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Comparative Antioxidant Properties of Water Extracts from Different Parts of Beefsteak Plant (*Perilla frutescens*)

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ABSTRACT

Water extracts from beefsteak plant stems (BS), leaves (BL), and ripe seeds (BR) were examined for their antioxidant activities and phenolic compounds. Based on superoxide anion radicals scavenging activity, the BS extract (61.8%) is the most effective one followed by BL and BR extract (60.8% and 33.3%, respectively). The antioxidant activity determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method revealed that the BS extract had the highest activity on DPPH free radicals (54.8%). In addition, the extracts of BS, BL and BR were effective in reducing capacity and chelating effect on ferrous ions in comparison with the control. Total phenolic compound and flavonoid content of the BS extract was found to be superior to other beefsteak plant extracts. The results indicated that the BS extract of beefsteak plant has effective antioxidant functions, especially in superoxide anion radical and DPPH radical scavenging activities and reducing power. It was of our paramount interest to further identify the specific antioxidant components in BS which may be a new health-care food supplement or functional food for special use in the future.

Key words: beefsteak plant, antioxidant activity, water extract

INTRODUCTION

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. Reactive oxygen species (ROS) produced by ultraviolet, sunlight, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as carcinogenesis and cellular degeneration related to aging[(1)]. Superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radical is normally formed first and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. Furthermore, these ROS can slow down aging and prevent Alzheimer's disease[(2)]. Note that almost all organisms are able to defend antioxidant and repair oxidative damage in the systems that evolved, as these systems prevent the damage completely. Antioxidants are substances that can delay or prevent oxidative cellular oxidizable substrates. Principal sources of antioxidant include herbs, spices, and medicinal plants. Natural antioxidants from dietary plants are reported to prevent oxidative damage by free radical and active oxygen, and they also prevent the occurrence of disease, aging, and cancer[(3)].

Beefsteak plant is an annual herbaceous plant, belonging to the family Labiatae, commonly known as perilla (*Perilla frutescens*). Its leaves are often used in a variety of Asian foods to add both flavor and color. It is also grown ornamentally in gardens. In general, the stems, leaves, and seeds of beefsteak plant are used individually in Chinese medicine to treat a variety of diseases[(4)]. The stem of the plant is traditionally used as an analgesic and anti-abortive agent. The leaves are said to be helpful for asthma, colds and flus, and regulate stomach function. On the other hand, the seeds are employed for dyspnea and cough relief, phlegm elimination, and bowel relaxation[(4)]. Considerable attention has been given to the anti-inflammatory, anti-allergic and anti-tumor promoting substances contained in beefsteak plants[(5-8)]. Recently, extracts of beefsteak plants have been studied as a treatment for allergic rhinitis. Beefsteak plants contain high level of rosmarinic acid which appears to have anti-inflammatory and anti-allergic actions[(9-12)]. Although animal studies hint that beefsteak plants might also be useful for a different

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type of allergy, such as severe, rapid reaction known as anaphylaxis, commonly associated with shellfish, peanut, and bee-sting allergies[6,11], there are only a few reports on antioxidant activities of leaves[13,14] and seeds[15,16] from beefsteak plant. Due to the increasing interest in the relationship between antioxidants and diseases, there is a need to get an overall measure of the antioxidant activity of extracts from beefsteak plant.

In this study, we evaluated the possible antioxidant effects of water extracts from different parts of beefsteak plant stems (BS), leaves (BL), and ripe seeds (BR) in different *in vitro* antioxidant tests including superoxide anion radical and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging, reducing power, metal chelating activities, total phenolic compounds and flavonoids. This work has examined the influence of beefsteak plant on human beings and provides an important background for further study.

MATERIALS AND METHODS

I. *Chemicals*

Ascorbic acid and ferric chloride were purchased from Fluka (Switzerland). Gallic acid, potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

II. *Preparation of the Extract*

The beefsteak plant stems (BS), leaves (BL), and ripe seeds (BR) were purchased locally (Goangder Tarng Ginseng Co., Taoyuan, Taiwan). The dried beefsteak plants were ground in a mortar, and extracted twice using 50 mL of distilled water (70°C) under reflux for 4 h. The supernatant was separated from the solid residue by paper filtration. The extracts were combined and evaporated at 60°C under reduced pressure. All dried extracts were stored at 4°C until use.

III. *Superoxide Anion Radical Assay*

Measurement of superoxide anion radical scavenging ability of the extracts was done based on the method described by Lin *et al*.[17] with slight modification. The reaction mixture contained the same volume of 120 μM PMS, 936 μM NADH, water extract, and 300 μM NBT in a total volume of 1 mL of phosphate buffer (100 mM, pH 7.4). After 5 min of incubation at ambient temperature, absorbance of the resulting solution was measured at 560 nm. The superoxide anion radical activity was calculated as scavenging effect (%) = (1 - absorbance of sample/absorbance of control) × 100.

IV. *DPPH Radical Assay*

Scavenging effect on DPPH radical was measured by the method of Lin *et al*.[17]. Briefly, 0.1 mL of a 1 mM methanol solution of DPPH was incubated with varying concentrations of the water extract. After a 30 min incubation period at room temperature, absorbance of the resulting solution was read at 517 nm. The 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity was expressed as the inhibition percentage calculated as (1 - absorbance of sample/absorbance of control) × 100.

V. *Reducing Power Assay*

The reducing power was determined according to the method of Lin *et al*.[17]. The water extract (0.25 mL) was mixed with 0.25 mL of 200 mM sodium phosphate buffer (pH 6.6) and 0.25 mL of 1% potassium ferricyanide. Then the mixture was incubated at 50°C for 20 min. After 0.25 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (0.5 mL) was mixed with 0.4 mL of deionized water and 0.1 mL of 0.1% ferric chloride solution, allowed to stand for 10 min, and the absorbance was measured at 700 nm. Higher absorbance indicated higher reducing power.

VI. *Chelating Effect on Ferrous Ions*

The chelating effect of ferrous ions was estimated by the method of Lin *et al*.[17]. Briefly, the water extracts were added to a solution of 2 mM $FeCl_2$ (0.02 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.04 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the mixture was measured at 562 nm. Chelating effect was calculated using the equation: (1 - absorbance of sample/ absorbance of control) × 100.

VII. *Determination of Antioxidant Contents*

Contents of total phenolic compounds in the extracts were estimated by a colorimetric assay based on procedures described by Slinkard and Singleton[18] with some modifications. Basically, water extract solution (0.05 mL) was mixed with 0.05 mL of Folin-Ciocalteu's phenol reagent. Then, 0.5 mL of a 15% sodium carbonate solution was added to the mixture and then it was adjusted to 1 mL with 0.4 mL of distilled water. The reaction was allowed to stand for 10 min with intermittent shaking, after which the absorbance was read at 725 nm. Gallic acid was used for constructing the standard curve (5-35 μg/mL; $y = 0.0964x - 0.0519$; $R^2 = 0.9856$) and the results

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were expressed as μg of gallic acid equivalents per mL of extract.

Flavonoid contents in the extracts were determined by a colorimetric method described by Kim *et al*.[19] with some modifications. The water extract (0.05 mL) was mixed with 0.4 mL of distilled water and 0.02 mL of a 7.5% sodium nitrite solution, followed by 15% aluminum chloride solution (0.02 mL). After 6 min, 0.2 mL of 1 M sodium hydroxide and 1 mL of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. Rutin was used for constructing the standard curve (10-100 μg/mL; $y = 0.0118x - 0.0108$; $R^2 = 0.9989$) and the results were expressed as μg of rutin equivalents per mL of extract.

VIII. *Statistical Analysis*

All data were presented as mean ± standard deviation (S.D.) of triplicate parallel measurements. Statistical analysis was performed using Student's t-test, and p value < 0.05 was regarded as significant and p value < 0.01 very significant.

RESULTS AND DISCUSSION

Many studies have shown that natural antioxidants are closely related with their biofunctionalities, such as the reduction of chronic diseases like mutagenesis, carcinogenesis, and DNA damage, etc. Natural oxidants are also related to inhibition of pathogenic bacteria growth, which are often associated with the termination of free radical propagation in biological systems[20,21]. Thus, antioxidant capacity is widely used as a parameter to characterize food or medicinal plants and their bioactive components. Gallic acid is a biological lipid antioxidant that prevents the formation of free radicals from lipid peroxidation and has been shown to be an antimutagen or anticarcinogen in *Salmonella* tester strains[22] as well as in human leucocytes *in vitro*[23]. In the present study, the antioxidant activities of beefsteak plant were evaluated in a series of *in vitro* test: superoxide anion radical and DPPH radical scavenging, reducing power, metal chelating activities, and total phenolic compounds and flavonoids.

I. *Superoxide Anion Radical Scavenging Activity*

Superoxide anion, a reduced form of molecular oxygen, has been implicated in the initiating oxidation reactions associated with aging[24]. Also, it has been implicated in several pathophysiological processes, due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Superoxide anions play an important role in formation of other reactive oxygen species such as singlet oxygen, hydrogen peroxide, and hydroxyl radical, which induce oxidative damage in DNA, lipids, and proteins[25,26]. Also, superoxide anion is an oxygen-centered radical with selective reactivity. These species are produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome c. In the present study, all extracts from different parts of beefsteak plant effectively scavenged superoxide anion radical in a concentration-dependent manner (Figure 1). These results showed BS and BL had the highest activities upon the elimination of superoxide anion radical than that of ascorbic acid ($p < 0.05$), but the scavenging activity of BR was the same as that of gallic acid. Scavenging effect of water extracts from different parts of beefsteak plant and standards (ascorbic acid and gallic acid) on the superoxide anion radical decreased in the following order: BS > BL > BR > gallic acid > ascorbic acid, with 61.8%, 60.8%, 33.3%, 28.5% and 19.5%, at the concentration of 5 μg/mL, respectively. These results clearly suggested that antioxidant activity of the extracts from different parts of beefsteak plant were also related to their ability to scavenge superoxide anion radical. The result was in agreement with that reported by Nakamura *et al*.[13], who demonstrated that the scavenging activity of rosmarinic acid from the leaves of perilla was greater than that of ascorbic acid or other phenolcarboxylic acids and rosmarinic acid effectively exhibited antioxidative activity in the biological systems through the scavenging of O_2^-, one of the precursors of ROS. Additionally, Robak and Gryglewski[27] reported that antioxidant properties of some flavonoids are effective mainly via the scavenging of superoxide anion radical.

II. *Scavenging Effect on DPPH Radical*

Antioxidant properties, especially radical-scavenging activities, are very important due to the

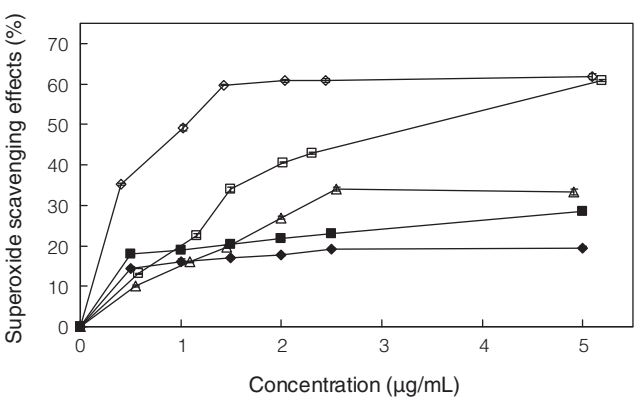

Figure 1. Scavenging effect of water extracts from beefsteak plant on superoxide anion radical. Each value is expressed as mean ± standard deviation (n = 3). (◇, BS; □, BL; △, BR; ◆, ascorbic acid; ■, gallic acid).

deleterious role of free radicals in foods and biological systems. The reduction of DPPH absorption is indicative of the capacity of the extracts to scavenge free radicals, independently of any enzymatic activity. The method widely used to predict the ability of flavonoids to transfer H atoms to radicals is based on the free radical, 1,1-diphenyl-2-picrylhydrazyl in the DPPH assay. The antioxidants were able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine. Figure 2 illustrates the DPPH radical scavenging ability of different parts of beefsteak plant and standards. Ascorbic acid and gallic acid were used as reference radical scavengers. The extract of BS showed excellent DPPH radical scavenging activity that was enhanced with increasing concentration. Scavenging effects of the extract of beefsteak plant and standards on the DPPH radical decreased in the order: BS > gallic acid > ascorbic acid, i.e. 54.8%, 47.2% and 34.0%, at the concentration of 10 μg/mL, respectively. However, the DPPH radical scavenging activities of BL and, BR were low. Some investigators[28] have reported that the change of the content of the flavonoids and phenolic acids in the leaves and seeds of *P. frutescens* L. at different growth stages were determined. In addition, Yamamoto *et al*.[29] have studied that fresh leaves are a more useful source of phenolic compound because the quantity of rosmarinic acid in fresh leaves is 10 times larger than that in the seeds.

III. *Reducing Power*

The antioxidant activities of natural components might have a reciprocal correlation with their reducing powers. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant sample. Reducing power of a compound served as a significant indicator of its potential antioxidant activity. Reducing powers of water extracts from different parts of beefsteak plant, ascorbic acid and gallic acid were enhanced by increasing concentration of samples (Figure 3). Specifically, reducing powers of water extracts of BS were higher than those of BR and BL at 2.5 - 62.5 μg/mL. As seen in Figure 3, reducing powers of the extracts from different parts of beefsteak plant were in the following order: BS (1.96) > BL (1.69) > BR (1.49) at the concentration of 37.5 μg/mL. On the other hand, reducing powers of ascorbic acid and gallic acid were 1.97 and 1.96, respectively. Reducing powers of the extracts from different parts of beefsteak plant and standards were similar statistically. The water extracts of BS, BL and BR showed significant effects on the reducing capacity at all amounts. Reducing power of a compound might serve as a significant indicator of its potential antioxidant activity[30].

The antioxidant activity has been reported to be concomitant with the development of reducing ability[31]. Therefore, the antioxidant activity of the extracts might partially be a result of its reducing ability. Okuda *et al*.[32] mentioned that the reducing ability of tannins prevented liver injury by inhibiting the formation of lipid peroxides. Furthermore, reductones such as ascorbic acid can react directly with peroxides and also with certain

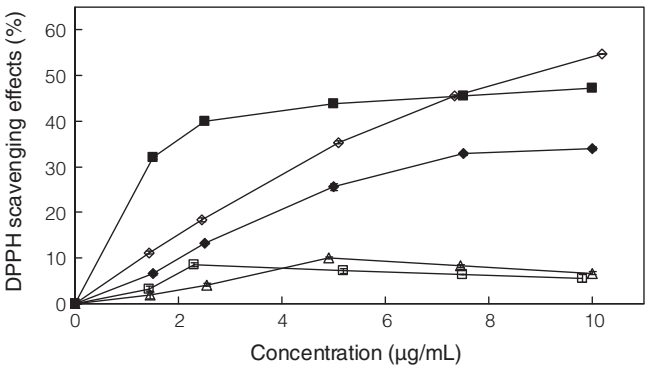

Figure 2. Scavenging effect of water extracts from beefsteak plant on DPPH radical. Each value is expressed as mean ± standard deviation (n = 3). (◇, BS; □, BL; △, BR; ◆, ascorbic acid; ■, gallic acid).

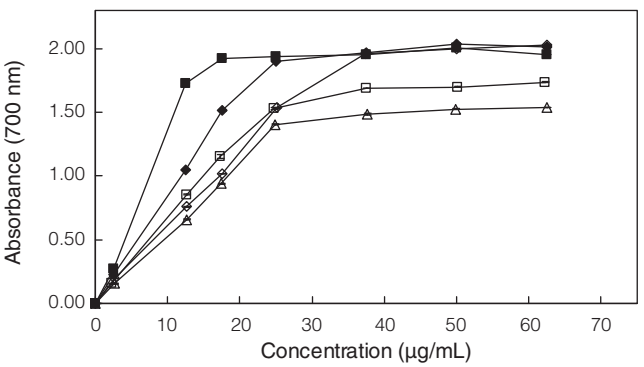

Figure 3. Reducing power of water extracts from beefsteak plant. Each value is expressed as mean ± standard deviation (n = 3). (◇, BS; □, BL; △, BR; ◆, ascorbic acid; ■, gallic acid).

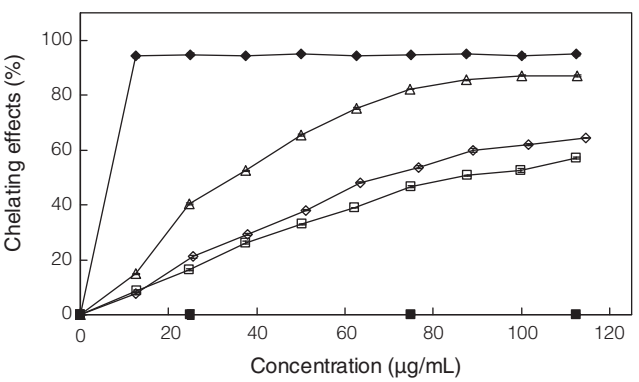

Figure 4. Chelating effect on ferrous ions of water extracts from beefsteak plant on ferrous ion. Each value is expressed as mean ± standard deviation (n = 3). (◇, BS; □, BL; △, BR; ◆, EDTA; ■, citric acid).

precursors, thereby prevent peroxide formation. The reducing ability of various extracts might be due to its hydrogen-donating capacity, as described by Shimada *et al.*[33]. Therefore, the extracts might contain reductones, which could react with free radicals to stabilize and terminate radical chain reactions.

IV. *Chelating Effect on Ferrous Ions*

Ferrozine could quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation was disrupted as the red color of the complex is decreased. Measurement of the rate of color reduction therefore allowed estimation of the chelating effect of the coexisting cheater. Figure 4 shows the chelating effects of the BS, BL and BR extracts on ferrous ions. The percentages of chelating activities increased as water extracts concentration increased. Similarly, absorbance of Fe^{2+}-ferrozine complex decreased linearly based on the concentration (from 12.5 to 75 μg/mL). Specifically, the BR extract showed a sharp increase in scavenging ability in a concentration-dependent manner. The difference among all beefsteak plant concentrations and control values were statistically significant ($p < 0.01$). In addition, the BR extracts exhibited 82.2% chelation of ferrous ion at 75 μg/mL concentration. On the other hand, the percentages of chelating capacity of the extracts of BS and BL were 53.7% and 46.6%, respectively. Both BS and BL extracts showed moderate ferrous ion chelating ability that reached 57.1 - 64.5% at 112.5 μg/mL. The chelating effects of beefsteak plant extract were in the order of BR > BL ≈ BS. However, EDTA showed an excellent chelating ability of 94.2% at 12.5 μg/mL, while citric acid was not a good chelating agent for ferrous ions. Chelating effect on ferrous ions of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Chelating ability was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation[34]. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential thereby stabilize oxidized form of the metal ion[35]. Since ferrous ions were the most effective pro-oxidants in food system[36], the moderate to high ferrous-ion chelating abilities of various extracts from beefsteak plant would be beneficial. These assays were used to establish the abilities of water extracts from different parts of beefsteak plant to chelate and had important applications for the pharmaceutical and food industries.

V. *Amount of Total Phenolic Compounds and Flavonoids*

Although the biological activity and superior safety of beefsteak plant are well documented, few reports on the quantitative presence of total phenolic and flavonoid in beefsteak plant are available[14,28]. As shown in Table 1, the extract of BS was found to have the highest phenolic content [594.0 ± 4.7 μg (gallic acid equivalents) / mL (extract)] among all beefsteak plant extracts evaluated, followed by 576.7 ± 2.1 μg/mL and 515.8 ± 1.6 μg/mL of BL and BR, respectively. On the other hand, total flavonoid content in the extract of BS was also found to be superior to the other beefsteak plant extracts [939.5 ± 10.2 μg (rutin equivalents) / mL (extract)]. The lowest flavonoid content was exhibited in the water extract of BR (133.1 ± 10.0 μg/mL). Polyphenolic compounds played an important role in stabilizing lipid oxidation associated with its antioxidant activity[37,38]. The total flavonoid concentrations equivalents were used for the measurements of water extract concentrations. Based on this study, we proposed that the potent free radical-scavenging and antioxidative activity of beefsteak plant might result from its high contents of phenolic and flavonoid type compounds. This result was in agreement with the report by Meng *et al.*[14], who demonstrated high correlation between *o*-dihydroxylated polyphenolic compounds and antioxidant activity. In contrast, the main phenolic compound has been proven to be rosmarinic acid. There are small amounts of flavonoids and phenolic acids such as catechin, apigenin, luteolin, caffeic acid, and ferulic acid found in the leaves and seeds of beefsteak plant[28,39-41]. In general, medicinal plants of the same scientific name differ in composition of effective components and their contents, depending on its origin and growth conditions[28]. In addition, the extraction method is another major factor to determine the composition of effective components and their contents in the resulting plant extract. Flavonoids have

Table 1. Contents of total phenolic compounds and flavonoids in water extracts from beefsteak plant

Extract	Total phenolic compounds [gallic acid equivalents (μg/mL)]	Total flavonoids [rutin equivalents (μg/mL)]
BS	594.0 ± 4.7[a]	939.5 ± 10.2
BL	576.7 ± 2.1	927.0 ± 11.7
BR	515.8 ± 1.6	133.1 ± 10.0

[a]Data expressed as mean ± standard deviation of three samples analyzed separately.

been proven to display a wide range of pharmacological and biochemical actions, such as antimicrobial, antithrombotic, antimutagenic and anticarcinogenic activities [42,43]. In food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides. Therefore, flavonoids show antioxidative efficiency in oils, fats and emulsions[44-47].

CONCLUSIONS

The different parts of beefsteak plant are used individually in Chinese medicine to treat a variety of diseases. Compared to leaf and ripe seeds, little is known about the antioxidant activities of stems. Our results clearly showed that the extract of stems (BS) had strong superoxide anion radical and DPPH radical scavenging activities as compared to the water-soluble natural antioxidants (e.g. ascorbic acid and gallic acid). In addition, the potent antioxidative activity of beefsteak plant might result from its high contents of polyphenolic compounds. The water extract from different parts of beefsteak plant, as an easily accessible source of natural antioxidant, could be used as a health-care food supplement and in the pharmaceutical industry. However, the antioxidant constituents in the extracts of beefsteak plant, especially stems extract (BS), need to be further evaluated.

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