

Protein-Tyrosine Phosphatase 1 β Inhibitory Activity Potential in Vegetables' Juice

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ABSTRACT

Aim: Insulin resistance and leptin resistance are hallmarks in common for development of type 2 diabetes mellitus (T2DM) and obesity. Both these conditions are associated with increased activity and expression of Protein-Tyrosine Phosphatase 1 β (PTP 1 β). Therefore, inhibition of PTP 1 β activity or down-regulation of its expression has become an attractive area of therapeutics development in order to ameliorate phenomenon of insulin and leptin resistance. This research analysed and assessed PTP 1 β inhibitory activity in thirteen easily available and commonly consumed vegetables' juice. **Materials and Methods:** Percentage yield of the juice, total polyphenol and total protein concentrations were estimated in fresh juice of vegetables. PTP 1 β inhibitory activity by vegetables' juice in cytosolic supernatant of goat liver and skeletal muscle was determined. **Results:** Wide variations in juice yield, total polyphenol and protein concentration in vegetables' juice were found. Juice of Ridge Gourd (RG) and Yellow Pumpkin (YP) were rich source of both, polyphenol and protein. Green Cucumber (GC), Bottle Gourd (BG), Snake Gourd (SG), RG, Radish (RD), Carrot (CT), Noolkol (NK) and Yellow Cucumber (YC) displayed reversible inhibition of PTP 1 β . Juice of vegetables YP, Ivy Gourd (IG), Chayote (CH), Ash Gourd (AG) and Banana Stem (BS) displayed irreversible PTP 1 β inhibition. Based on the order of PTP 1 β inhibition potential in both liver and skeletal muscle, juice of RD and BG were identified most potent. **Conclusion:** This research finds vegetables' juice as potent source of PTP 1 β inhibitor and may offer cost effective therapeutic potential in reducing risk of insulin resistance and development of T2DM.

Key words: Vegetables juice, radish, bottle gourd, protein-tyrosine phosphatase 1 β inhibition, insulin resistance, type 2 diabetes mellitus

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INTRODUCTION

Irrespective of developed and the developing countries, prevalence of type 2 diabetes mellitus (T2DM) and obesity is increasing worldwide¹. Recently, India has been outlooked as diabetes time bomb, reason being the embracement of the worst of both the Eastern and the Western way of lifestyle in the course of economic growth and modernization². Developed nations on the one hand have adequate medical care and infrastructure facilities, population of the developing countries on the other hand, are poorly equipped in terms of medical care, infrastructure, economy and even

knowledge about the disease. For increased adoption of maladaptive diet in modern world is the major cause of world wide epidemic 'diseases of modern civilization' like T2DM and obesity, realignment of eating habits may offer cost-effective preventive and therapeutic measures than resorting to drug therapy³. Therefore, development of suitable and affordable nutritional and dietary strategies for these disorders has become an important area of research.

Defective insulin secretion, reduced insulin sensitivity and increased insulin resistance results increase in postprandial and fasting blood glucose level. Persistent hyperglycemia leads to increased generation of free radicals and consequently oxidative stress. These aspects are important metabolic defects responsible for the onset of T2DM, obesity and development of related disease complications. Recent advances in nutrition and

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diet research reveals that increased consumption of vegetables may contribute to the improvement of these metabolic derangements^{4,5,6,7,8}. Vegetables have been researched out to offer anti-diabetic activity by multiple mechanisms^{4,5,9}. In order to manage better metabolic control in diabetic patients and prevent development of chronic diabetic complications, 'eating vegetables before carbohydrates' is emerging as an exciting meal plan⁸.

Infact, insulin resistance and leptin resistance are hallmarks in common for development of T2DM and obesity. Both these conditions are associated with increased activity and expression of Protein-Tyrosine Phosphatase 1 β (PTP 1 β)¹⁰. Therefore, inhibition of PTP 1 β activity or down-regulation of its expression has become an attractive area of therapeutics development in order to ameliorate phenomenon of insulin and leptin resistance.

Bitter gourd (*Momordica charantia*) is a well known oriental vegetable and its consumption has been advocated beneficial for diabetes patients since ancient times. This vegetable displays antidiabetic activity through multiple mechanisms⁹. Recently, various fractions of bitter gourd have been demonstrated to inhibit activity and expression of PTP 1 β and increase insulin sensitivity in insulin resistant db/db mice⁷. However, it is important to mention here that despite being a potent antidiabetic vegetable, its bitter taste mars public preference and the risk of hypoglycemia, coma, and convulsions in children warrants its careful use⁹. Therefore, search of non-bitter vegetables with antidiabetic activities may provide alternate and excellent therapeutic opportunity. In this research, we analyse and assess liver and skeletal muscle PTP 1 β inhibitory potential of thirteen vegetables' juice.

MATERIALS AND METHODS

Chemicals: Folin-Coicalteu reagent, Gallic acid, Bradford's reagent, Bovine Serum Albumin (BSA), HEPES [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], Phenylmethylsulfonyl fluoride (PMSF), Ethylenedisodiumtetraacetic acid (EDTA), p-Nitrophenylphosphate (pNPP) substrate was procured from Sigma-Aldrich chemicals (St. Louis, MO, USA). Other chemicals of analytical grade were purchased from Indian manufacturers.

Vegetables: Vegetables presented in Table 1 were obtained from local vegetable markets of Hyderabad (India).

Preparation of vegetables' juice: Unpeeled vegetables were thoroughly washed under running tap water. Each vegetable was tasted first for bitter taste before processing and discarded if any. Paste of chopped and weighed

amount of vegetables were prepared in food grade blender. The paste was transferred onto a clean, sterile muslin cloth and squeezed to obtain juice. Volume of the obtained juice was measured using a measuring cylinder to calculate percentage yield. Clear supernatant of the juice was obtained by centrifugation (5000 rpm for 30 min at room temperature) as described earlier⁵ for further analysis.

Analysis of total polyphenolic and protein content:

Total polyphenol and total protein content in the juice were analyzed as follows:

Total polyphenols: Total polyphenol content was measured using Folin-Coicalteu reagent⁵. In brief, fresh juice (25 μ L) was reconstituted in 2.5 mL distilled de-ionized water, followed by the addition of Folin-Coicalteu reagent (1 N, 250 μ L) and Sodium carbonate (20% w/v Na₂CO₃, 250 μ L). Mixture was incubated at room temperature (60 min). Absorbance (765 nm) was recorded spectrophotometrically on microplate reader (SpectraMax plus³⁸⁴, Molecular Devices, Sunnyvale, CA, USA). Total polyphenolic content was expressed as micrograms of Gallic Acid Equivalent per milliliter of the juice (μ g GAE mL⁻¹).

Total proteins: Protein content in fresh juice was analysed using Bradford's reagent⁵. Briefly, 10 μ L of juice was incubated with 240 μ L of Bradford's reagent for fifteen minutes and absorbance (595 nm) was measured as above. Total protein content was expressed as micrograms of BSA Equivalent per milliliter of juice (μ g BSAE mL⁻¹).

Preparation of PTP 1 β : Goat liver and skeletal muscle tissues from freshly slaughtered animal were obtained from slaughter house in cold Phosphate Buffer Saline (PBS). Tissues were washed thoroughly in PBS, minced and homogenized (1:1, w/v) in HEPES buffer (50 mM-HEPES, 50 mM-NaCl, 0.5 mM-EDTA, 0.1 mM-PMSF, pH7.2) and centrifuged (Eppendorf centrifuge 5430R, Eppendorf AG, 22331 Hamburg, Germany) for 60 min at 15,000 rpm and 2°C. Supernatant as a source of PTP1 β was collected and stored at -80°C. Protein concentration in cytosolic supernatant was estimated applying Bradford's method⁵.

PTP 1 β inhibition assay: PTP 1 β activity assay was adopted from Klomann *et al.*⁷ and modified to suit 96-well plate assay. Fresh juice (20 μ L) of vegetable was reconstituted in HEPES buffer (50 μ L) and incubated for 2 min with 50 μ L of cytosolic supernatant of liver (95 μ g BSAE mL⁻¹ protein) and skeletal muscle (88 μ g BSAE mL⁻¹ protein). Subsequently, 50 μ L substrate (10 mM, pNPP) was added and release

Table 1: Name and identification of vegetables used in the present study

Name	Botanical name	Family	Parts used
Green cucumber (GC)	<i>Cucumis sativus</i> L.	Cucurbitaceae	fruit
Bottle gourd (BG)	<i>Lagenaria siceraria</i> (Molina) standl.	Cucurbitaceae	fruit
Ash gourd (AG)	<i>Benincasa hispida</i> (Thunb.) COGN.	Cucurbitaceae	fruit
Yellow pumpkin (YP)	<i>Cucurbita maxima</i> (L.) Duchesne	Cucurbitaceae	fruit
Snake gourd (SG)	<i>Trichosanthes cucumerina anguina</i> (L.) Haines.	Cucurbitaceae	fruit
Chayote (CH)	<i>Sechium edule</i> (Jacq.) Sw.	Cucurbitaceae	fruit
Ridge gourd (RG)	<i>Luffa acutangula</i> (L.) Roxb.	Cucurbitaceae	fruit
Radish (RD)	<i>Raphanus sativus</i> L.	Brassicaceae	root
Ivy gourd (IG)	<i>Coccinia grandis</i> (L.) Voight	Cucurbitaceae	fruit
Carrot (CT)	<i>Daucus carota</i> L.	Apiaceae	root
Noolkol (Kohlrabi) (NK)	<i>Brassica oleracea</i> var. <i>gongylodes</i> L.	Brassicaceae	stem
Banana (BS)	<i>Musa paradisiaca</i> L.	Musaceae	stem
Yellow Cucumber (YC)	<i>Cucumis melo</i> var. <i>chito</i> (Morren) Naudin	Cucurbitaceae	fruit

of p-nitrophenol was measured spectrophotometrically (405 nm) after 5 min (BioTek synergy4 multimode microplate reader, BioTek Instruments Inc, Winooski, VT, USA). Suitable blank in the absence of enzyme was prepared with each vegetable's juice in order to correct background absorption. To determine 50% enzyme inhibitory concentration (IC₅₀), various dilutions of vegetables' juice were analyzed and results were expressed in terms of total polyphenol (µg GAE mL⁻¹) and total protein (µg BSAE mL⁻¹) concentration in diluted juice. Percentage inhibitory activity was calculated applying following formula:

$$\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{juice}}}{\text{Absorbance}_{\text{control}}} \times 100$$

where, 'A' is absorbance value.

Statistical analysis: Unpaired (two-tailed) t-test with Welch's correction was applied to find differences in the activity potential of juice between liver and skeletal muscle. Suitable regression analysis was applied to find IC₅₀ values. Two-tailed Pearson correlation analysis was done to find influence of total polyphenol and total protein in the juice on IC₅₀ value. Degree of significance was calculated at $p < 0.05$.

RESULTS

The yield of juice in vegetables varied from 33% to 56% (mL 100 g⁻¹). YP yielded lowest amount of juice and BS the highest (Table 2). BS is a porous soft stem used to prepare curry. Its juice is also used by elder people in India as coolant. The lowest yield of juice in YP obtained in our study might be due to its thick peel. CT is a hard root and IG is a small fruit vegetable. Similarly, CH is a solid medium size fruit. Therefore, yield of low quantity of juice in these vegetables is obvious. The concentration of total polyphenol and total protein content also varied greatly (Table 2). Lowest polyphenol content was observed in CH, BS and CT. Juice from YP, RG, BG and NK showed higher concentration of polyphenols.

Table 2: Percentage yield of juice in vegetables and chemical analysis

Vegetables	Yield (%) (mL 100g ⁻¹)	Total polyphenols (µg GAE mL ⁻¹)	Total protein (µg BSAE mL ⁻¹)
GC	41 ± 16.01	143.8 ± 0.01	633.1 ± 0.04
BG	47.6 ± 13.72	314.6 ± 0.04	513.0 ± 0.02
YC	47.5 ± 8.20	232.7 ± 0.06	305.1 ± 0.04
RG	42.6 ± 13.51	313.3 ± 0.02	1255.6 ± 0.10
SG	42.8 ± 6.73	137.1 ± 0.02	760.8 ± 0.03
YP	32.6 ± 9.03	303.8 ± 0.04	1170.1 ± 0.01
CH	38.5 ± 5.13	75.4 ± 0.03	1200.5 ± 0.05
AG	40.5 ± 4.49	193.3 ± 0.00	590.6 ± 0.09
RD	45.1 ± 1.27	187.9 ± 0.01	629.4 ± 0.04
IG	38.7 ± 8.16	193.7 ± 0.05	571.1 ± 0.01
CT	36.1 ± 2.51	85.6 ± 0.02	752.3 ± 0.08
NK	40.0 ± 5.35	477.3 ± 0.07	853.3 ± 0.23
BS	56.3 ± 4.67	79.9 ± 0.02	ND

ND: Not detected, value represent mean ± SD of 3-6 analysis done in triplicate

Similarly, wide variations in total protein content in different vegetables' juice were also observed in our study. Protein content could not be detected in BS juice. Juice of YP, CH and RG were observed rich source of protein. Juice of vegetables RG and YP were found rich source of both, polyphenol and protein. Although, CH juice was a rich source of vegetable protein, polyphenol content was observed lowest.

Figure 1 presents PTP 1β inhibitory activity of undiluted fresh juice. It was observed that, fresh juice of BG, YC, SG, RD and NK inhibited PTP 1β more than 50% in both the liver and skeletal muscle. Juice of YP, IG and BS displayed less than 25% of enzyme activity, juice of BS being the least potent in inhibiting PTP 1β of either source. Primary screening with fresh juice (Fig. 1) revealed that juice of AG, BG and RD were equipotent in inhibiting PTP 1β in liver and skeletal muscle. Furthermore, the selectivity of vegetables juice in inhibiting PTP 1β of skeletal muscle was significantly high in RG ($p < 0.0001$), SG ($p < 0.0001$), YC ($p < 0.0001$), CH and YP ($p < 0.0002$), GC ($p < 0.0004$) and IG ($p < 0.0038$) than liver. On the other hand, selectivity of PTP 1β inhibition towards liver was significantly high in NK ($p < 0.004$) and CT ($p < 0.002$).

The concentration dependent (polyphenol) inhibitory activity pattern of PTP 1β in liver and skeletal

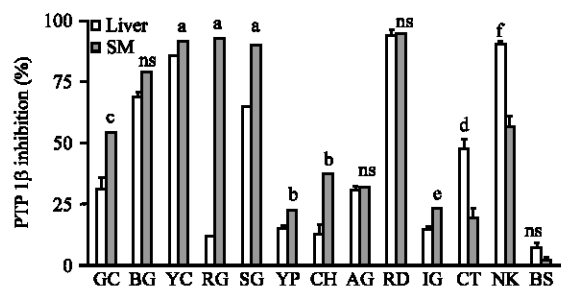


Fig. 1: Inhibition of PTP 1 β in liver and skeletal muscle (SM) by undiluted fresh juice of vegetables. Data represent pooled mean \pm SD of three experiments. Unpaired (two-tailed) t-test with Welch's correction was applied to find differences in the activity potential of juice between liver and skeletal muscle. ^a $p < 0.0001$, ^b $p < 0.0002$, ^c $p < 0.0004$, ^d $p < 0.002$, ^e $p < 0.0038$, ^f $p < 0.004$, NS = Not significant

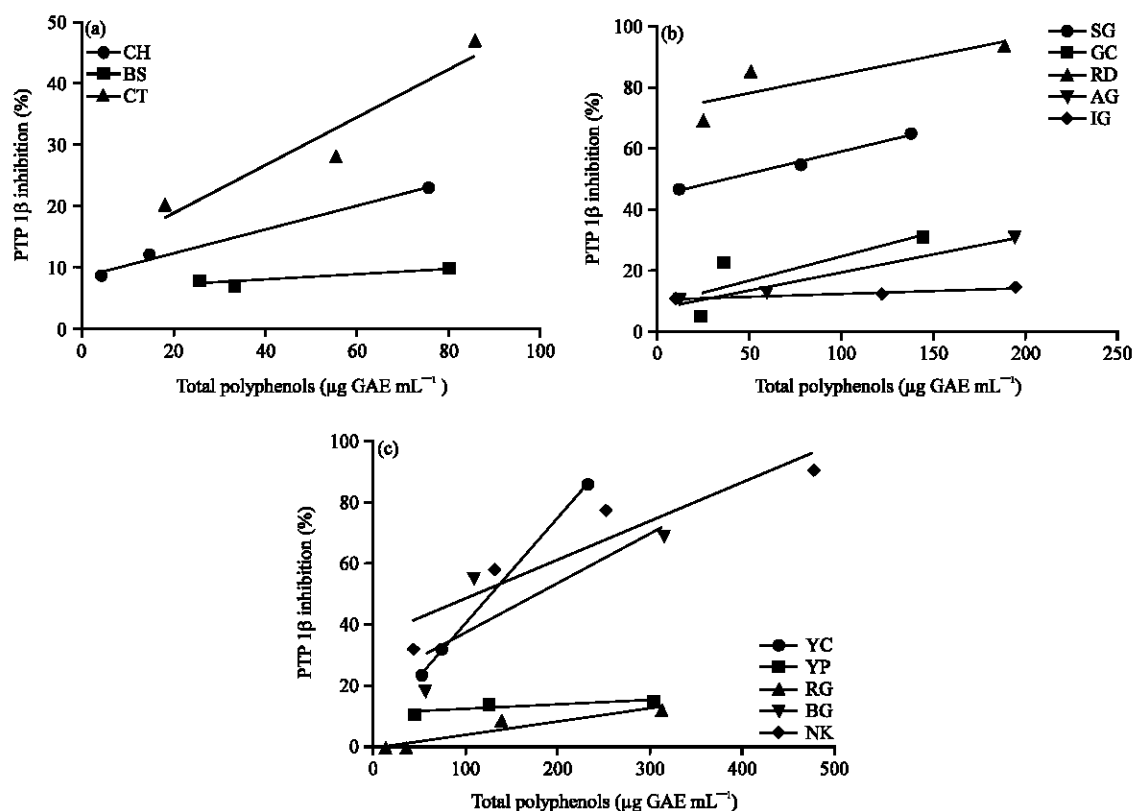


Fig. 2(a-c): Concentration dependent (in terms of total polyphenol) inhibitory pattern of PTP 1 β inhibition in liver by fresh juice of vegetables. Values represent mean of triplicates

muscle by vegetables' juice is presented in Fig. 2(a-c) and Fig. 3(a-c), respectively. Different trends and wide variations in activity potentials of vegetables juice was detected in this analysis. It was observed that CT, CH (Fig. 2a); SG, GC, RD and AG (Fig. 2b) and YC, RG, BG and NK (Fig. 2c) displayed concentration dependent inhibitory pattern for liver PTP 1 β , however juice of BS (Fig. 2a), IG (Fig. 2b) and YP (Fig. 2c) did not respond to

the dilution in terms of decreasing polyphenol concentration. Similarly, juice of CT, SG and GC (Fig. 3a); RD and IG (Fig. 3b) and YC, RG, BG and NK (Fig. 3c) displayed concentration dependent change in activity pattern in inhibiting skeletal muscle PTP 1 β . The juice of CH and BS (Fig. 3a); AG (Fig. 3b) and YP (Fig. 3c) did not display dilution dependent change in inhibiting PTP 1 β in skeletal muscle.

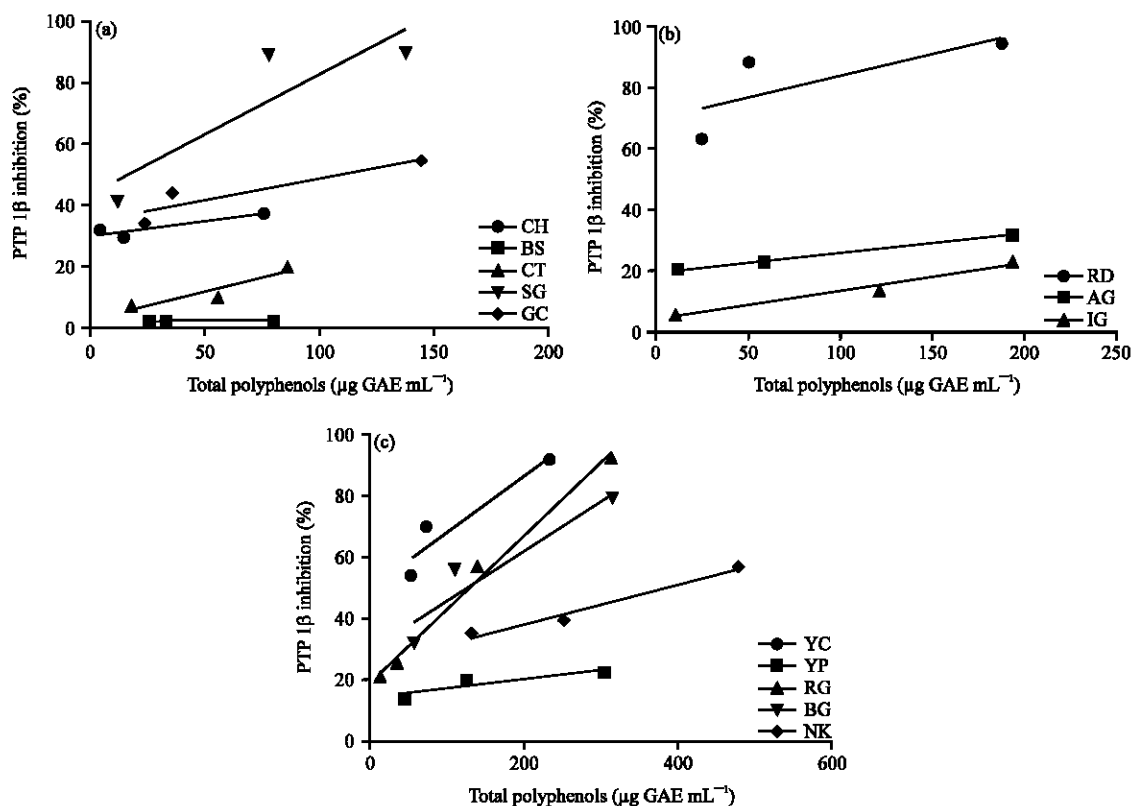


Fig.3 (a-c): Concentration dependent (in terms of total polyphenol) inhibitory pattern of PTP 1 β inhibition in skeletal muscle by fresh juice of vegetables. Values represent mean of triplicates

Table 3: IC₅₀ of fresh juice of vegetables in terms of total polyphenol ($\mu\text{g GAE mL}^{-1}$) for inhibition of PTP 1 β in liver and skeletal muscle (SM) in decreasing order of the potency

IC ₅₀ in Liver	RD	SG	NK	CT	YC	BG	CH	GC	AG	RG
	3.7	22.4	75.8	125.5	128.5	137.9	213.6	257.6	363.6	1192.3
IC ₅₀ in SM	RD	YC	SG	BG	RG	GC	CT	NK	IG	
	6.3	39.0	89.0	102.0	130.2	209.2	262.6	388.6	497.3	

Table 4: IC₅₀ of fresh juice of vegetables in terms of total protein ($\mu\text{g BSAE mL}^{-1}$) for inhibition of PTP 1 β in liver and skeletal muscle (SM) in decreasing order of the potency

IC ₅₀ in Liver	NK	RD	YC	BG	AG	CT	GC	CH	RG	
	5.6	21.4	162.7	302.9	773.6	906.3	946.1	2240.3	4211.3	
IC ₅₀ in SM	YC	RD	SG	BG	GC	NK	RG	IG	AG	CT
	1.0	74.4	180.9	223.6	510.2	710.5	760.8	886.4	913.6	2169.8

Similar was the case when enzyme inhibitory activity was expressed in terms of protein concentration of the juice for liver PTP 1 β (Fig. 4a-c) and skeletal muscle PTP 1 β (Fig. 5a-c). Except juices of SG (Fig. 4a), IG (Fig. 4b) and YP (Fig. 4c), the juice of other vegetables displayed concentration dependent inhibition for liver PTP 1 β . On the other hand however, except CH and YP (Fig. 5c) skeletal muscle PTP 1 β inhibitory activity decreased with decreasing concentration of protein by other juices.

Capacity of vegetables' juice in inhibiting liver and skeletal muscle PTP 1 β (in decreasing order) either in terms of polyphenol concentration or protein concentration is presented in Table 3 and 4, respectively. It was found that juice of RD was most potent in inhibiting both liver and skeletal muscle PTP 1 β in terms of IC₅₀ ($\mu\text{g GAE mL}^{-1}$, Table 3) and ranked second in the order of its potential in terms of protein IC₅₀ ($\mu\text{g BSAE mL}^{-1}$, Table 4). Juice of vegetable, BG was also found potent in inhibiting PTP 1 β of both the sources

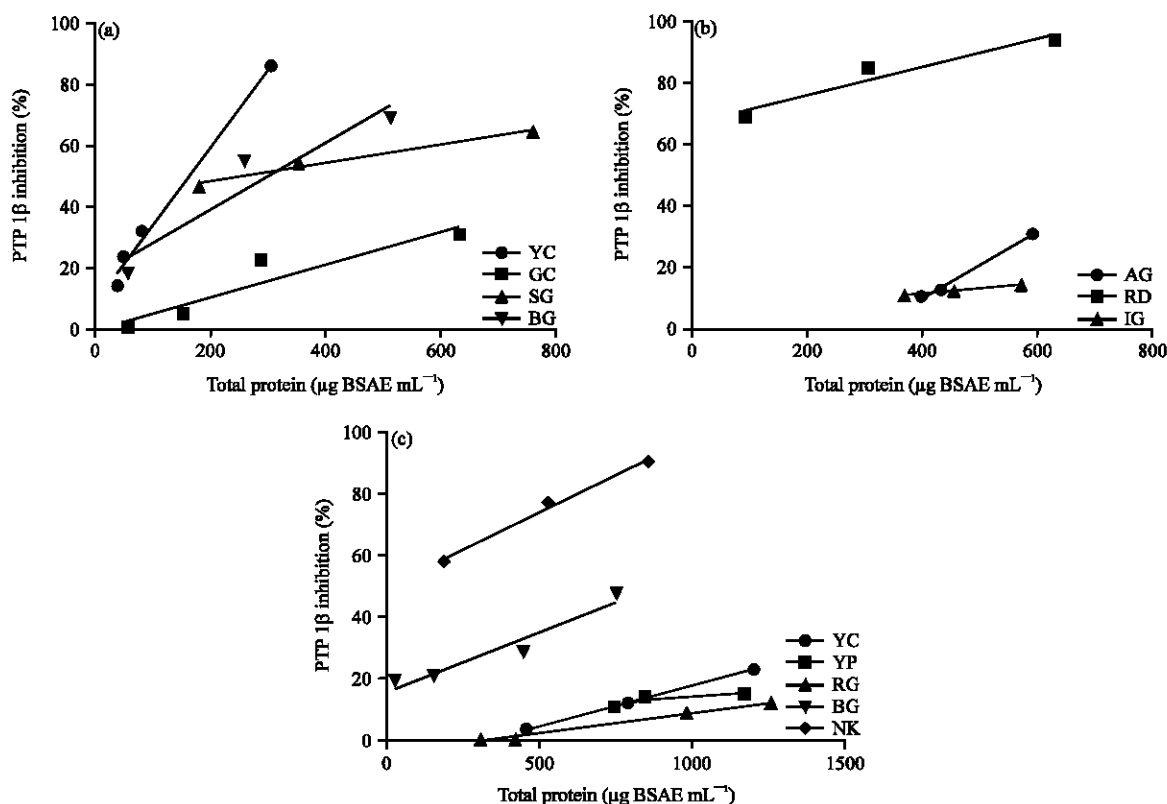


Fig. 4(a-c): Concentration dependent (in terms of total protein) inhibitory pattern of PTP 1 β inhibition in liver by fresh juice of vegetables. Values represent mean of triplicates

and ranked fourth in terms of protein concentration (Table 4). Juice of vegetables RG, AG, IG and CT were found less potent than other vegetables' juice (Table 3 and 4).

One of the interesting observation in our study was that inhibition of liver PTP 1 β was significantly ($p < 0.007$) influenced by protein concentration in the vegetables' juice (Fig. 6b). Concentration of total polyphenol in vegetables' juice could not influence significantly the PTP 1 β inhibitory potential (Fig. 6a).

DISCUSSION

We selected easily available vegetables consumed by the common people in day-to-day life. Wide variations in yield of the juice, presence of total polyphenols and total proteins among vegetables' juice were observed. Furthermore, PTP 1 β inhibitory potential and pattern in liver as well as skeletal muscle also differed greatly. Simultaneously, we could not observe substantial association between polyphenol or the protein content in vegetable's juice with PTP 1 β inhibitory activity except for the association between total protein and IC₅₀ in liver. Therefore, influence of only total polyphenol or total protein content present in vegetables juice can not

explain their enzyme inhibitory potential. The juices used in our study were freshly drawn and represent natural therapy. The scientific basis of natural therapy plant-based medicines in particular, is based on the fact that mixture of compounds present in plant-based medicine impart therapeutic efficacies by stimulating multiple therapeutic targets either synergistically, agonistically or antagonistically¹¹. Therefore, therapeutic effect of natural medicines may not be governed by a single component; rather it may be explained on the basis of wholistic therapeutic approach, for whole might display more therapeutic potential than sum of the parts¹².

We observed that inhibitory activity of YP and IG juice did not respond to the dilution factor either in terms of protein concentration or polyphenol for the liver PTP 1 β . Similar was the observation with juice of YP and CH for skeletal muscle PTP 1 β inhibition. In terms of polyphenol concentration, juice of BS could not display concentration dependent change in PTP 1 β inhibition in liver and skeletal muscle. Furthermore, similar was the trend obtained for AG juice in terms of polyphenol concentration for skeletal muscle PTP 1 β inhibition. It appears therefore, that phytochemicals present in respective vegetables' juice might be inhibiting

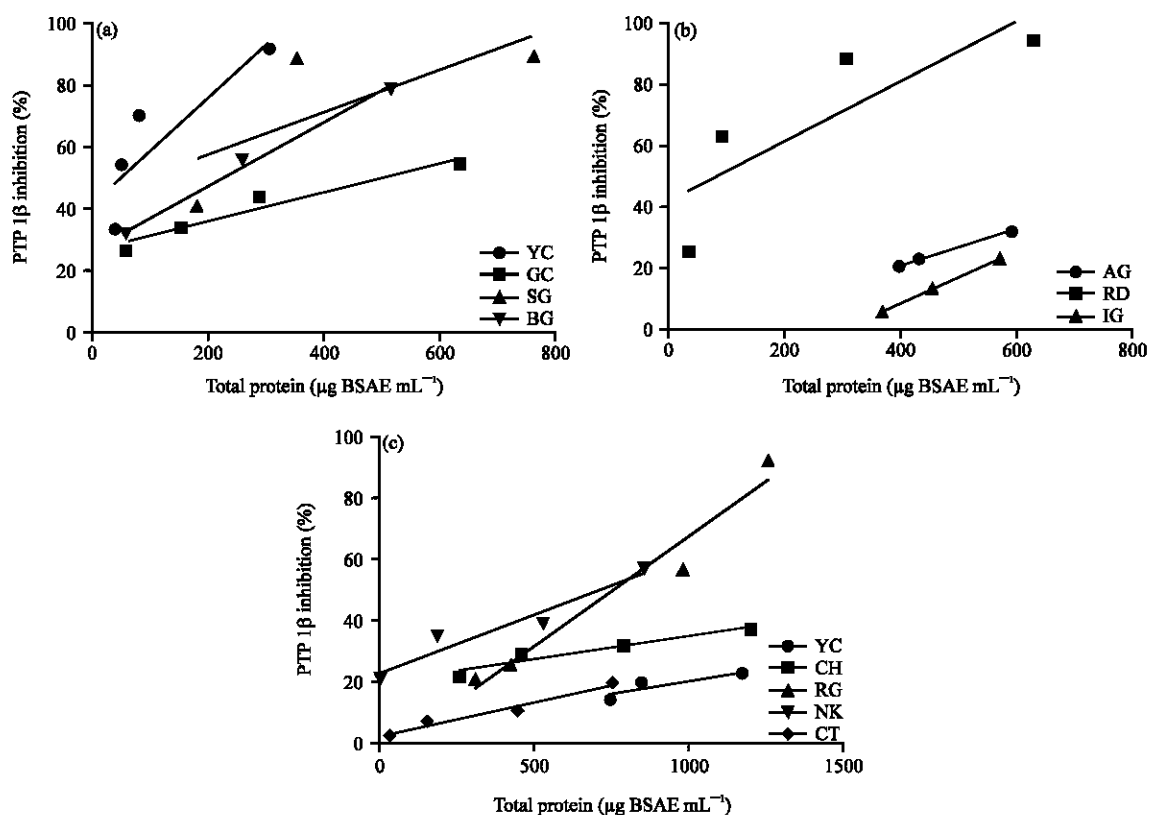


Fig. 5(a-c): Concentration dependent (in terms of total protein) inhibitory pattern of PTP 1 β inhibition in skeletal muscle by fresh juice of vegetables. Values represent mean of triplicates

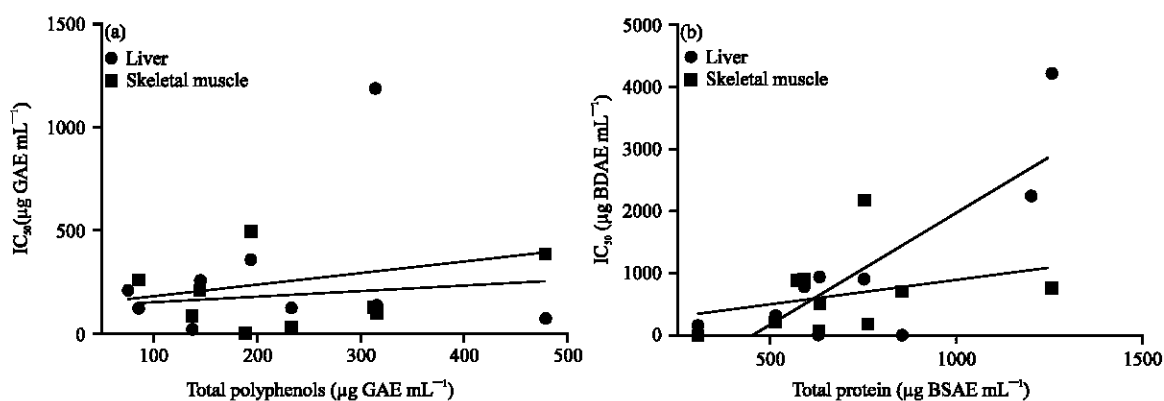


Fig. 6 (a-b): Influence of presence of total polyphenol (a) Total protein (b) Present in vegetables juice on IC_{50} of PTP 1 β inhibition in different tissues. Two-tailed Pearson regression analysis was applied to find correlations, (a) Pearson $r = 0.1972$, $p = 0.5850$ for liver and Pearson $r = 0.1894$, $p = 0.6255$ for skeletal muscle, (b) Pearson $r = 0.8136$, $p < 0.0076$ for liver and Pearson $r = 0.3126$, $p = 0.3793$ for skeletal muscle

the enzyme irreversibly in respective tissues. PTP 1 β inhibitory activity decreased with dilution of other vegetables' juice. Therefore, those vegetables' juice could be categorised as reversible inhibitor of the enzyme.

Variation in selectivity of vegetables' juice in inhibiting liver and skeletal muscle PTP 1 β were also noticed in our study. Such variation have been reported by other investigators also⁷.

Liver PTP 1 β plays an important role in glucose and lipid metabolism¹³. The muscle specific PTP 1 β plays an important role in decreasing glucose uptake by muscles, decreasing systemic insulin sensitivity, glucose tolerance ability and plays a major role in regulating insulin action and homeostasis¹⁴. Furthermore, PTP 1 β is also identified as a negative regulator of insulin and leptin signalling and hence its inhibition is also emerging as potential therapy for diabetes and obesity¹⁵. Proof of principles demonstrate that partial and reversible inhibition of PTP 1 β is beneficial in the treatment of insulin resistance, diabetes and obesity^{13,14,15}. Vegetables' juice have been reported recently to mitigate starch-induced postprandial glycemic excursion⁴ and sweet-beverages induced development of impaired glucose tolerance and hyperglycemia-induced physiological imbalances⁵. Analysis of this research identified vegetables, selectively and differently inhibiting PTP 1 β of liver and skeletal muscle. This information may help further analyse vegetables' juice and identify active principles. Nonetheless, RD and BG may become vegetables of choice, based on their superiority in ranking order of inhibiting liver as well as skeletal muscle PTP 1 β .

Dietary intake and nutritional status are important and independent risk factors influencing aetiology of 'diseases of modern civilization', such as diabetes, obesity, heart disease and cancer¹⁶. Vegetables have been used by mankind for health benefits since ancient times and are often recommended by naturopaths. However, their arbitrary use can turn toxic and dangerous also. Complex tetracyclic triterpenoid cucurbitacin compounds found in plants belonging to Cucurbitaceae family, are responsible for bitter taste in vegetables. These compounds when absorbed into the blood may cause hepatitis, pancreatitis, cholecystitis and even renal damage leading to multiorgan dysfunction¹⁷. It is advisable therefore, to avoid consumption of bitter tasting vegetables.

CONCLUSION

PTP 1 β plays an important role in glucose and lipid metabolism, alteration in adiposity, negative regulation of insulin and leptin signalling, increasing insulin resistance and decreasing insulin sensitivity. Inhibition of PTP 1 β therefore may provide multiple beneficial effects in disorders of glucose and lipid metabolism. This research provides evidence that vegetables are potent natural source of PTP 1 β inhibition and may become cost effective therapeutics in correcting metabolic disturbances of T2DM, obesity and cardiovascular disorders. To the best of our knowledge, this is the first report identifying PTP 1 β inhibitory potential in raw vegetables juice.

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