Galiazzo, F, Federici, G. (1984). Detection of glutathione transferase activity on polyacrylamide gels. Anal Biochem, 143, 226–230.