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Studies on phytoenzymes as mimics of mammalian drug metabolising enzymes

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Abstract
Drug metabolism and pharmacokinetics is an integral part of the drug discovery and development process. The pharmacokinetic (absorption, distribution, metabolism and excretion) properties of a drug allow the drug development scientist to understand the safety and efficacy data required for regulatory approval. Further, the knowledge of mammalian enzymes involved in the metabolism of a new chemical entity is of prime importance. In this regard, plant metabolism of organic compounds is similar to the mammalian metabolism in terms of the processes for detoxification of contaminants, the specific enzymatic pathways, the structure of the enzymes involved and the removal of the compounds from the susceptible organelles. In our studies, we isolated phytoenzymes (esterases and glutathione-S-transferases, GSTs) from plant cells and investigated their applicability as tools for drug metabolism studies. A total of 32 plants were screened for esterases activity and 14 plants for GSTs activity. In case of esterases, the activity was compared to human plasma esterases and also the effect of esterase inhibitor on the activity was studied. Overall, the presence of phytoenzymes and their catalytic similarities to the mammalian drug metabolising enzymes suggest that plants can be potential models for mammalian drug metabolism. Further, phytoenzymes can be considered as a potential surrogate for reducing the number of animals utilized in the drug discovery process.

Keywords: Plant metabolism, phytoenzymes, esterases, glutathione-S-transferases

Introduction
Drug metabolism and pharmacokinetics (DMPK) is an integral part of the process of drug discovery and development. Drug metabolism is the enzymatic biotransformation of drugs to water soluble metabolites which can be readily excreted from the body. Biotransformation is catalysed by enzymes that are present in the liver and other tissues. *In vitro* PK i.e. absorption, distribution, metabolism and excretion *(*ADME) screening and *in vivo* PK profiling studies play a key role in the drug discovery process by providing a basis for choosing new chemical entities (NCEs) and lead compounds that have desirable drug metabolism, PK or safety profiles [1,2]. The results of *in vitro* studies provide a rationale for selection of animal species for the toxicology studies, prediction of possible drug-drug interactions and for phenotyping studies. They also allow one to study the metabolic stability, compare the metabolite profiles, predict the induction/ inhibition potential by the NCEs, generate the metabolite/s on a large-scale and assess species differences in metabolism of NCEs. The ADME properties of a drug allow the drug development scientist to understand the safety and efficacy data required for regulatory approval. Hence, the knowledge of enzymes involved in the metabolism of an NCE is of prime importance [3].
The capability of plants to biosynthesize various secondary metabolites like alkaloids, glycosides, terpenes, steroids, flavonoids, tannins, etc; is an evidence of the presence of extensive endogenous biotransformation pathways occurring in the plants. The enzymes involved in the biosynthesis of the secondary plant metabolites also allow the plant to potentially metabolize many xenobiotics to which the plant may be inescapably exposed. Plant metabolism of organic compounds is quite similar to the mammalian liver function in terms of

the processes for detoxification of contaminants, the specific enzymatic pathways, the structure of the enzymes involved and the removal of the compounds from the susceptible organelles. One of the major differences is that, as opposed to excretion in mammals, the ultimate fate of plant metabolism/ metabolites is their storage in vacuoles or lignified tissues. Plants have been described as 'green livers' and also act as a global sink for environmental pollutants through the metabolism of xenobiotics. Plants contain several families of enzymes like the cytochrome P450 monooxygenases, carboxylesterases, glutathione transferases and UDP-glucuronosyltransferases, O-glucosyl and O-malonyl transferases as well as N-glucosyl and N-malonyl transferases [4-6]. On the basis of this reported knowledge, we have explored plants for their potential to metabolize xenobiotics.

Among the phytoenzymes mentioned above, we selected esterases and glutathione transferases for our studies. In our preliminary studies, we isolated phytoenzymes from plant cells and investigated their applicability as tools for drug metabolism studies. Esterases are phase I hydrolytic enzymes that hydrolyze xenobiotics with functional groups such as a carboxylic acid ester, amide, thioester, phosphoric acid ester and acid anhydride. They play an important role in the metabolism of a number of compounds used as drugs in humans and also toxic xenobiotics including organophosphorous insecticides [2]. Glutathione-S-transferases (GSTs) are phase II enzymes and are responsible for conjugation of a tripeptide glutathione to an electrophilic substrate to form polar S- glutathione adducts. In plants, the tau and phi classes are predominant and play a role in primary and secondary metabolism, cell signalling and stress tolerance [7].

Materials and methods

Chemicals and reagents

p-Nitrophenyl acetate, 1-chloro-2,4-dinitrobenzene, dithiotreitol were purchased from Himedia. p-Nitro phenol, glutathione, ethylene diamine tetraacetic acid, di- sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, hydrochloric acid and sodium hydroxide were purchased from S. D. Fine Chemicals. Acetonitrile, methanol and ortho-phosphoric acid were procured from Merck. Calcium chloride dihydrate and phenylmethylsulphonylfluoride were purchased from Thomas Baker and Spectrochem, respectively. Aspirin was obtained from West Coast Lab. All other chemicals used in the present study were of analytical grade (AR).

Drug free human plasma was obtained as a gift sample from King Edward Memorial Hospital, Parel, Mumbai. The plant parts (seeds, leaves, fruits, vegetables, etc.) required for the study were either purchased from the local market or procured from the botanical garden of Bombay College of Pharmacy or grown in the laboratory itself.

Isolation of esterases containing fraction

The plant parts i.e. the germinating seeds/ vegetables/ fruits/ flowers were rinsed with distilled water and then precooled in the refrigerator for about 10-15 minutes. This was then blended for 5 minutes in a blender (food processor) surrounded with ice to ensure that no heat was generated during this process. The blend was then stored at -80 °C overnight. Ten grams of plant material was weighed and four times volume of 0.1 M tris-HCl buffer, pH 6.85, containing 2 mM EDTA and 10 mM $CaCl_2$ was added. The mixture was crushed in the blender for 2 minutes at 0-4˚C. The mixture was then centrifuged at 20,000 × g for 20 minutes at 0-4˚C in a refrigerated centrifuge (Biofuge Stratos). Supernatant was decanted and collected while the precipitate was discarded. The supernatant was then stored at -86˚C until further use [8].

Isolation of glutathione-S-transferases containing fraction

Liquid nitrogen was used to disrupt the cell wall and release the cytoplasmic contents. Liquid nitrogen was added to the plant part and allowed to evaporate after which the frozen plant part was crushed to a fine powder using a mortar and pestle. The powder was stored at -86 °C overnight. Fifteen grams of the crushed powder was weighed to which three times volume of 0.25 M Tris-HCl buffer, pH 7.8, containing 1 mM EDTA and 0.5 mM dithiothreitol was added. The mixture was ground for 2 minutes at 0-4˚C. The mixture was then centrifuged at 20,000 × g for 15 minutes at 0-4˚C in a refrigerated centrifuge (Biofuge Stratos). The precipitate was discarded and the supernatant was collected. To the supernatant, solid ammonium sulphate was added over a period of 15 min with continuous stirring to bring it to 40 % saturation. This was subjected to centrifugation (20,000 x g, 20 minutes at 0-4 °C). The precipitate was discarded and the supernatant was bought to 70 % saturation by further slow addition of solid ammonium sulphate and was then centrifuged at 20,000 x g for 20 minutes at 0-4 °C. The precipitate was resuspended in 5 mL 0.25 M Tris-HCl buffer, pH 7.8 buffer and was stored at -86 °C until further use [9].

Protein content estimation

The protein content of plant esterases and the plant GSTs fractions was determined by Biuret method using Liquixx Total Protein kit (ERBA). Reagent and the protein standard (Bovine Serum Albumin, 6g/dL) were available in the kit. The absorbance of standard and test was measured at 546 nm on a UV spectrophotometer, with distilled water as a blank. The protein content was calculated by using the formula,

$$\text{Total protein content} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} * \text{Concentration of standard (60 mg/mL)} \quad \text{(Equation 1)}$$

In vitro plant esterases assay

The enzyme rich fraction containing esterases was incubated at ambient temperature with p-nitrophenyl acetate (PNPA) (Final concentration 1 mM) in 0.1 M sodium phosphate buffer containing 0.01 mM EDTA, pH 6.5. After 100 µL of enzyme was added to the sample cuvette, the reaction was initiated with PNPA (substrate). The incubation details are given in Table 1.

Table 1: Incubation details for esterases activity by U.V. Spectrophotometry

	A	B
0.1 M Sodium phosphate buffer, pH 6.5	2970 µL	2870 µL
PNPA (100 mM)	30 µL	30 µL
Plant esterases	-	100 µL
Total	3000 µL	3000 µL

A: Reference cell, B: Sample cell

The rate of hydrolysis to p-nitrophenol (PNP) was measured spectrophotometrically by an increase in absorbance at 400 nm over a time course of 10 mins. The percent conversion to product over 10 minutes was calculated as,

$$\%\ conversion = \frac{\text{Final molar concentration of metabolite formed in 10 minutes (mM)}}{\text{Initial molar concentration of substrate}} * 100$$

(Equation 2)

An extinction coefficient value of 10.9 $mM^{-1}cm^{-1}$ for p-nitrophenol was used for calculation of concentration in the incubation [10].

Phenylmethylsulphonylfluoride (PMSF), a known non-specific irreversible inhibitor of esterases, was pre-incubated with enzyme for 30 minutes. The rate of hydrolysis of PNPA to PNP was measured in presence and absence of inhibitor by determining the increase in absorbance at 400 nm over a time course of 10 mins [10]. The incubation details are given in Table 2.

Plasma esterases assay in presence of inhibitor

Table 2: Incubation details for plant esterase assay in presence of inhibitor

	Control incubation		PMSF incubation	
	Reference	**Test**	**Reference**	**Test**
0.1 M Sodium phosphate buffer, pH 6.5	2980 µL	2880 µL	2960 µL	2860 µL
PMSF 75 mM (stock solution)	-	-	20 µL	20 µL
Enzyme	-	100 µL	-	100 µL
Pre- incubated for 30 minutes				
PNPA 150 mM (stock solution)	20 µL	20 µL	20 µL	20 µL
Total	3000 µL	3000 µL	3000 µL	3000 µL

The percent residual activity in presence of PMSF was calculated using the following formula,

$$\%\ \text{Residual activity (PMSF)} = 100 - \%\ Activity\ (PMSF)$$

(Equation 3)

$$\%\ Activity\ (PMSF) = \frac{\%\ \text{PNP formed in PMSF incubation}}{\%\ \text{PNP formed in control incubation}} * 100$$

(Equation 4)

***In vitro* plant glutathione-S-transferases assay**

The enzyme rich fraction containing GSTs was incubated at ambient temperature along with 1-chloro-2, 4-dinitrobenzene (CDNB) and glutathione (GSH) in 0.1 M potassium phosphate buffer pH 6.5. When 25 µL of enzyme was added to the sample cuvette to initiate the reaction. CDNB (substrate) was converted into 2, 4-dinitrophenyl-*S*-glutathione (DNP-S-GLUT) (metabolite). The incubation details are given in Table 3.

Table 3: Incubation details for GSTs activity by U.V. Spectrophotometry

	Blank		Test	
	A	**B**	**A**	**B**
CDNB (12 mM)	100 µL	100 µL	100 µL	100 µL
GSH (30 mM)	-	100 µL	100 µL	100 µL
Volume of enzyme (µL)	-	-	-	25 µL
0.1 M phosphate buffer	2900 µL	2800 µL	2800 µL	2775 µL
Total	3000 µL	3000 µL	3000 µL	3000 µL

The rate of formation of DNP-S-GLUT was measured spectrophotometrically by change in absorbance at 340 nm over a time course of 10 mins. The percent conversion was calculated using Equation 2. An extinction coefficient value of 2.79 $mM^{-1}cm^{-1}$ for DNP-S-GLUT was used for calculation of concentration in the incubation [11].

Results and discussion

The list of plants which were screened for esterase activity and GST activity are shown in Table 4 and Table 5, respectively.

Table 4: List of plants screened for esterase activity

Name	Biological name	Family
Mustard seeds	*Brassica nigra*	Brassicacea
Castor seeds	*Ricinus communis*	Euphorbiaceae
Sunflower seeds	*Helianthus annus*	Fabaceae
Urad seeds	*Vinga mungo*	Apiaceae
Coriander seeds	*Coriandrum sativum*	Fabaceae
Masoor seeds	*Lens culinaris*	Fabaceae
Pea seeds	*Pisum sativum*	Cucurbitaceae
Pumpkin seeds	*Cucurbita pepo*	Pedaliaceae
Seasame seeds	*Seasamum indicum*	Amaryllidaceae
Ginger rhizome	*Zingiber officinale*	Lauraceae
Cinnamon bark	*Cinnamomum verum*	Brassicacea
Onion seeds	*Allium cepa*	Zingiberaceae
Soybean sprout	*Glycine max*	*Fabaceae*
Matki sprout	*Vigna aconitifolia*	*Fabaceae*
Hyacinth bean sprout	*Lablab purpureus*	*Fabaceae*
Wheat sprout	*Triticum aestivum*	*Poaceae*
Peanuts sprout	*Arachis hypogaea*	*Fabaceae*
Sorghum sprout	*Sorghum bicolor*	*Poaceae*

Moong bean sprout	*Vigna radiate*	*Fabaceae*
Black eyed peas sprout	*Vigna unguiculata*	*Fabaceae*
Kidney beans sprout	*Phaseolus vulgaris*	*Fabaceae*
Brown chickpeas sprout	*Cicer arietinum*	*Fabaceae*
Garlic clove	*Allium sativum*	Amaryllidaceae
Chikoo	*Manilkara zopota*	Sapotaceae
Spinach leaves	*Spinacia oleracea*	Amaranthaceae
Fenugreek leaves	*Trigonella foenum-graenum*	Fabaceae
Neem leaves	*Azadirachta indica*	Meliaceae
Curry leaves	*Helichrysum italicum*	Asteraceae
Coriander leaves	*Coriandrum sativum*	Apiaceae
Pumpkin flesh	*Curcurbita mamixa*	Cucurbitaceae
Orange pulp	*Citrus sinensis*	Rutaceae
Orange peel	*Citrus sinensis*	Rutaceae

Table 5: List of plants screened for GST activity

Name	Biological name	Family
Moong (seed)	*Vigna radiata*	Fabaceae
Masoor (seed)	*Lens culinaris*	Fabaceae
Matki (seed)	*Vigna aconitifolia*	Fabaceae
Bitter gourd (fruit)	*Monrdica charsntia*	Cucurbitaceae
Urad (seed)	*Vigna mungo*	Fabaceae
Chana (seed)	*Cicer arietinum*	Fabaceae
Vatana (seed)	*Pisum sativum*	Fabaceae
Kidney beans (seed)	*Phaseolus vulgaris*	Fabaceae
Black vatana (seed)	*Pisum arvense*	Fabaceae
Onion (seed)	*Nigella sativae*	Ranunculacea
Mohari (seed)	*Brassica nigra*	Brassicaceae
Wheat (seed)	*Triticum aestivum*	Poaceae
Sorghum (seed)	*Sorghum bicolor*	Poaceae
Chawli (seed)	*Vigna unguiculata*	Fabaceae

Esterase activity

The overall comparison of protein content of different plant sources and human plasma is illustrated in Figure 1.

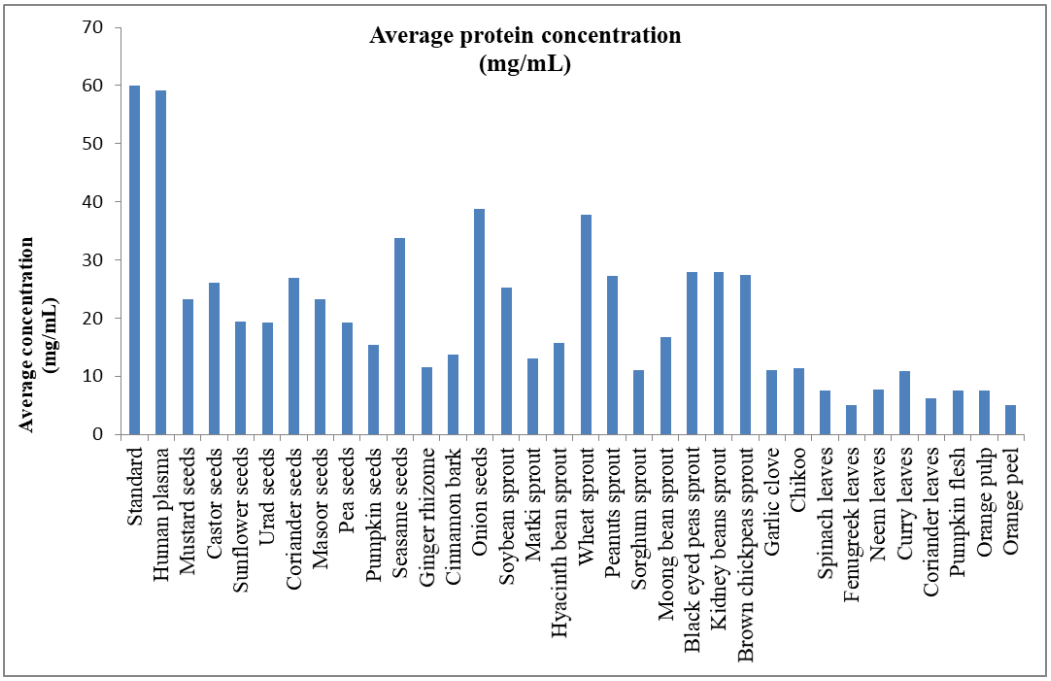

Fig 1: Overall comparison of protein content of different plant sources and human plasma (Esterase study)

The highest protein content was found in onion seeds whereas, the lowest protein content was found in orange peel.

In the present study, 32 plants (Table 4) and human plasma were investigated for presence of esterase activity. The esterase activity was determined using para-nitrophenyl acetate as a substrate, and the ability of plant fraction to hydrolyze and form para nitrophenol. The esterase activity in different plant parts, derived on the basis of the % conversion of PNPA to PNP per mg of the protein present in the enzyme fraction was compared and is represented in Figure 2.

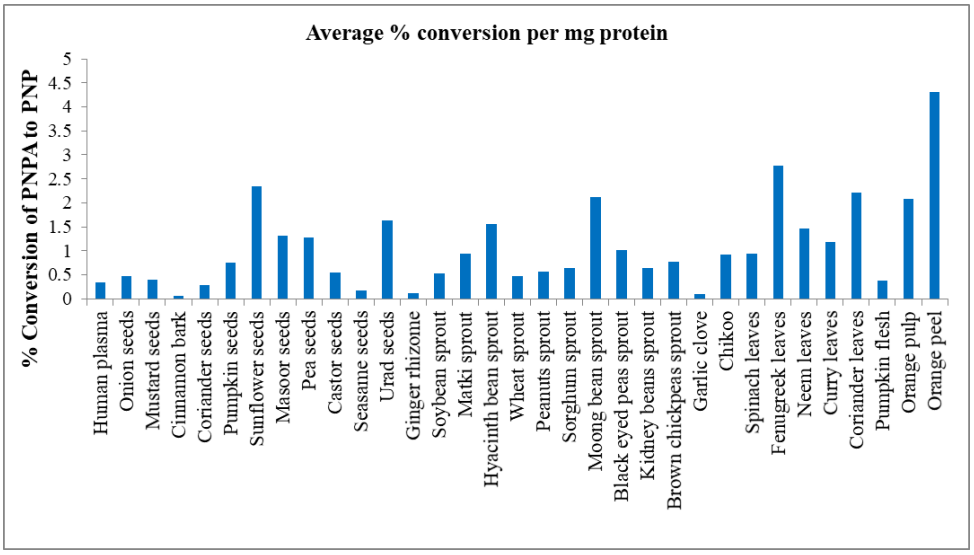

Fig 2: Overall comparison of percent conversion of PNPA to PNP per mg of protein in different enzyme fractions

On comparison based on the activity normalized to 1 mg of protein, orange peel showed the highest activity followed by fenugreek leaves, sunflower, pumpkin and moong seeds. Urad seeds, black eyed peas, pea seeds, chickpea and masoor seeds showed similar activities while sesame seeds, garlic clove, cinnamon bark and ginger rhizome showed the lowest activity.

Amongst all the plants studied, onion seeds had the highest protein content (38.85 mg/mL) which was almost 8 times that of orange peel (4.99 mg/mL) which had the least protein content. Contrary to this, the activity per mg protein in onion seeds (4.31) was $1/9^{th}$ of the activity in orange peel (0.48). This non-correlation of protein content with activity suggests the presence of protein other than esterases in the isolated enzyme fractions.

To compare plant esterases properties with mammalian esterases an assay was carried out in the presence and absence of inhibitor. PMSF was pre-incubated with enzyme for 30 minutes and the rate of hydrolysis to PNP was then measured spectrophotometrically by an increase in absorbance at 400 nm over a time course of 10 mins. The rate of hydrolysis was measured in presence and absence of inhibitor. The overall comparison of % residual activity after incubation in presence of inhibitor is illustrated in Figure 3 for the 10 selected plants.

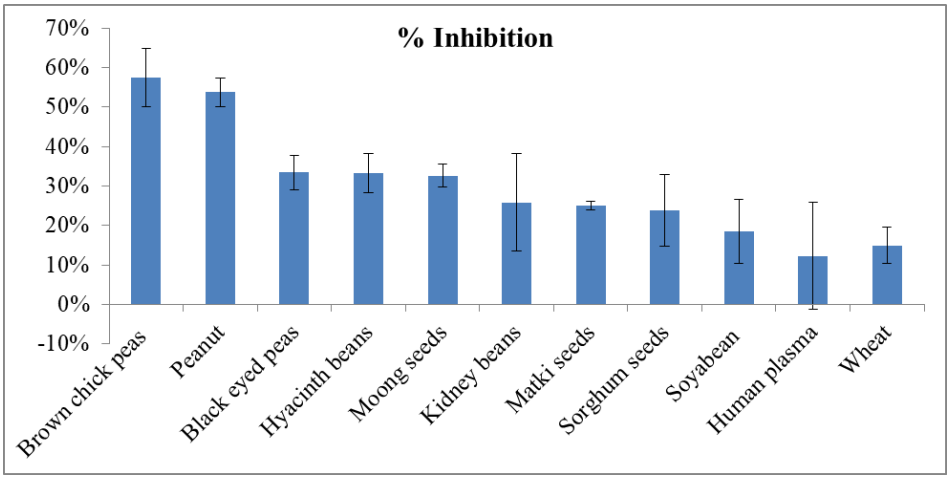

Fig 2: Overall comparison of % residual esterase activity after incubation in presence of inhibitor

Difference in residual activity was observed. The highest inhibition was observed in brown chickpeas and peanut sprouts whereas, the lowest was observed in human plasma and wheat sprout. Variation in residual activity observed in all sources may be due to different types of esterases present and the varying specificity of PMSF towards different types of esterases.

As compared to plant parts such as bark, rhizome, leaves and fruits, esterase activity was found to be higher in most of the seeds studied. During germination, esterases such as lipases and nucleases play an important role by participating in processes necessary for generation of ATP, cell division and growth. Chlorophyllase, one of oldest known esterase helps mature plants synthesize chlorophyll which in turn helps in harvesting energy [12].

GSTs activity

Overall protein content in plant parts evaluated for GSTs activity is illustrated in Figure 4.

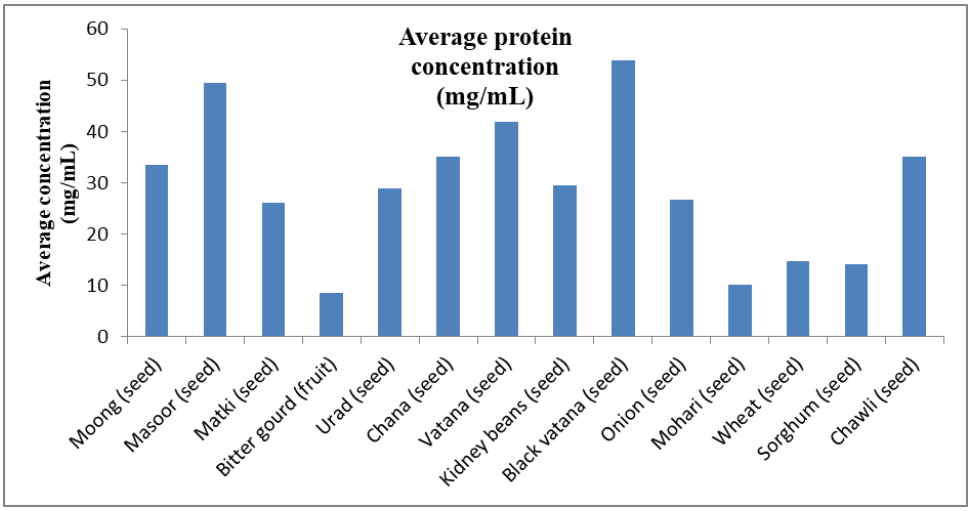

Fig 4: Overall comparison of protein content of different plant sources (GST study)

Highest protein content was seen in black vatana followed by masoor, vatana and chawli seeds while the least was seen in bitter gourd fruit. The GSTs activity in 14 different plant materials was determined using CDNB as a substrate, and the ability of plant fraction to form the conjugate DNP-S-GLUT. The GSTs activity in different plant parts, derived on the basis of the % conversion of CDNB to DNP-S-GLUT per mg of the protein present in the enzyme fraction was compared and the same is represented in Figure 5.

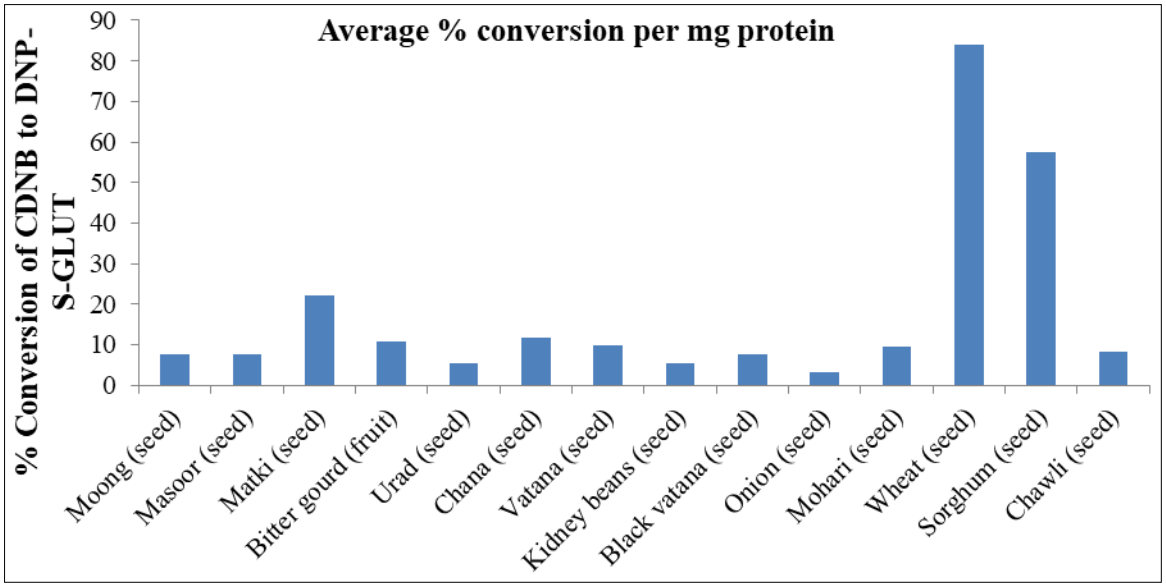

Fig 5: Comparison of percent conversion of CDNB to DNP-S-GLUT per mg protein

Wheat seeds showed highest activity followed by sorghum, matki, chana, bitter gourd fruit, vatana, mohari, chawli, black vatana, masoor, moong, urad, kidney bean and onion seeds which showed the least activity. It was found that even though the protein content of wheat seeds (14.76 mg/mL) was almost 4 times less as compared to black vatana seeds (53.85 mg/mL), the percent conversion per mg of protein in wheat seeds was 83.90 which was almost 8 times greater than that shown by black vatana seeds which was 7.70.

GST containing fractions isolated from several leaves, fruits and flowers showed no activity. The probable reason behind this could be that

a) The method followed for isolation of enzyme was not efficient enough to isolate the enzyme from fruits, flowers, and leaves

b) The tannins and polyphenols present in growing plants may act as an enzyme inhibitors

c) The enzyme is not present in these plant parts.

In this study, we have maintained constant temperature, pH, substrate concentration and volume of crude enzyme fraction during the determination of catalytic activity. Upon evaluating the relative levels of esterases and GSTs in plant parts procured from different sources, differences in the relative levels of both the enzymes were observed. The probable reason for this may be the variation in protein content of the plant parts from different sources. Further, the different plants may possess different isoforms of esterases and GSTs and these may show different selectivity towards the substrate used.

Overall, the presence of phytoenzymes and their similarities to the mammalian drug metabolism suggest that plants can be potential models for mammalian drug metabolism. However, differences between plant and mammalian enzymes were also observed especially with respect to the sensitivity of plant esterases to inhibition by PMSF. These similarities and differences can be the basis for the development of reliable tools using phytoenzymes for performing *in vitro* studies, metabolic profiling and also for synthesizing specific

metabolites. Isolation of phytoenzymes is much easier as well as economical as compared to obtaining enzymes from animal origin. Thus, phytoenzymes can be further evaluated as a potential surrogate for reducing the number of animals utilized in the drug discovery process.

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Note
Abhishek Rathod, Bhavesh Deore, Gaurav Bhalgat and Girish Gurbani have contributed equally to the isoation of esterases and GSTs and further studies for determining the enzyme activity in the isolated fractions. Deepali Desle has compiled the data and has contributed in the writing this paper. The studies were conducted under the guidance of Krishna Iyer.

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