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Abstract

Research on antioxidant potential from vegetables is increasingly focused on their effects on human health. However, relatively little work has been done to investigate the antioxidant effect of crude extract and/or different fractions from *Brassica rapa* L. In the present study, the antioxidant potential of crude extract and its fractions from *Brassica rapa* L. fruit part was tested for glutathione peroxidase (GPx), superoxide dismutase (SOD) enzymes and total antioxidant status (TAS) in blood samples. Our results reveal the fact that crude extract and each analyzed fraction (i.e. aqueous, ethyl acetate and chloroform) showed a concentration dependent effects on GPx, SOD and TAS in respect with saline solution (0.9% NaCl) used as negative control and vitamin C, as positive control. Therefore, GPx levels showed a highest value in crude extract and chloroform fraction (6981 U/L both at 10 mg/ml), SOD levels showed the same results in aqueous and ethyl acetate fractions (220 U/ml both at 10 mg/ml) and TAS in crude extract and all three fractions (i.e. aqueous, ethyl acetate and chloroform, 1.68 mmol/L at 10 mg/ml for all three fractions) in respect with saline solution ($p < 0.05$). Furthermore, vitamin C showed the highest values on all three analyzed enzymes (8769 U/L for GPx, 223 U/ml for SOD and 1.8 mmol/L for TAS at 100 µg/ml). Our investigations have been proved to be promising in terms of future potential applications of crude extract and its fractions as components in a range of phytochemicals composition and/or different pharmaceutical usage, owing to their antioxidant potential.

Keywords: *Brassica rapa* L., glutathione peroxidase, superoxide dismutase, total antioxidant status, enzymes.

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Rezumat

Cercetările asupra potențialului antioxidant din legume prezintă o importanță crescută a efectelor sale asupra sănătății umane. Cu toate acestea, puține studii au fost realizate pentru a investiga efectul antioxidant al extractului crud și/sau a diferitelor fracțiuni din *Brassica rapa L.* În studiul de față, potențialul antioxidant al extractului crud și a fracțiunilor acestuia din fructul *Brassica rapa L.* a fost testat pentru enzimele glutatión peroxidaza (GPx), superoxid dismutaza (SOD) și statusul total antioxidant (STA) în probe de sânge. Rezultatele noastre au arătat faptul că atât extractul crud cât și fiecare fracțiune analizată (apoasă, de acetat de etil și cloroformică) au arătat efecte dependente de concentrație asupra GPx, SOD și STA în comparație cu soluția salină (0,9% NaCl) folosită ca și control negativ și vitamina C, ca și control pozitiv. Prin urmare, nivelurile GPx au prezentat valori crescute în extractul crud și fracțiunea cloroformică (6981 U/L ambele la 10 mg/ml), nivelurile SOD au prezentat aceleași rezultate în fracțiunea apoasă și cea de acetat de etil (220 U/ml ambele la 10 mg/ml) și STA în extractul crud și toate cele trei fracțiuni (apoasă, de acetat de etil și cloroformică; 1,68 mmol/L la 10 mg/ml pentru toate fracțiunile) în comparație cu soluția salină ($p < 0,05$). Mai mult, vitamina C a prezentat cele mai mari valori asupra celor 3 enzime analizate (8769 U/L pentru GPx, 223 U/ml pentru SOD și 1,8 mmol/L pentru STA la 100 μg/ml). Investigațiile noastre se dovedesc a fi promițătoare în ceea ce privește viitoarele aplicabilități ale extractului crud și fracțiunile acestuia ca și componenți în diferite preparate fitochimice și/sau de uz farmaceutic datorită potențialului lor antioxidant.

Cuvinte cheie: *Brassica rapa L.*, glutatión peroxidaza, superoxid dismutaza, status total antioxidant, enzime.

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Introduction

In recent years, there has been an increasing trend towards the exploration of safer and effective antioxidants and functional ingredients from natural dietary sources like vegetables (1, 2). The antioxidant effect of vegetables like broccoli raab (*Brassica rapa L.*), also called turnip top, one of the oldest cultivated vegetables in southern Italy, is mainly due to the occurrence of phenolic compounds (3). However, few reports dealing with the obtaining of different fractions and further determination of the compounds with antioxidant potential can be found mainly due to the low efficiency (in terms of quantities) in their extraction that did not encourage further investigations of these useful and promising fractions (4).

These fractions comprise both phenolic compounds and organic acids (4, 5), which contribute to their organoleptic features (6), despite being applied in the quality control of several matrices (7, 8). The polyphenol composition of several materials from members of *Brassica* genus, or their byproducts, has been described (9, 10), including that of *B. oleracea* var. *costata* (11, 12), *B. oleracea*

var. *Acephala* (13) and *B. rapa* var. *rapa* (8, 14), referring distinct profiles between them. Recent publications report the possible antioxidant potential of these species (8, 13, 15).

In this regard, both epidemiological studies and experimental research indicated that regular intake of cruciferous vegetables may reduce risk for many chronic diseases (16). On the other hand, low consumption of these vegetables may reduce serum antioxidant capacity (17). However, how these fractions can interfere into oxidative process is still under debate.

In the present study, antioxidant potential of crude extract and its fractions from *Brassica rapa L.* on GPx, SOD and TAS in blood sample, using saline solution (NaCl, 0.09%) and vitamin C as controls was carried out.

Materials and Methods

Sample preparation

Brassica rapa L. fruit parts were collected from the local supermarkets (about 5 kg), and immediately frozen and kept at -180°C. Identification was carried out by the Department of Phar-

macy, Faculty of Biological Sciences, Kohat University of Science and Technology, Pakistan. A specimen has been kept in our laboratory for future reference. Fruit pulp at room temperature were mashed and stored in a tightly closed container for future use. Five milliliters of blood was taken from the healthy volunteers after signing the informed consent. An informed consent was obtained from the patients in accordance with the Helsinki Declaration and guidelines of the local Committee of Ethics of the Department of Pharmacy, Faculty of Biological Sciences, Kohat University of Science and Technology, Pakistan.

Preparation of fruit extracts

Mashed fruit was soaked in 5 l of 70% aqueous methanol for 3 days, occasional shaking. Then was filtered through a muslin cloth and further through a Whatman qualitative (20–25 µm) filter paper. The procedure was repeated twice and the combined filtrate was evaporated on a rotary evaporator under reduced pressure to a thick, semi-solid mass of dark brown color, which represented crude extract (Br-Cr). Fractionation of the crude extract was obtained by standard phytochemical procedures using different organic solvents. About 50 g from the crude extract was dissolved in 50 ml distilled water. Then the extract was introduced into a separating funnel. Ethyl acetate (50 ml) was added into the same separating funnel. The mixture was shaken vigorously for about 30 min, until the two layers separate. The upper layer of ethyl acetate was acquired and the same procedure was repeated twice and all the ethyl acetate layers were collected and concentrated in a rotary evaporator to obtain the ethyl acetate fraction (BR-Eth). A quantity of 50 ml of chloroform was added to the remaining layer and the same process was repeated, obtaining the chloroform fraction in the end (BR-Chl). The yield of both fractions was 20% and 25%, respectively, while the remaining layer was the aqueous layer (BR-Aq).

Chemicals and reagents

Saline solution (NaCl, 0.09% w/v) and vitamin C were purchased from Sigma Chemical

(St. Louis, MO), kits for superoxide dismutase (SOD), glutathione peroxidase (GPx), and total antioxidant status (TAS) from Redox (UK) and solvents (HPLC grade) from Mallinckrodt Chemicals (St. Louis, Missouri). Stock solutions of all the chemicals were made in distilled water and the dilutions were made fresh in normal saline (NaCl, 0.9% w/v) in the same day of the experiment. All samples, solutions, and buffers were prepared using Milli-Q water.

Further to our experiments, crude extract and its various fractions obtained from *Brassica rapa* L. were tested on the three antioxidant enzyme levels in human blood.

Saline solution used as negative control, and vitamin C as positive control were separately analyzed from crude extract and its various fractions.

Glutathione peroxidase activity determination

GPx levels were determined using commercially available kits from RANDOX, UK and assays were carried out using a spectrophotometer DU 800 (Beckmann, USA). After adding all the chemicals, crude extract and different fractions obtained were added before the addition of substrate (i.e. t-Butyl-hydroperoxide). The absorbance at 340 nm was measured. GPx was measured by coupling the peroxidase reaction with the reduction of oxidized glutathione by glutathione reductase and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (18). Reduction of the substrate was followed by the decrease in absorbance of NADPH at 340 nm. Activity was evaluated using glutathione as the co-substrate (18).

Superoxide dismutase activity determination

SOD activity was determined using the same commercially available kits and spectrophotometer. SOD activity assay was conducted as described by Oyanagui (19). After adding all the chemicals, different fractions were added before the addition of substrate (i.e. 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium

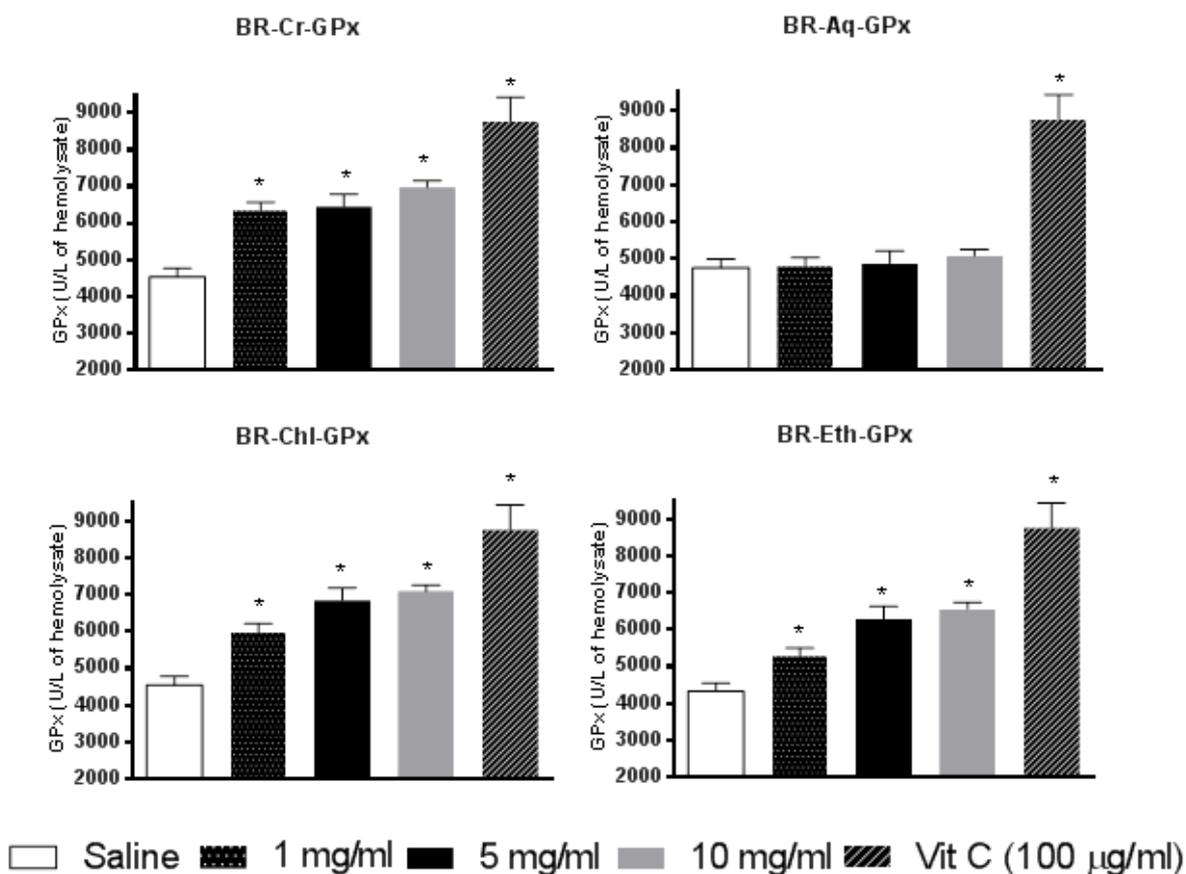


Figure 1. Comparative effects of all three doses (1, 5 and 10 mg/ml) of crude extract and BR-Cr, BR-Aq, BR-Chl and BR-Eth fractions of *Brassica rapa* L. on GPx levels in human blood (n=5 ml); *p<0.05 compared to saline.

chloride (INT)) to obtain a formazane dye. The absorbance at 505 nm was measured. The method involved xanthine and xanthine oxidase to generate superoxide radicals. The SOD activity was measured taking into consideration the degree of inhibition in this reaction. One unit of SOD can cause 50% inhibition of the rate of reduction of the INT under the condition of the assay.

Total antioxidant status determination

TAS was measured in human plasma using the same kits and spectrophotometer used for GPx and SOD determination. The assay was performed as described by Mitrevky and contributors (20).

After adding all the chemicals, crude extract and all three fraction were added before the addition of substrate (2,2' azino-bis-[3-ethylbenz-thiazoline-6-sulfonic acid] (ABTS)) at the end. The concentration was determined by measuring absorbance at 600 nm. The amount of ABTS⁺ produced is monitored by reading the absorbance at 600 nm. Under these conditions, the antioxidants in the plasma cause suppression of the absorbance at 600 nm to a degree that is proportional to their concentration. The final plasma antioxidant concentration was obtained (mmol/L) by multiplying the absorbance of the sample to the specific factor obtained from the standard and the blank.

Statistical analysis

All the aforementioned experiments were conducted in triplicate. Statistical comparisons were performed by one-way analysis of variance (ANOVA). In the case of the identification of statistical differences using ANOVA, the Student Newman-Keuls test was used to compare the fractions using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Probability values < 0.05 were considered to indicate significant difference.

Results

The antioxidant potential on GPx levels

The results on GPx levels are summarized in *Figure 1*. The enzyme showed a concentration dependent with each dose of crude extract and all three fractions from *Brassica rapa* L. (i.e. 1, 5 and 10 mg/ml) in respect with saline solution which showed approximately similar values (i.e. 4500 U/L in respect with crude extract and chloroform fraction and 4350 U/L in respect with ethyl acetate fraction and 4830 U/L with aqueous fraction) and vitamin C (i.e. the same values at 8769 U/L at 100 $\mu\text{g/ml}$) in respect with crude extract and all three fractions (*Figure 1*).

Interestingly, at 1 mg/ml, the highest values of GPx was obtained in crude extract (6230 U/L, $p=0.05$, $SD=2.43$), at 5 mg/ml in chloroform fraction (6730 U/L, $p=0.04$, $SD=4.16$) and at 10 mg/ml in both crude extract and chloroform fraction from *Brassica rapa* L. (each at 6981 U/L, $p=0.03$, $SD=2.98$).

The antioxidant potential on SOD levels

Further investigations were performed to identify the levels of SOD in crude extract and each fraction. In particular, at 1 mg/ml highest values of SOD was obtained in chloroform and ethyl acetate fraction from *Brassica rapa* L. (both at 190 U/ml, $p=0.05$, $SD=1.89$), at 5 mg/ml in aqueous fraction (215 U/ml, $p=0.04$, $SD=1.61$) and at 10 mg/ml in aqueous and ethyl acetate fraction (both at 220 U/ml, $p=0.03$, $SD=2.13$) in respect with saline solution. A remarkable difference was seen in vitamin C

showing the same values (223 U/ml at 100 $\mu\text{g/ml}$) in respect with crude extract and all three fractions (*Figure 2*).

The antioxidant potential on TAS levels

The results of TAS levels exhibited a similar profile to that of GPx and SOD, respectively. At 1 mg/ml the highest values of TAS was obtained in ethyl acetate fraction (1.6 mmol/L, $p=0.05$, $SD=0.27$) at 5 mg/ml in ethyl acetate fraction (1.65 mmol/L, $p=0.04$, $SD=0.98$) and at 10 mg/ml in crude extract and all three fractions (1.68 mmol/L, $p=0.03$, $SD=1.46$). Saline solution showed the highest values of TAS in respect with ethyl acetate fraction (1.25 mmol/L). Nevertheless, the highest values were obtained for vitamin C which showed similar values (1.68 mmol/L at 100 $\mu\text{g/ml}$) in respect with crude extract and all three fractions (*Figure 3*).

In any case, the results provide enough data to demonstrate the antioxidant potential of crude extract and all three fractions (i.e. aqueous, ethyl acetate and chloroform) on GPx, SOD and TAS levels from blood samples.

Discussion

Vegetables serve as a good source of antioxidant apart from nutritive contribution of carbohydrates, proteins and lipids. There is an extensive body of literature accumulated in the recent years suggesting the role of *Brassica rapa* L. in health management and especially lowering the risk of chronic disease and other age related disorders (9, 21-23).

The generation of free radicals is a vital phenomenon of the normal metabolism of human body. Normally, free radicals are neutralized by both enzymatic and nonenzymatic antioxidants present as an inbuilt antioxidant mechanism in the body and also by the dietary antioxidants supplemented in daily diets through fruits and vegetables (24). The major phytochemicals found in vegetables involved in stabilization of free radicals includes flavonoids, coumarins, tannins and other phenolic compounds. Strikingly, it

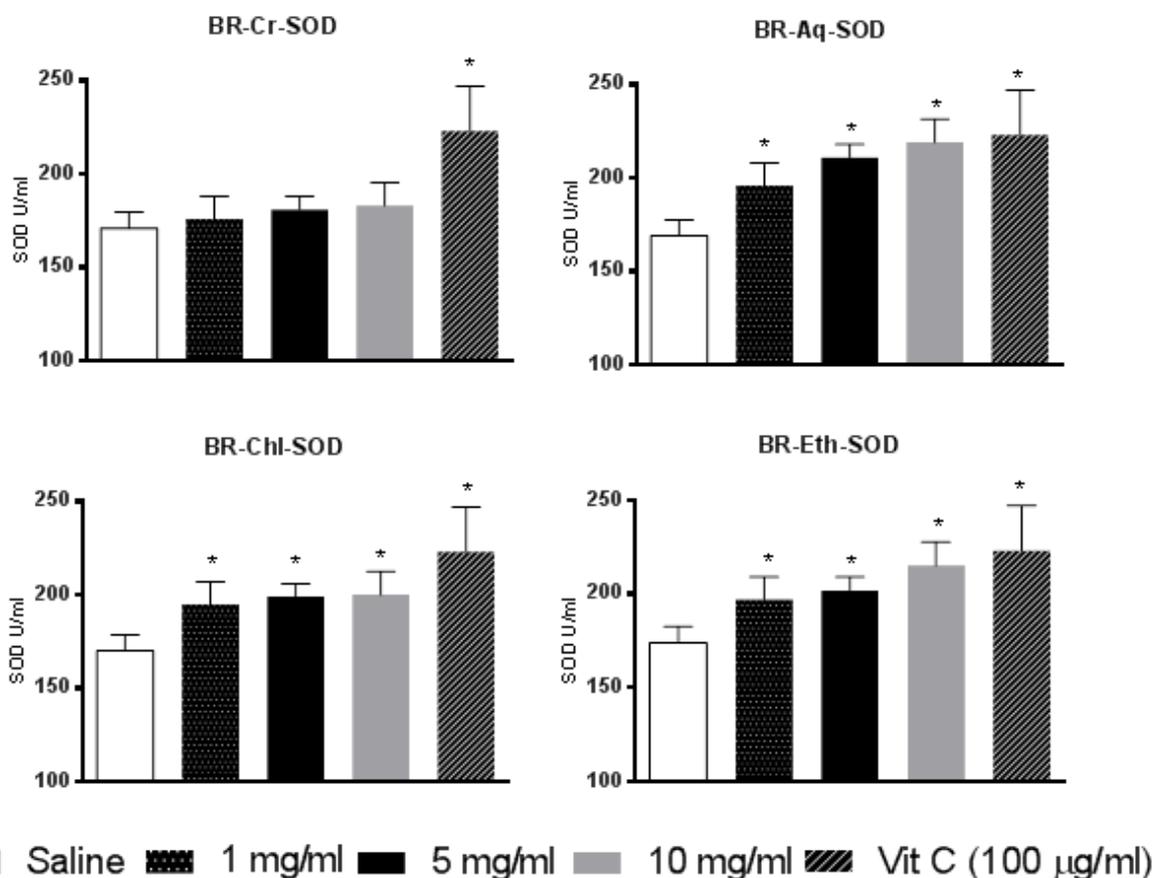


Figure 2. Comparative effects of all three doses (1, 5 and 10 mg/ml) of crude extract and BR-Cr, BR-Aq, BR-Chl and BR-Eth fractions of *Brassica rapa* L. on SOD levels in human blood (n=5 ml); *p<0.05 compared to saline.

has been indicated that these phytochemicals, have high free radical scavenging activities, which help to protect cells from oxidation damage induced by the free radicals and thereby help to reduce the oxidative stress (25-29).

In the present study, we choose to analyze aqueous, ethyl acetate and chloroform fractions from *Brassica rapa* L. based on the fact that they have been showed to present significant high activities in free radical scavenging and lipid peroxidation of turnip roots extraction in respect with these fractions vs. crude extract (3).

The observed antioxidant potential of both different fractions and crude extract from

Brassica rapa L. may be due the presence of above stated properties. However, our study does not attempt to identify the compounds responsible for observed antioxidant effects from *Brassica rapa* L. Furthermore, since *Brassica rapa* L. has not been investigated previously in terms of its pharmacological effects, we investigated its protective effects against two antioxidant enzymes (i.e. GPx, SOD) and TAS.

Experiments with different fractions of *Brassica rapa* L. on different antioxidant enzymes produced very interesting results. Compared with crude extract and chloroform fraction which showed the highest values, in contrary, the aqueous

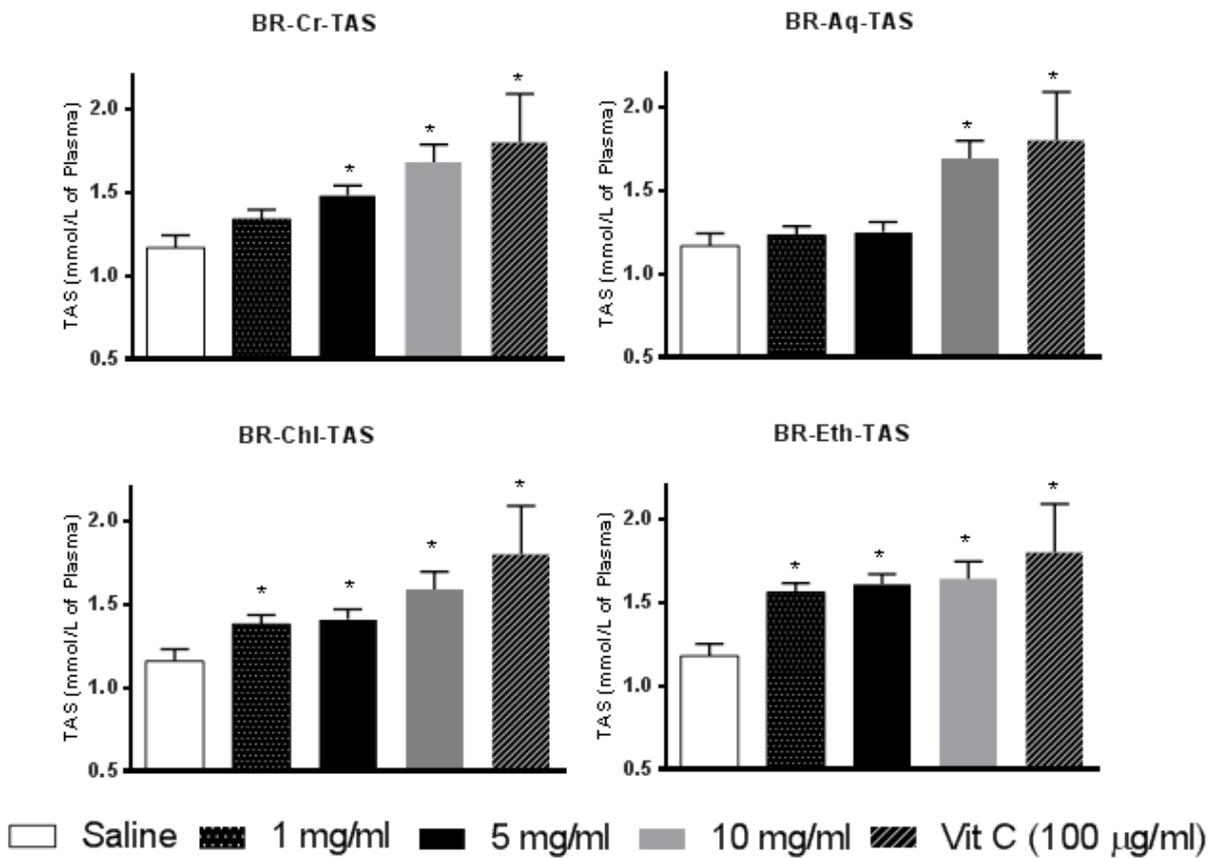


Figure 3. Comparative effects of all three doses (1, 5 and 10 mg/ml) of crude extract and BR-Cr, BR-Aq, BR-Chl and BR-Eth fractions of *Brassica rapa* L. on TAS levels in human blood (n=5 ml); *p<0.05 compared to saline.

fraction may contain some inhibitors of GPx. Interestingly, chloroform fraction seems to have concentrated it in only those compounds which are responsible for increasing of GPx levels, without any other inhibitors. Our results are in agreement with those reported by Nain and contributors (30) in which the authors found higher levels of GPx in streptozotocin induced diabetic rats, reducing the risk of diabetic complications. Shah & Verma (31) showed also similar results by treatment of butylparaben to mice for 30 days which resulted in significant elevation in hepatic lipid peroxidation and glutathione peroxidase antioxidants activities (31).

In comparison with crude extract and chloroform fraction, aqueous and ethyl acetate

fractions of *Brassica rapa* L. showed a higher increase in the SOD levels. The reason of the decrease in crude extract could be due to the fact that the crude extract became inactive in due course of processing. With fractionation, these inhibitors are likely to be diluted and thus having a slow effect on SOD elevating capability of fractions. These results suggest that SOD enhancing constituents of *Brassica rapa* L. are more distributed in aqueous and ethyl acetate fraction. Elegant work from Abraham and contributors (32) showed that ethyl acetate is the most optimal solvent for the extraction of compounds with antioxidant and anti-proliferative activities. Moreover, Mahdy and colleagues (33) which examined SOD from erythrocytes of the

Alzheimer' disease induced rats, showed an elevation of the value and further ameliorates the neurodegeneration characteristic of diseases.

Crude extract and all fractions of *Brassica rapa L.* (i.e. aqueous, ethyl acetate and chloroform) were found to improve TAS levels, albeit showing different potencies. These results suggest that TAS elevating compounds are distributed throughout the crude extract and all three fractions of *Brassica rapa L.* Saeed and contributors (34) found similar results using different extracts (i.e. methanol, n-hexane, chloroform, ethyl acetate, n-butanol and residual aqueous fraction) on superoxide anion and hydroxyl radicals and found significantly higher values, showing that these extracts act as an antioxidant agent due to its free radical scavenging and total antioxidant status.

Conclusions

Our results reveal the fact that crude extract and each analyzed fraction (i.e. aqueous, chloroform and ethyl acetate) from *Brassica rapa L.* showed concentration dependent effects on GPx, SOD and TAS in respect with both saline solution and vitamin C used as controls. GPx levels showed a highest value in crude extract and chloroform fraction (both at 10 mg/ml), SOD levels showed the same results in aqueous and ethyl acetate fractions (both at 10 mg/ml) and TAS in crude extract and all three fractions (i.e. aqueous, ethyl acetate and chloroform, at 10 mmol/L for all three fractions) although vitamin C showed the highest values on all three analyzed enzymes at 100 µg/ml.

The crude extract and different fractions analyzed in the present study, showed a higher antioxidant level through all three analyzed enzymes, and merits considerations to be used as future components in a range of phytochemicals composition and/or different pharmaceutical usage.

Conflicts of interest: None to declare.

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