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Inter-individual variation of normal and Down syndrome glutathione transferase in response to different phenolic compounds.

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ABSTRACT

Glutathione transferase (GST) catalyzes the conjugation of reduced glutathione (GSH) with a variety of xenobiotic compounds. Down syndrome (DS) considered as the most mental retardation genetic cause. GST was purified from the erythrocyte of two subjects subjected to genotype analysis normal (GSTT1⁺) and DS (GSTT1⁺& GSTM1⁺) erythrocytes by affinity chromatography. The effect of some bioactive compounds, eight different plant extracts and the daily supplement curcumin on the activity of GSTP from normal and DS was examined. Normal and DS GSTs were found to be different, 1) clove extract has inhibitory effect on normal GST (IC₅₀ value of 3.63 µg), while has no effect on DS GST. Eugenol (the clove major component) has no effect on normal and DS GSTs. 2) Tannic acid and quercetin have no effects on normal GST while they inhibit DS GST activity. Allyl-isthiocynate was not the most effective compound on DS GST activity (IC₅₀ = 7.66 µM). Tannic acid was 4.35 fold powerful inhibitor of DS GST activity (IC₅₀ = 1.76 µM) than allyl-isthiocynate. 2.5 µg of rocket extract (the most effective plant on DS GST activity) caused decrease in the Km and Vmax values from 2 to 0.19 mM and from 50 and 10 µmoles / min / mg protein, respectively, for glutathione substrate. While Vmax value was unchanged (20 µmoles / min / mg protein) with a Km equal 0.105 mM increased to 0.5 mM for CDNB.

Keywords: Down syndrome, Polyphenols, Antioxidant capacity, Glutathione transferase, Enzyme kinetic, Enzyme inhibition

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INTRODUCTION

Down syndrome (DS) considered as one of many causes of intellectual disability (ID) with a number of neurobiological disorders such as learning, memory deficits and Alzheimer disease (**Ruparelia, Pearn, & Mobley, 2013**). Elevated frequency of many health problems as congenital heart disease, hypotonia, leukemia, physical anomalies and motor disorders occur in DS people (**Lin et al. 2015**).

Oxidative stress is the imbalance between production of free radicals, reactive metabolites and the antioxidant defense systems. DS individuals have been associated with oxidative stress as a result of trisomy of 21st chromosome. Oxidative stress producing many morphological abnormalities, intellectual disability, premature aging, number of biochemical abnormalities and other immune disorders. The antioxidant defense systems acts against toxic effects of free radicals, reactive oxygen species (ROS) and their metabolites in the living organism. Reduced function of antioxidant defense system increases oxidative stress leads to structure disruption and dysfunctions of important biomolecules, such as proteins, lipids and nucleic acids affecting pathophysiology of organs and the whole organism (**Muchová et al. 2014**).

Glutathione transferases, GST, (EC 2.5.1.18) are a multifunctional super family of detoxification enzymes which catalyze the conjugation of glutathione to toxins and xenobiotics. GSTs play an important role in the detoxification of electrophilic compounds such as, toxins, drugs and carcinogens then exported from the cell through the GS-X pump in an ATP dependent manner. Enzymes of GST considered as an adaptive response to chemical challenge as they are induced by many chemopreventive agents (**Hayeshi, 2007**). The human cytosolic GSTs are dimeric proteins classified into seven gene-independent classes (Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta) depending on their amino acid sequence (**Parker et al. 2008**). The cytosolic GSTs are expressed in the different organs in the human body. It is also possible that one organ expresses more than one isoform of GST. Human erythrocytes express the Pi isoform of the enzyme (GSTP1) and also distributed in many tissues (**Van-Haafden and Haenen, 2003**). The potential role of GSTP1 in the disease makes this enzyme as a subject of many studies (**Parker et al. 2008 & Hamed et al. 2011**). Individual differences in expression of bio-transforming and detoxifying enzymes may lead to increased susceptibility to toxic agents. A genetic susceptibility toward certain pollutants might determine the individual risk against mutagenic pollutants (**Dirksen et al. 2004**). GSTM1 (class Mu) and GSTT1 (class Theta) genes are polymorphic in humans (**Chiang et al. 2007**). Genetic polymorphisms in the GST genes can affect the function of these enzymes, therefore are of special interest as candidate genes. The polymorphisms in GSTM1 and GSTT1 are due to homozygous gene deletions resulting in absence of the respective GST enzyme activity (**Agalliu et al. 2006**).

Research concerning the health benefits of a diet rich in natural products and consumption of food supplements has increased recently over the last 5–10 years all over the world. The Mediterranean diet, rich in flavonoids, considered as an effective way for improving the general health status. Also the French paradox is discussed with respect to the ability of polyphenols in reduction of the incidence of cardio-vascular disease in a population consuming a lipid rich diet. However, as a coin has two sides, there are also some problematic issues, regarding the toxicological aspects, there are complex issues regarding pharmacokinetic interactions leading to changes of important ADME (absorption, distribution, metabolism and excretion) processes under the effect of natural compounds from diet or food supplements (**Tribolo et al. 2008; Winterbone et al. 2009; Margina et al. 2015**). Natural polyphenol beneficial effects reflecting their ability to scavenge-free radicals generated endogenously or formed by xenobiotics. In addition to the antioxidant effect of the polyphenols, emerging evidence indicates that polyphenols could act as pro-oxidants initiating a reactive oxygen species (ROS) lead to DNA damage and apoptosis (**Hamed et al. 2014**).

A great number of natural plants have received attention as sources of bioactive compounds with antioxidant, antimutagenic and anticarcinogenic properties. Among the sources of natural antioxidants are primarily, plant phenolic and polyphenolic compounds that have an important role in human nutrition (**Karakurt and Adali, 2011**). Some plant extracts generally used in traditional medicine as the tea components (epigallocatechin gallate) are listed as cytochrome-P450 (CYP) enzymes inhibitors leading to drug metabolism disturbance. Fruit juices (e.g. grapefruit juice) having an important impact on the pharmacokinetic data and toxicity of drugs, as they can inhibit the activity of some enzymes involved in drug metabolism (**Margina et al. 2015**).

Theories advocating various vitamins, minerals, amino acids, enzymes and hormones supplementation in different quantities, are sources of considerable controversy in DS community (**Buckley and Sacks, 1998**). There are no studies of well-designed scientific on the use of alternative therapies in DS individuals. Antioxidants considered as a theoretical promise for cognitive, immune, premature aging problems and malignancy treatment in DS (**Roizen, 2005**). As a result concerns are raised regarding the potential interactions of supplements in patient's under-going chronic therapy which may lead to changes of bioavailability, metabolism, distribution, elimination and toxicity of the drugs. DS individuals with lower overall GST activity and slight differences in some kinetic characters are at greater risk from xenobiotic contamination compared to normal individuals. In this study, since children with DS are targeted for natural supplement use, eugrenol, allyl-isthiocynate, quercetin, tannic acid, eight different plant extracts belong to different families and the daily supplement powder curcumin were used to investigate their polyphenol, flavonoid, anthocyanin contents, antioxidant capacities and there in vitro effects on normal and DS erythrocyte GSTs activity.

MATERIALS AND METHODS

Chemicals

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH), Folin-Ciocalteu-phenol reagent (FC), bovine serum albumin (BSA), 2, 4-dithioeritol (DTT), reduced glutathione (GSH), and 1-chloro-2, 4-dinitrobenzene (CDNB) were purchased from Merck Company. SDS-molecular weight standard protein kit, ascorbic acid, eugrenol, allyl-isthiocynate, quercetin and tannic acid were purchased from Sigma Company and epoxy activated Sepharose 6B from Pharmacia Company. All other chemicals were of the highest purity commercially available. The daily supplement powder curcumin was purchased from NuTriVene, Longvida, Middle River, MD 21220 (International Nutrition, Inc.).

Subjects

This study was carried out on three DS (20–30 years) patients referred to the Clinic with Special Needs, National Research Centre, Giza, Egypt, and 3 healthy participants matched for age (30-35 years). Cytogenetic analysis (comprehensive history, clinical examination and karyotyping by G-banding technique) showed free non-disjunction trisomy 21 in the studied DS patients. A written consent was obtained from each participant, according to the guidelines of the ethical committee of the National Research Centre.

Genotype analysis

Genomic DNA was extracted from peripheral leukocytes of three different normal and three different DS samples by the generation DNA purification capture column kit (Gentra Company catalog No GC; 0050). Genomic DNA was analyzed with polymerase chain reaction technique (PCR) according to the method described by **Naoe et al. (2000)**. A 50 bp-10,000 bp DNA size-standard marker was used.

Plant materials

Collection of plants

Plant materials consisted of eight different seeds belong to different families, *Syzygium aromaticum* (cloves), *Psidium guajava* (guava), *Punica granatum* (pomegranate), *Raphanus sativus* (Radish), *Brassica juncea* (Mustard), *Eruca sativa* (rocket), *Cinnamomum verum* (cinnamon), *Cinnamomum camphora* (camphor) were collected from different localities and markets in Egypt, at various periods in 2013-2014. The plant seeds were botanically identified by the Botany Department, National Research Centre, Egypt.

Preparation of plant extracts

Tissue samples of each plant (seeds) were dried at room temperature and ground to a fine powder, using a mortar and pestle. The extraction was processed using 0.5 g of each sample in 5 ml of 70% ethanol at 55° C for 2 hrs. The extracted material was centrifuged at 1000 g for 10 minutes, filtered through Whatman No. 1 filter paper and saved at -4°C for further analyses.

Phytochemical analysis

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 $\mu\text{g} / \text{ml}$) as described by **Singleton and Rossi (1965)** 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 Ciocalteu'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 $\mu\text{g} / \text{ml}$) as described in the aluminum chloride colorimetric method (**Zhu et al. 2009**). 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 (**Fuleki & Francis, 1968**). 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 $\text{mol}^{-1} \text{cm}^{-1}$.

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 **Blois (1958)**. 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.

Purification of human erythrocyte GST using glutathione-Sepharose affinity chromatography

Venous blood (20ml) was collected in Ethylene diaminetetraacetic acid, sodium salt (EDTA) containing tube and centrifuged within 4 hr of sampling at 1030 xg for 10 minutes. 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 distilled water to yield a 50% hemolysate. Erythrocyte GSTP1 was purified to homogeneity using affinity chromatography on glutathione-Sepharose 6B. GST activity was determined by conjugation activity towards CDNB and absorbance at 280 nm was recorded (**Hamed et al. 2011**).

GST activity determination

Glutathione transferase activity was determined by measuring the increase in the concentration of the conjugation product of GSH and CDNB at 340 nm over 3 min at 25°C (**Habig et al. 1974**). 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 $9.6 \text{ mM}^{-1}\text{cm}^{-1}$. Protein was estimated using Coomassie brilliant blue G-250 and bovine serum albumin as standard (Bradford, 1976).

Effect of phenolic compounds and plant extracts on GST activity

Inhibitory effect of eugenol, allyl-isothiocyanate, quercetin and tannic acid were examined. Plant extracts rich in the examined phenolics were also screened for their effects on GST affinity purified from normal and DS erythrocytes. The concentration of inhibitor required to bring about 50% inhibition of GST activity, the IC_{50} value, was determined by plotting sigmoid dose response curves of enzyme activity versus log plant extract concentrations.

Kinetic studies

Kinetic of the purified normal and DS erythrocyte GSTs

All kinetic and inhibition studies were carried out using the purified preparations of the enzyme in the presence and absence of the rocket seed extract at a concentration which cause 50% inhibition of enzyme activity (IC_{50}). The apparent K_m and V_{max} 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 K_m and V_{max} 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 K_m values.

Effect of reduced glutathione (GSH) on the inactivation of the purified GST by rocket extracts and curcumin supplement

The possible role of the thiol group of GSH in the protection of erythrocyte GST from inactivation by rocket extracts and dietary curcumin supplement was investigated by incubating the enzyme with 2.5 μg of rocket extract and 2.3 ng of curcumin concentrations, 1 mM or 2 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 agents on the activity of the enzyme. Incubations were carried out with the enzyme in buffer alone. At fixed time intervals, an aliquot of the incubation mixture was withdrawn and assayed for GST activity.

High performance liquid chromatography (HPLC)

HPLC method for the analysis of allyl isothiocyanate was developed in the rocket (*E. sativa*) seed extract according to the method of Pelosi et al. (2014).

Statistical analysis

All data are reported as mean \pm SD for $n = 2-4$ independent experiments. The Student's t test was performed to examine the difference between means.

RESULTS

Seeds of eight plants belong to four plant families (Myrtaceae, Lythraceae, Brassicaceae & Lauraceae) and the antioxidant daily supplement curcumin (Zingiberaceae) (optimized curcumin extract from *Curcuma longa*) are believed to have powerful medicinal properties (Dahiya et al. 2014; Diego Francisco Cortés-Rojas et al. 2014) were chosen for this study. The plant extracts were prepared and their phenolic, flavonoid, anthocyanin contents and antioxidant capacity were determined.

Total phenolic content (TPC)

Total concentrations of phenolic compounds in extracts of the eight plant seeds were expressed as mg of total phenolics in g of extracts as gallic acid equivalents (Table 1). Among the studied plants, *P. granatum* (pomegranate) and *C. verum* (cinnamon) extracts exhibited the highest phenolic contents (144 ± 36 and 114.5 ± 64 mg gallic acid/g seed). The two extracts of Myrtaceae (guava and cloves) have the lowest content of phenolic compounds (11.5 ± 2.1 and 13.5 ± 4.8 mg gallic acid / g seed). *C. camphora* (camphor), *R. sativus*

(radish) and *E. sativa* (rocket) extracts exhibited relatively high amounts of phenolic compounds (71.6 ± 12.7 , 41 ± 4.2 and 35 ± 10.4 mg gallic acid/g dry seed, respectively) compared to the extracts of the other studied plants.

Total flavonoid content (TFC)

The highest content was found in *P.granatum* (pomegranate) extract with 10 ± 3.1 mg rutin / g dry seed. Both plant extracts of **Lauraceae family** have almost the same flavonoid contents (3.4 ± 0.68 and 3.33 ± 1.3 mg rutin / g dry seed for *C. camphora* (camphor) and *C. verum* (cinnamon) extracts, respectively). Total flavonoids of other plant extracts represented the following decreasing order: *E. sativa* (rocket) > *S. aromaticum* (cloves) > *R. sativus* (radish) > *P. guajava* (guava) (Table 1).

Table 1: Total phenolic (TP), total flavonoid (TF) and total anthocyanin (TA) contents of eight different seeds and their DPPH free radical scavenging activity

Family	Plant	TPC (mg gallic/ g seed)	TFC (mg rutin/ g seed)	TAC (mg / g seed)	DPPH * IC ₅₀ (mg dry seed /ml)
Myrtaceae	<i>S. aromaticum</i> cloves	13.5 ± 4.8	1.68 ± 0.18 (12.4)%	6.76 ± 4.3 (50.1%)	0.222 ± 0.1
Myrtaceae	<i>P. guajava</i> guava	11.5 ± 2.1	0.73 ± 0.3 (6.3%)	1.22 ± 0.7 (10.6%)	5.45 ± 1.6
Lythraceae	<i>P. granatum</i> pomegranate	144 ± 36	10 ± 3.1 (6.94%)	33 ± 2.5 (23%)	1.85 ± 0.3
Brassicaceae	<i>R. sativus</i> radish	41 ± 4.2	0.98 ± 0.26 (2.4 %)	13.6 ± 5.7 (33.2%)	2.48 ± 0.69
Brassicaceae	<i>B. juncea</i> mustard	18.7 ± 6.8	0.69 ± 0.27 (3.7 %)	ND	6.63 ± 0.81
Brassicaceae	<i>E. sativa</i> rocket	35 ± 10.4	2.1 ± 0.43 (6 %)	22.7 ± 4.6 (65%)	2.29 ± 1.2
Lauraceae	<i>C. verum</i> cinnamon	114.5 ± 64	3.33 ± 1.3 (2.9 %)	65 ± 42 (56.8%)	1.43 ± 0.068
Lauraceae	<i>C. camphora</i> camphor	71.6 ± 12.7	3.4 ± 0.68 (4.75 %)	37.2 ± 0.32 (52%)	2.085 ± 0.79
	Ascorbic acid	-	-	-	0.625 ± 0.016

-Values are expressed as mean ± SE of triplicate experiments

- ND: not detected under experiment conditions.

- IC₅₀ (amount of extract which cause 50% inhibition of DPPH free radical scavenging activity)

- Values between parrenthes are the percent of TFC and TAC /TPC

Total anthocyanin content (TAC)

Results in Table 1 showed that extracts of *C. verum* (cinnamon) exhibited the highest anthocyanin content (65 ± 42 mg anthocyanin / g seed) compared to the other studied plants. Total anthocyanin content of the other extracts was ranging from 37.2 ± 0.32 to 1.22 ± 0.7 mg anthocyanin / g seed. The highest values of total anthocyanin were in the following decreasing order: *C. camphora* (camphor) > *P.granatum* (pomegranate) > *E. sativa* (rocket) > *R. sativus* (radish) > *S. aromaticum* (cloves) > *P. guajava* (guava). Anthocyanins could not be detected in *B. juncea* (Mustard) extracts. According to the percentage of anthocyanin yield relative to total phenolics, these results indicated that *E. sativa* (rocket) extracts contained the highest percentage of anthocyanin yield (65%) relative to total phenolics, followed by cinnamon (56.8%), camphor (52%) and cloves (50.1%).

The antioxidant activities using DPPH free radical scavenging activity method

Antioxidant activity of the present studied plant extracts was investigated by using DPPH scavenging inhibition method. The IC₅₀ values for DPPH % scavenging activity were determined from the % inhibition

versus log plant extract concentration curve using vitamin C as a standard. Plant extracts IC₅₀ values for DPPH percent scavenging activity are shown in Table 1. The data showed that *S. aromaticum* (cloves) seed extracts have a powerful antioxidant capacity where IC₅₀ value of 0.22 ± 0.1 mg dry seed/ml compared to the other seed extracts, even powerful than the standard vitamin C (IC₅₀ = 0.625±0.0163). On the other hand, the lowest antioxidant capacity was given by *B. juncea* (Mustard) extracts (6.63 ± 0.81 mg / g seed). According to the calculated IC₅₀ of the effective plant water extracts in DPPH radical scavenging ability, they exhibited the following decreasing order: *C. verum* (cinnamon) > *P. granatum* (pomegranate) > *C. camphora* (camphor) > *E. sativa* (rocket) > *R. sativus* (radish) > *P. guajava* (guava).

Genotype analysis

PCR technique was used for the determination of the presence or absence of GSTT1 and GSTM1 in genomic DNA, with the primers for GSTT1 and GSTM1, using β-globin as internal control for DNA degradation, the presence of DNA in all samples under investigation was indicated. DS samples representing the following genotypes: the double null of GSTT1 and GSTM1 (GSTT1⁻ & GSTM1⁻), GSTT1 present (GSTT1⁺) and GSTM1 present (GSTM1⁺) and GSTM1 present (GSTM1⁺) were used in this study. Two of the normal erythrocytes having GSTT1 genotypes and the other one have the double null of GSTT1 and GSTM1 were used in this study. Two samples from normal and DS having GSTT1 genotype (GSTT1⁺) and GSTT1⁻, GSTM1⁺, respectively, were chosen for the further GST purification, characterization and inhibition studies.

Erythrocyte GST purification

In the present study, a simple reproducible procedure for the purification of GSTP1 from human red blood cells was used. The normal and DS enzyme exhibited almost the same chromatographic behavior on GSH-Sepharose affinity column with specific activity of 24.6 and 31.24 units/mg protein for normal and DS, respectively. One major band could be detected for normal and DS purified erythrocyte GST as judged by 7% PAGE (data not shown).

Table 2: Effects of plant extracts and some of their bioactive compounds on the activities of the affinity purified GST from normal and DS GSTP1

Plant name	IC ₅₀		Bioactive compound	IC ₅₀	
	normal	DS		normal	DS
<i>S. aromaticum</i> cloves	3.63 mg	15.8 mg	Eugrenol	NI	NI
<i>P. guajava</i> guava	NI	NI			
<i>P. granatum</i> pomegranate	1.48 mg	39.8	Tannic acid	NI	1.76 μM
<i>R. sativus</i> radish	NI	5.75 mg			
<i>B. juncea</i> mustard	NI	NI			
<i>E. sativa</i> rocket	NI	2.51 mg	Allyl-isthiocynate	NI	7.66 μM
<i>C. verum</i> cinnamon	1.122 mg	NI	Quercetin	NI	25.7 μM
<i>C. camphora</i> camphor	4.37 mg	NI			
Curcumin	14.9 μg	2.29 ng			

IC₅₀: amount of extract which cause 50% inhibition

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

Effect of some phenolic bioactive compounds on normal and DS GST activities

The inhibitory effect of some natural occurring compounds (eugenol, quercetin, allyl isothiocyanate and tannic acid) on the affinity purified normal and DS GST activities were investigated (Table 2). Not all the

studied compounds have shown considerable inhibition on the GST activities. Comparing the used compounds for their IC_{50} values, tannic acid was the most effective inhibitor of DS GST activity ($1.76 \mu\text{M}$), followed by allyl isothiocyanate ($7.66 \mu\text{M}$) and quercetin ($25.7 \mu\text{M}$). Eugenol has no inhibitory effect on both of normal and DS GST. However, normal GST was not inhibited by all the tested compounds.

Effects of plant extract rich in the previously examined phenolics on normal and DS purified erythrocyte GST activity

All the studied plant extracts have shown considerable inhibition on GST activity and the percent inhibition of GST enzymes was calculated in the presence of varied extract concentrations (2–6 $\mu\text{g/g}$ seed). Comparing all the studied plants for their inhibitory capacity IC_{50} values as shown in Table 2, *C. verum* (cinnamon) and *P.granatum* (pomegranate) extracts were the most effective inhibitors of normal erythrocytes GST activity toward CDNB, with almost comparable values ($1.122 \mu\text{g}$ and $1.48 \mu\text{g}$, respectively). Of all the plant extracts tested in our study, curcumin extracts showed the highest inhibitory effect on DS GST activity, with a value of 2.29 ng dry seed ; followed by *E. sativa* (rocket) extracts with IC_{50} value of $2.51 \mu\text{g}$ dry seed. GST affinity purified from normal erythrocytes inhibited by cloves and camphor extracts with IC_{50} value of 3.63 and $4.37 \mu\text{g}$, however, the three extracts of the family Brassicaceae showed no inhibition on normal GST. By the same way, the two extracts of the family Lauraceae showed no inhibition on DS GST. Specifically, the famous antioxidant extracts guava and mustard have no effects on both normal and DS GST activities (Table 1).

HPLC analysis

HPLC analysis for *E. sativa* showed the presence of gallic acid (0.192 mg per g of dry seed), protocatechuic acid (1.024 mg/g), catechin (1.2 mg/g), gentesic acid (0.647 mg/g), vanillic acid (0.315 mg/g), syringic acid (0.051 mg/g) and caffeic acid (0.03 mg/g) in the rocket ethanol extract. A concentration of 1.03 mg/g seed was identified ($128.7 \mu\text{g/ml}$) for allyl isothiocyanate in the ethanol extract of rocket (*E. sativa*) seed. HPLC fingerprint profile showed the presence of an unknown peak component with high concentration (11.6 fold increases) compared to the examined allyl isothiocyanate content (Table 3).

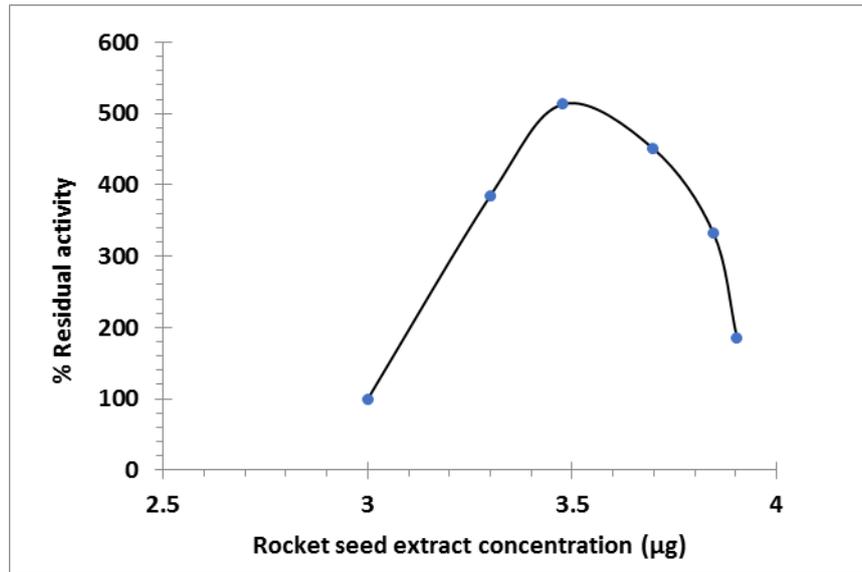
Effect of *E. sativa* (rocket) on variable substrate concentration of the erythrocyte purified GST

The effect of rocket extract concentrations on two DS samples (GSTT1⁻ & GSTM1⁻) and (GSTT1⁺ & GSTM1⁺) genotypes was determined (Fig.1). Completely different behavior was observed, where rocket extract increased GST activity by more than 4 folds for the GSTT1⁻ & GSTM1⁻ genotype sample. The reverse was true for GSTT1⁺ & GSTM1⁺ genotype where GST activity was completely inhibited (more than 80% of activity). Rocket extracts inhibited DS (GSTT1⁺ & GSTM1⁺) GST activity with 50% inhibitory concentration of $2.5 \mu\text{g}$. GST activity was assayed with variable concentrations of either CDNB or GSH. Figs. 2 and 3 showed the Lineweaver–double reciprocal plots with different concentrations of CDNB and GSH. When GSH was used as the varying substrate, the K_m and V_{max} values were decreased in the presence of rocket extract from 2 to 0.19 mM and from 50 and $10 \mu\text{moles / min / mg protein}$, respectively. While, by using rocket extract, the V_{max} value was unchanged ($20 \mu\text{moles / min / mg protein}$) with a K_m equal 1mM increased to 0.5 mM for CDNB (Fig 2).

Effect of GSH addition on the inactivation of DS GST by rocket and curcumin extracts

Effect of GSH on the inhibitory capacity of the most effective extracts, rocket and curcumin was investigated on DS GST activity (Fig. 4). GSH at 1 and 2 mM protected the DS GST enzyme from inactivation by the powerful inhibitor curcumin ($IC_{50} = 2.29 \text{ ng}$). However, 2 mM GSH could not protect DS GST from rocket extract ($IC_{50} = 2.51 \mu\text{g}$) inactivation as shown in Fig 2. GSH alone enhanced the activity of the normal enzyme in the absence of an inhibitor. This trend was not observed when the DS enzyme was incubated with 1 mM GSH.

(a)



(b)

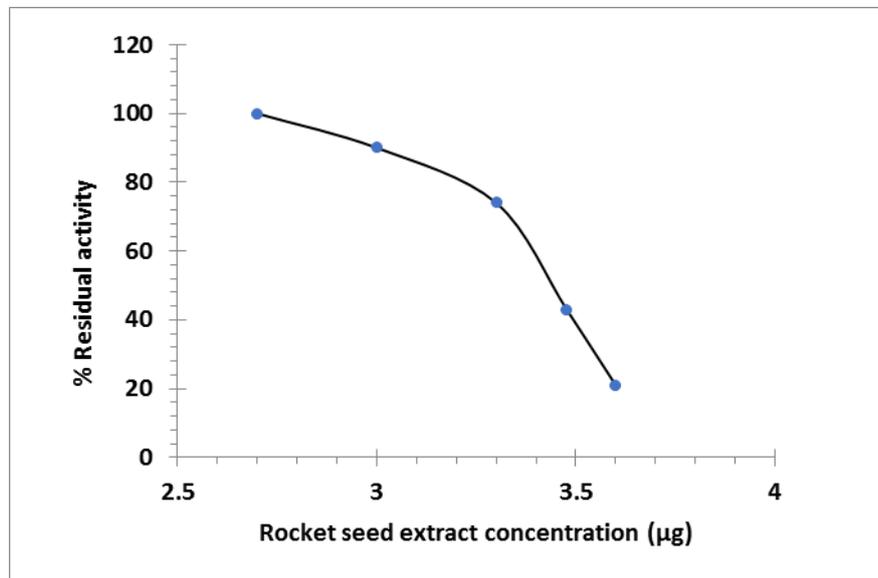
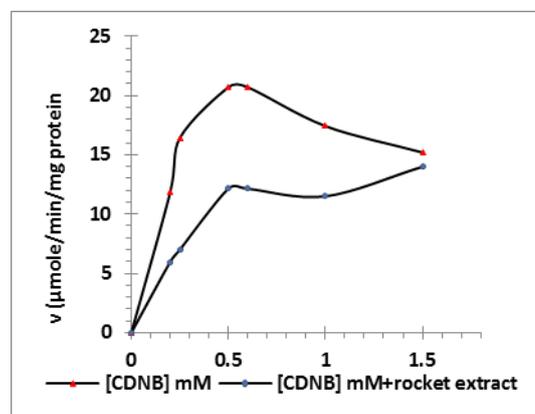


Fig. 1: Effect of rocket extract on GST activity of two genetically different DS samples a: (GST T1⁻& GSTM1⁻) and b: (GST T1⁺& GSTM1⁺).



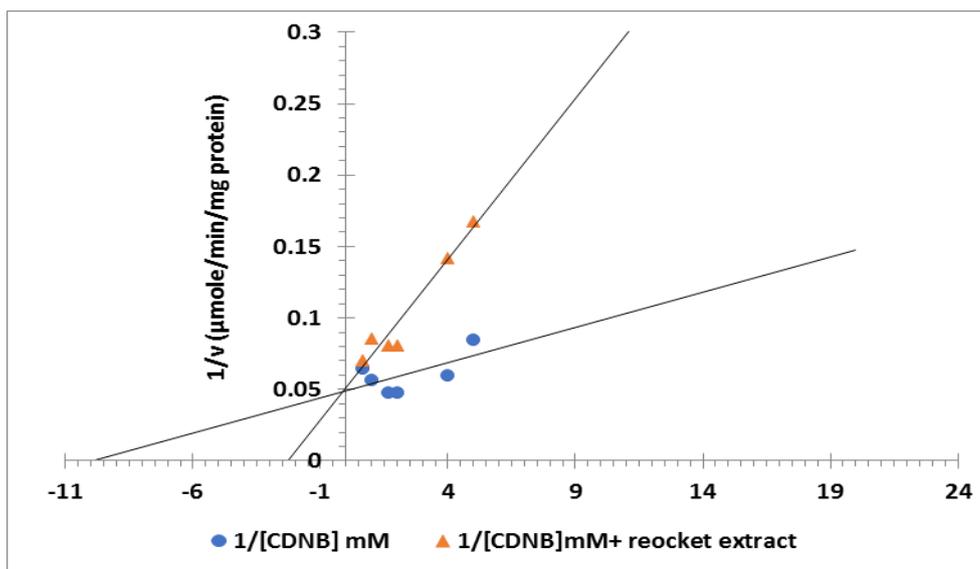


Fig. 2: Lineweaver–Burk graph relating DS GST activity to GSH concentrations (0.25- 2 mM) at fixed CDNB concentration (1mM) in the presence and absence of 2.5 mg of rocket extract (rocket extract for 50% inhibition of GST activity).

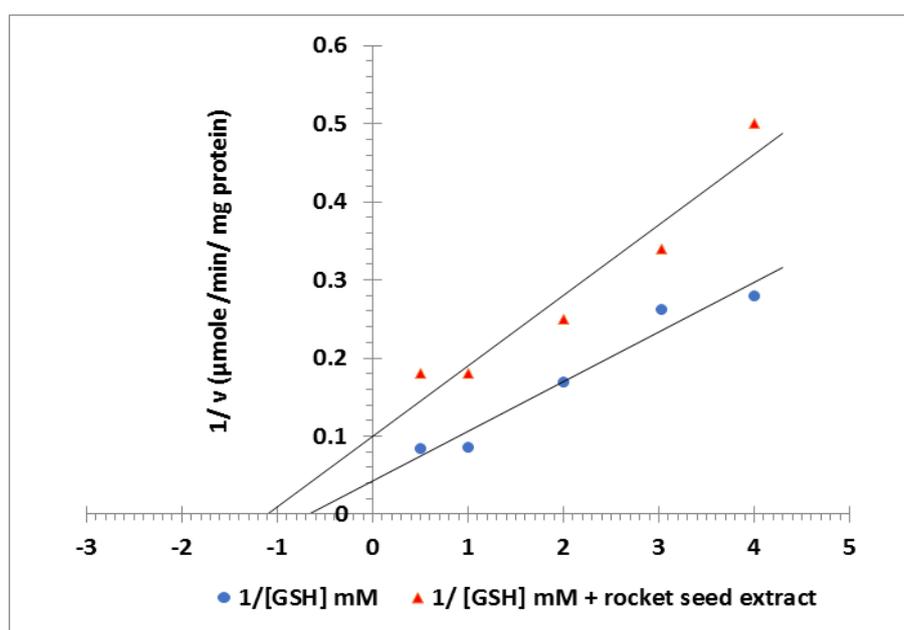
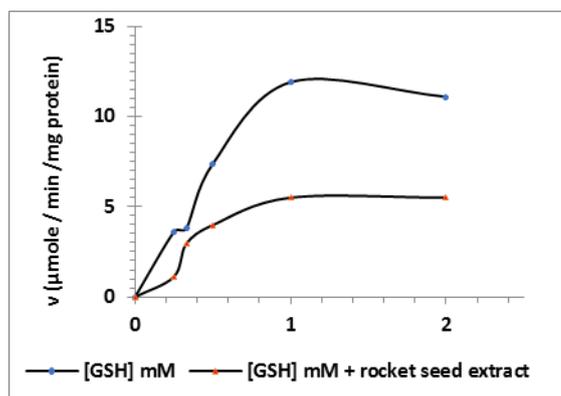


Fig. 3: Lineweaver–Burk graph relating DS activity to CDNB concentrations (0.1- 1 mM) at fixed GSH concentration (1mM) in the presence and absence of 2.5 mg of rocket extract (rocket extract for 50% inhibition of GST activity).

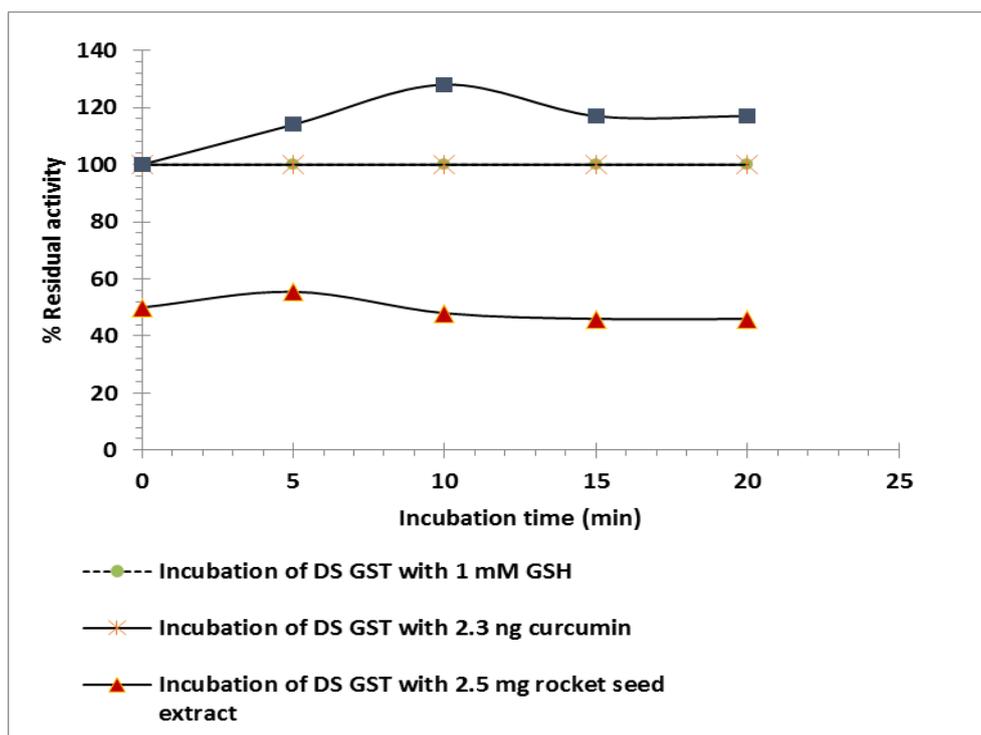


Fig. 4: Effect of incubation for 20 min with 1 & 2 mM GSH concentrations on normal (GSTT1⁺) and DS (GST T1⁺& GSTM1⁺) GST activity in the presence and absence of 2.5 mg rocket extract and 2.3 ng of curcumin.

DISCUSSION

In the present study we were interested in the evaluation of the effect of four structurally different phenolic compounds as eugenol, tannic acid, allyl-isthiocyanate and quercetin on the activity of the affinity purified (GSTP1) GST enzyme from normal and DS erythrocytes.

Phytochemical analysis of eight plant extracts belong to different families containing in the examined phenolics and curcumin supplement were used to examine the in vitro effects and variation in activity of the affinity purified (GSTP1) enzyme from normal and DS erythrocytes. As a state we purified GST from normal (GSTT1⁺) and DS (GSTT1⁺& GSTM1⁺) erythrocytes using a simple reproducible procedure. Affinity purification step of human erythrocytes employed the presence of one major GST form binds to GSH affinity column (GSTP1). In erythrocyte, GSTP1 is the most abundant form of transferase representing 95% of entire GST pool (Noce et al. 2012). In erythrocyte and lymphocytes, GSTT1 is expressed and hence acts in the hematopoietic system (Dirksen et al. 2004); GSTM1 was not expressed in human erythrocytes.

In principle, differences in the biotransformation enzymes levels between individuals can be of genetic and environmental origin (van Iersel et al. 1999). Inherited differences in individual drug metabolizing enzymes are typically differences in a single gene. The influence of this difference on the pharmacokinetic and pharmacodynamic effects of xenobiotics is determined by the importance of these polymorphic enzymes on the activation and deactivation of such substrates (Henríquez-Hernández et al. 2012). Normally, in a frequency of at least 1% of the population, polymorphism is a sequence of variations including deletions, insertions, nucleotide substitutions and mutations occurs (Miller et al. 2001). Most human GSTs harbor polymorphism, primarily, single nucleotide polymorphisms (SNPs) and less frequently, the deletions polymorphism. Polymorphisms have been identified in the GSTM1, GSTT1 and GSTP1 genes in different populations (Beeghly et al. 2006). The three important phase II enzymes GSTM1, GSTT1 and GSTP1 are involved in protection from oxidative stress. They utilize as substrates a wide variety of products of oxidative stress. GSTM1 is involved in the polycyclic aromatic hydrocarbons detoxification (Henríquez-Hernández et al. 2012). GSTT1 catalyze the detoxification of halo-methanes in the human erythrocytes, and metabolize a number of chemicals such as dichloromethane, methyl chloride, methyl bromide and ethylene oxide (Henríquez-Hernández et al. 2012).

In DS individuals, increased oxidative stress has been confirmed in many studies. Decrease in the level of reduced glutathione (GSH), the important redox marker, was found in individuals with DS, along with increased level of malondialdehyde, the marker of oxidative damage to lipids, marker of aging and erythrocyte lipofuscin in DS individuals (**Hamed et al. 2011 & Muchová et al. 2014**).

A great attention has been focused on the protective biochemical function of naturally occurring antioxidants and on the mechanisms of their actions in the biological systems. Phenolic compounds are widely distributed in plants and play an important role in the prevention of oxidative damage in living systems (**Usoh et al. 2005**). There is an association between the consumption of phenolic acid-rich foods or beverages and the prevention of many diseases. These phenolic compounds have good antioxidant and chemoprotective properties in vitro experiments which may have beneficial effects in vivo as dietary antioxidants (**Yeh et al. 2009**). Flavonoids are a large class of polyphenols, secondary plant metabolites, involved in the plant physiology, growth, development and defense mechanisms. Dietary intake of flavonoids is correlated with important health benefits (**Margina et al. 2015**).

The most important feature discussed for polyphenols is their antioxidant activity (**Spanou et al. 2012; Stagos et al. 2012**); however, flavonoids as many other antioxidants can act under certain conditions as prooxidants, e.g. in systems containing redox active metals (copper, iron, etc.). The prooxidant activity of dietary flavonoids is linked to the total number of hydroxyl groups in the molecule. The prooxidant ability of dietary flavonoids could directly influenced by the total number of hydroxyl groups in the molecule. Prooxidant activity increases the risk of radical/non-radical reactive species (hydrogen peroxide, hydroxyl radicals) production. In Fenton reactions: multiple hydroxyl groups, especially in the B-ring, or 2, 3-double bond and 4-oxo arrangement of flavones can act as prooxidants. The extent to which flavonoids are able to act as anti- or pro-oxidants is poorly understood (**Procházková et al. 2011**).

Eugenol considered as an anti-mutagenic agent in vitro and an anti-genotoxic in vivo. Eugenol potentially forms a quinone methide species by one- or two-electron oxidative pathways that are capable of alkylating cellular proteins and thiols. GSTs are sensitive to covalent modification by quinones and the resulted irreversible inactivation. In man, ingestion of eugenol resulted in decrease in Alpha-class GSTs in plasma. This can be explained in vivo by an irreversible GST inhibition by the oxidation product of eugenol, probably a quinone methide (**Rompelberg et al. 1996**). Eugenol itself did not irreversibly inhibit the purified GST isoenzymes, but in the presence of tyrosinase, eugenol irreversibly inhibited the human GST isoenzymes A 1, M 1 and P1. In this respect the GST of the Pi-class was the most sensitive enzyme. The human GSTA2, which does not possess any cysteine residues, was not inactivated, suggesting that a cysteine residue is presumably the target site. Similar results were obtained with caffeic acid and dopamine (**Ploemen et al. 1993 & 1994**). Modification of the highly reactive cysteine residue in Pi-class GST results in enzyme inactivation (**Tamai et al. 1990**). The GST Pi cysteine residues may undergo a (reversible) oxidative inactivation by the formation of an inter-subunit disulfide between the position 47 and 101 of the cysteines. Mass spectrometry evidence was found that human GSTP1 may be inhibited via three mechanisms: nucleophilic addition of quinones and oxidation of cysteine residues, a covalent subunit cross-linking was also observed (**Rompelberg et al. 1996**).

Curcumin exerted its antioxidant effect directly as a chemical antioxidant and by modulating cellular defenses. Although curcumin has not been shown directly to be a substrate for GSTP1, it has been shown indirectly to inhibit GSTP1. Curcumin contains two electrophilic (a, b-) unsaturated carbonyl groups, which can react with nucleophilic compounds such as glutathione (GSH) forming the GSH–curcumin conjugates. The presence of GSTP1 accelerated the initial rate of GSH-mediated consumption of curcumin in 10 mM potassium phosphate, pH 7.0, and 1 mM GSH. GSTP1 catalyzed the reverse reaction leading to the formation of curcumin from GSH adducts. GSTP1 kinetics indicated substrate inhibition (apparent K_m for curcumin of 25.9 M, and apparent K_i for curcumin of 0.893 M) (**Awasthi et al. 2000**). The antioxidant properties of curcumin are thought to be due to its b-diketone moiety, cleavage of the C–C bond at the methylene carbon between two carbonyls in the b-diketone moiety, and formation of relatively stable free-radicals due to its extended conjugated double bond structure (**Awasthi et al. 2000**).

A number of phenolic substances were isolated from a variety of spice sources, including phenolic acids, flavonoids, phenolic diterpenes and volatile oils. Species like; *Cinnamomum zeylanicum*, *Coriandrum sativum*, *Cuminum cyminum*, *Crocus sativus*, which are rich in phenolic constituents such as phenolic acids, stilbenes, tannins, lignans and lignin, demonstrated good antioxidant activity, stabilizing lipid peroxidation and inhibit

various types of oxidizing enzymes (Gallo et al. 2010 & Deepak, 2013). We chose some plants known as medicinal plants having different polyphenol compounds. Spices as clove, oregano, mint, thyme and cinnamon, have been employed as food preservatives and as medicinal plants mainly due to its antioxidant and antimicrobial capacities (Diego Francisco Cortés-Rojas et al. 2014). The naturally occurring phenolic compound, eugenol, is a major component of clove oil and is also present in oils of cinnamon, basil and nutmeg (Rompelberg et al. 1996). Quercetin is a flavonoid, one of the most abundant polyphenolic groups in plants (Ramos, 2007). The anti-inflammatory action of many species of cinnamon has been attributed to the polyphenolic component such as tannins and procyanidins (Rathi et al. 2013). Tannins are plant polyphenolic compounds consumed as food and beverages by human. Tannic acid is a common hydrolyzable tannin found in tea, coffee, red wine, grains, nuts and immature fruits and it has been used as a food additive (Karakurt and Adali, 2011). Guava contains broad spectrum of phytochemicals including minerals, enzymes, proteins, terpenoid alcohols and triterpenoid acids, alkaloids, glycosides, steroids, flavanoids, tannins and saponins. Guava is high in lutein, zeaxanthine and lycopene and is very rich in antioxidants and vitamins (Shruthi et al. 2013). Radish anthocyanins have been characterized and the presence of pelargonidin 3-sophoroside-5-glucoside derivatives acylated with p-coumaric, caffeic and ferulic acids have been investigated (Tatsuzawa et al. 2008). Rocket species are rich in glucosinolates (GLSs). The whole order Brassicales is characterized by the presence of these bioactive substances which have an important role in determining the characteristic flavour of Brassica vegetables. The characteristic spicy flavor and the putative anti-carcinogenic properties of rocket plant may be related to the presence of glucosinolates and their associated hydrolytic products, in particular isothiocyanates. This glucosinolates and its derived isothiocyanates may in part explain the characteristic pungent taste and flavor of rocket species that is very different from other Brassicaceae. The organo-sulfur compound, allyl isothiocyanate, is responsible for the pungent taste of mustard, radish, horseradish, and wasabi (Everaerts et al. 2011). Also pomegranate is an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins (ETs), proanthocyanidin compounds, hydrolyzable tannins, mainly punicalin, pedunculagin, punicalagin and minerals (Lansky and Newman, 2007; Viuda-Martos et al. 2011 & Endo et al. 2012). Curcumin (diferuloylmethane), a yellow pigment of turmeric with antioxidant properties act as cancer preventative in animal studies. The present data showed that *S. aromaticum* (cloves) seed extracts have a powerful antioxidant capacity where IC_{50} value of 0.22 ± 0.1 mg dry seed/ml, compared to the other seed extracts, even powerful than the standard vitamin C ($IC_{50} = 0.625 \pm 0.0163$). Dahiya et al. (2014) reported that buds oil of *S. aromaticum* has biological activities, such as antimicrobial, insecticidal and antioxidant properties. In the present results, high antioxidant capacity observed with *S. aromaticum* may be attributed to the high level of eugenol contained in *S. aromaticum* seed extracts.

GSTs function is to catalyze the conjugation of the sulfur atom of glutathione to an electrophilic center of endogenous and exogenous toxic compounds, increasing their solubility and excretion (Habig and Jakoby, 1981). Most of GSTs composed of two identical subunits. A complete active site of each subunit contains composed of one site of binding for GSH (G site) and one site which binds a number of hydrophobic substrates (H site) adjacent to the G site (Wilce and Parker, 1994). Glutathionylation of proteins is considered as a primary line of defense. This reversible modification reaction can be performed non-enzymatically, but it has proposed that GSTP1 may enhance this modification of thiol proteins during oxidative stress and/or nitrosative stress (NS). Because of their GSH high affinity and specificity, GSTs are structurally well equipped to accommodate diverse GSH-bound substrates such as glutathionylated proteins. GSTP1 can potentially serve as a cellular NO donor and/or carrier (Vasieva, 2011), regulating the activities of other thiol redox-modifying enzymes (e.g. glutathione reductase (GR) (Mazzetti et al. 2015).

Our results (Table 1& 2) indicated that, pomegranate and cinnamon extracts exhibited the highest phenolic, flavonoid and anthocyanin contents, they also represented the most affected extracts on normal GSTP1 activity inhibition. However, no inhibitory capacity of normal GST activity could be detected for rocket extracts; they were almost the only extracts having effect on DS GST activity. GST of normal erythrocytes inhibition was accompanied with a highest phenolic, flavonoid and anthocyanin contents. Inhibition of normal GSTP1 could be mainly due to the high phenolic contents of the examined extracts. These results suggested that, high polyphenolic content of plant extracts may explain the high GST inhibitory activity of these plant extracts, as reported for the GST inhibitory effects of naturally occurring plant polyphenols in Zabri Tan et al. (2011). Inhibitory effects of the plant polyphenols such as tannins, quercetin, ellagic acid, ferulic acid, curcumin, caffeic acid, stilbene, and chlorogenic acid against GSTs have been demonstrated. Plant extracts that are high in polyphenols are known to have important inhibitory effect on GST (Yeh et al. 2009). This fact was not true in case of DS GST. There are several reasons to explain the ambiguous relationship between the

phenolics and flavonoids and their inhibitory potency. The total phenolics content did not include all the possible inhibitors; the synergism among inhibitors in the mixture accounted for the inhibition but was not only dependent of the concentration of individual inhibitors but also on the structure and interaction among them (Bangou et al. 2011). Van Zanden et al. (2004) study showed the structure relationship of flavonoids in GST inhibitory activities. The present results demonstrated that rocket extracts contained some compounds with DS GST inhibitory activities.

In the present study, normal and DS GSTs were quite different in their responses to the studied plants, 1) clove extract has inhibitory effect on normal GST (IC_{50} value of 3.63 μ g), while has no effect on DS GST (Table 2). Unexpected, eugenol, the major component of clove, has no inhibitory effect on both of normal and DS GSTs. 2) Tannic acid and quercetin have no effects on normal GST while inhibit DS GST activity. These compounds considered as effective constituents of *P. granatum* (pomegranate) and *C. verum* (cinnamon) the most affected plants on normal GSTP1-1. Allyl-isthiocynate considered as one of the important compounds of *E. sativa* (rocket), the most effective plant on DS GST activity. However, allyl-isthiocynate was not the most effective compound on DS GST activity (IC_{50} = 7.66 μ M) but tannic acid was 4.35 fold powerful inhibitor of DS GST activity (IC_{50} = 1.76 μ M) than allyl-isthiocynate.

In this study, genotype analysis of two different DS subjects was determined. Interestingly, the two genetically different DS samples, the GSTT1 & GSTM1 double null and the GSTT1⁺& GSTM1⁺ present are different in their response. Rocket extracts increased GST activity in (GSTM1⁻), however, a decrease in GST activity was observed in (GSTM1⁺). These may explain the different responses observed for normal and DS GSTs to the studied plants in this study and common compounds, suggesting structural differences between the two GST enzymes. By another hand, genotype difference between normal (GSTT⁺) and DS (GSTT⁺ GSTM1⁺) samples, also, the two examined DS samples (GSTT⁺ and GSTT⁻) may explain response differences between subjects.

Between humans, large inter-individual differences in response to xenobiotic compounds have been documented (Court et al. 2001). It has become feasible to perform more complete/in-depth analysis of the genomic components contributing to inter-individual differences in humans (Jetten et al. 2015). Large degree of differences in toxicity responses are shown within the human population. Jetten et al. (2014) study, evaluate whether inter-individual variation in baseline enzyme activity (EA)/gene expression (GE) levels in liver predispose for the variation in toxicity responses by assessing dose-response relationships for several prototypical hepato-toxicants. GSTM1 is involved in the phase-II metabolism of acetaminophen (APAP), one of the most commonly used analgesics/antipyretics (Zhao and Pickering, 2011). GSTM1 is known to be highly polymorphic within the human population (Gattas and Soares-Vieira, 2000). GSTM1 causes variability in APAP-metabolism that is dependent on the gene expression (GE) responses of CYP enzymes within an individual (Jetten et al. 2014). in vivo, one of the cytochrome-P450 (CYP) enzymes, CYP1A1 levels are known to vary 20–130-fold between individuals, while a 21-fold inter-individual variation of CYP1A1 enzyme activity (EA) has been reported (Jetten et al. 2014).

Lineweaver–Burk plot (Fig.2) indicated that rocket extract increased the K_m value while V_{max} value remained unchanged (20 μ mol/min/mg protein) in the presence and absence of extract, suggested this extract to be competitive inhibitor with respect to CDNB. In the double reciprocal plot (Fig. 3), rocket extract decreased both the V_{max} of the enzyme from 100 to 10 μ mol/min/mg protein and K_m value from 2 to 0.91 mM. Consequently, rocket extract has shown uncompetitive inhibition on DS GSTP1 activity with respect to GSH, as revealed in our observations. As a rule, when an inhibitor has a greater affinity for the enzyme-substrate complex than the free enzyme itself, the K_m decreases after inhibitor binding, these results suggested that rocket extract can bind only to the complex formed between GST enzyme and CDNB (the ES complex). The present K_m values were different from that carried out by Hamed et al. (2011) on the human erythrocyte enzyme purified from DS children (K_m GSH was 0.318 mM and k_m CDNB was 1.307 mM). Kinetic studies with purified GST show high differences in the specific activity for a same substrate as also for different substrates, K_m values for CDNB can differ 100 or more fold depending on the GST isoform. Comparative values of these kinetic constants are difficult to obtain because the assay conditions used by the investigators are different (Hamed et al. 2014). Cotton extracts (*G. hirsutum*) inhibited erythrocyte GST (hGSTP1) competitively with respect to GSH and CDNB. The mechanism of inhibition of GST by plant phenolics has not been fully elucidated however, the presence of polyhydroxylations in plant polyphenols may be important for GST inhibition (Hamed et al. 2014). Mutations in a gene resulted in the corresponding enzyme having an increased

Michaelis constant, or K_m , (decreased binding affinity) for a coenzyme with a lower rate of reaction. Some mutations affect the conformation of the protein, therefore, causing an indirect change in the enzyme binding site (Ames et al. 2002).

Effect of GSH on the inhibitory capacity of the most effective extracts, rocket and curcumin was investigated on DS GST activity (Fig. 4). GSH at 1 and 2 mM was shown to protect the DS GST enzyme from inactivation by the powerful inhibitor curcumin. However, 2 mM GSH could not protect DS GST from rocket extract inactivation.

Hamed et al. (2014) results indicated that, addition of reduced GSH could reverse the inactivation inhibitory effects of both *G. hirsutum* (cotton) extracts and gossypol on the erythrocyte hGSTP1 enzyme. GSTs M1, M2 and P1 were inactivated by the two polyphenolic compounds ellagic acid and curcumin (Hayeshi et al. 2007). The time-dependent inactivation observed indicates irreversible inhibition of these isoforms due to covalent binding. Glutathione and dithiothreitol can protect GSTP1 from ethacrynic acid- inactivation due to their thiol groups. Under normal physiological conditions (glutathione concentration 1-10 mM), glutathione may be expected to reverse any covalent binding of ethacrynic acid to GSTP1, and GST inhibition 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 and/or low levels of glutathione, covalent inhibition of GSTP1 might be predominant. Thus GSTP1 inhibition by covalent binding of ethacrynic acid would be reversed by glutathione; but when glutathione levels are low the covalent inhibition might be predominant, resulting in a completely different time course for the inhibition (Ploemen et al. 1994). Fraction of curcumin should be rapidly transformed into GSH-conjugates and this curcumin-GSH conjugation is reversible. These conjugates may serve a carrier in biliary transport. The reverse reaction would be favored in relatively GSH deficient bile, so curcumin may be available for reabsorption and recycling (Awasthi et al. 2000).

CONCLUSION

Different compounds are metabolized by various GST enzymes to varying degrees, suggested that reliable measurement of variability can only be compound specific. Since patient with DS are targeted for vitamin supplement use, especially at an early age, this may well constitute a major obstacle to DS supplement therapy. In this study we evaluate whether inter-individual variation in GST activity /GSTM1& GSTT1 allelic variant (generic enzyme variability data) in normal and DS erythrocytes for the variation in responses to some plant extracts characterized by their common medicinal effects beside investigation of some of their phytochemical characterizes. It is important to emphasis that in DS people, problems and differences at the molecular biology level is enormous, and so a great deal of work remains to be done.

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