Original article

Evaluation of antityrosinase and antioxidant activities of *Raphanus sativus* root: comparison between freeze-dried juice and methanolic extract

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Abstract:

Two types of extracts (freeze-dried juice and methanolic extract) from the root of Thai radish *(Raphanus sativus L.)* were evaluated for antityrosinase and antioxidant activities to determine their potential as a skin-whitening and anti-aging agent in cosmetic applications. The contents of total phenolics, total flavonoids and L-ascorbic acid (as per 1 mg of the dried extract) were found to be 10.09, 0.51 and 24.11 µg for the freeze-dried juice and 6.59, 0.33 and 8.28 µg for the methanolic extract, respectively. The freeze-dried juice showed higher potency of tyrosinase inhibition (IC_{50} = 3.09 mg/ml) than the methanolic extract (IC_{50} = 9.62 mg/ml). Also, the scavenging effects of the freeze-dried juice on DPPH radical, superoxide anion radical and singlet oxygen were greater than the methanolic extract, with the respective IC_{50} values of 0.64, 4.20 and 1.42 mg/ml for the freeze-dried juice and 1.25, 6.28 and 2.40 mg/ml for the methanolic extract. The higher contents of phenolic compounds and L-ascorbic acid in the freeze-dried juice appeared to be responsible for its greater antityrosinase and antioxidant activities. However, the activities of both extracts were much less than that of the reference antityrosinase agent (purified licorice extract) and the pure antioxidants (L-ascorbic acid and Trolox©) used as positive controls. Measurements of LDH leakage from fibroblast cells indicated that both extracts exhibited only mild cytotoxicity. Thus, provided that a more refined extraction process is developed with further evaluation, the extract of *R. sativus* root appeared to be a good candidate for application as a natural skin whitening/skin anti-aging agent due to its abilities to inhibit tyrosinase and scavenge several types of reactive oxygen species.

Keywords: Antityrosinase; Antioxidant; Freeze-dried juice; Methanolic extract; *Raphanus sativus* root

Introduction

Over the past few years, increasing attention has been paid to herbal plants for developing into modern medicine and cosmetic products. The skin whitening and anti-aging products containing natural ingredients from plants have become very popular due to their mild effects on the skin, which make them suitable for long term application.

Radish (*Raphanus sativus* L., family Cruciferae) is grown mainly for its edible, fleshy root. The root can be eaten raw, cooked or preserved in salt. Its seedlings, known as radish sprouts, are also used as vegetables [1]. In East Asia, the crushed root is used as a poultice to treat rheumatic pain, burns or bruises. Its squeezed juice is taken as a remedy for cough and diarrhea [2].

Apart from polysaccharides, proteins and vitamin C, the fresh radish roots contain many phenolic compounds such as kempferol, cyanidin, gentisic acid, hydrocinnamic acid, vanillic acid, pelargonidin, luteolin, myricetin and quercetin [3-7]. The aqueous extract of radish roots and leaves exhibited antimutagenic and antimicrobial activities *in vitro* [5,8]. The pungent principle extracted from radish root is *trans*-4-methylthio-3-butenyl-isothiocyanate which also possesses antimicrobial [9] and antimutagenic activities [10]. The root extract has also been shown to protect against paracetamol-induced lipid peroxidation and hepatotoxicity in albino rats [11,12].

However, its many other beneficial properties, especially for cosmetic and dermatological applications, are not widely known or studied. Traditionally, Thai women have used slices of fresh white radish roots for the treatment of melasma but there has not been any systematic study to support this evidence such as antityrosinase evaluation. One study recently reported that the methanolic extract of the radish sprout exhibited hydroxyl radical scavenging potency 1.8-fold higher than that of L-ascorbic acid [13]. It is suggested that flavonoids, together with sinapinic acid esters, may significantly contribute to the antioxidant activity of radish roots and sprouts [13,14]. Also, black radish, which is a variety of *R. sativus,* possesses antioxidant and free radical scavenging properties [15,16]. It is very likely that the Thai radish roots may possess significant antityrosinase and antioxidant activities which will have beneficial effects on the skin.

Therefore, the objectives of this study were to investigate the *in vitro* antityrosinase and antioxidant activities of the fresh white radish roots. Two different types of *R. sativus* L. root extracts, i.e., the freeze-dried juice and the methanolic extract were evaluated for their inhibitory effect on mushroom tyrosinase and their scavenging activity on DPPH, superoxide anion and singlet oxygen. The results were compared with that of well-known whitening agent and antioxidant like licorice extract, L-ascorbic acid and Trolox©. In addition, the chemical contents of the two extracts in terms of total phenolic compounds, total flavonoids and L-ascorbic acid were determined to find correlation with their antityrosinase and antioxidant activities. Cytotoxicity of the two extracts was also investigated using LDH assay in normal human fibroblast cell cultures. The data obtained would provide information regarding the feasibility of developing the radish root extract as a source of cosmetic ingredient with a skin-whitening and/or anti-aging property.

Materials and Methods

Materials

All fresh radish roots (*Raphanus sativus* L.) were purchased from a local market (Phak Doctor© brand). Folin-Denis reagent and L-3,4-dihydroxyphenylalanine (L-DOPA) were from Fluka Chemie, Germany. Sinapic acid, quercetin dihydrate, mushroom tyrosinase, 2,2-diphenyl-1-picryl-hydrazyl stable radical (DPPH) and lactate dehydrogenase toxicology assay kit (Tox-7) were from Sigma-Aldrich, USA. 6-Hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid (Trolox©), L-ascorbic acid and licorice extract (PT-40) were from Calbiochem (USA), DSM Nutritional Product (Switzerland) and Maruzen Pharmaceutical (Japan), respectively. Other chemicals and reagents were analytical grade and used as received.

Preparation of plant extracts

Freeze-dried juice: Fresh roots of *R. sativus* (1 kg) were squeezed. The juice was filtered through No. 1 Whatman paper and lyophilized using Dura-Dry II MP lyophilizer (FTS Systems, USA.)

Methanolic extract: The method was modified from Yoshiaki *et al.* [17]. Fresh roots of *R. sativus* (2.4 kg) were cut into small pieces and macerated three times with methanol for 7 days at room temperature. The resultant methanol solution was evaporated using a rotary evaporator at 35 °C. The residual water in the extract was further removed by lyophilization to obtain dried powder. Both extracts were kept in a well-closed container with silica gel at -20 °C for further studies.

Determination of total phenolic content

The total phenolic content of the extracts was determined using Folin-Denis' method as modified by Swin and Hills [18] and expressed as µg of sinapic acid equivalent per mg of dried sample. All measurements were done in triplicate.

Determination of total flavonoid content

The total flavonoid content in the extract was analyzed by colorimetry [19] and expressed as µg of quercetin equivalent per mg of dried sample. All measurements were done in triplicate.

Determination of L-ascorbic acid content

The L-ascorbic acid or vitamin C content in the extracts was determined by direct titration with 0.1N iodine, previously standardized with arsenic trioxide, using starch T.S. as indicator [20]. Five replicates of each extract were titrated.

Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined by DOPAchrome method using L-DOPA as a substrate [21]. The test samples consisted of extract solutions at various concentrations in 20% v/v propylene glycol in water whereas L-ascorbic acid and licorice extract were used as reference tyrosinase inhibitors. All tests were performed in triplicate.

Determination of the scavenging effect on DPPH, superoxide anion and singlet oxygen

Various concentrations (n = 3) of each of the *R. sativus* extract were tested for the ability to scavenge DPPH [22], superoxide anion [23] and singlet oxygen [24]. The results (calculated as average IC_{50}) were compared to that of L-ascorbic acid and Trolox©, the two most common reference antioxidants.

Cytotoxicity of Raphanus sativus L. extracts

The cytotoxic effect of the two extracts was tested in fibroblasts by measuring the activity of lactate dehydrogenase (LDH), an intracellular enzyme which leaks into the environment upon damage of the cell membrane. LDH catalyzes the reduction of nicotinamide adenine dinucleotide (NAD) to NADH, which reacts with yellow tetrazolium dye to give red formazan [25]. Briefly, normal human fibroblasts were first seeded in 96-well plates at 5 x 10^3 cells per well in 200 µl DMEM medium and incubated for 48 hr at 37 °C under 5% CO_2. For total (100%) LDH release, the cultures were removed from the incubator and 20 µl of LDH assay lysis solution was added to each well (no test extract). The plate was shaken and returned to the incubator for 45 min to allow for precipitation. The supernatant was subsequently analyzed for the LDH content according to the procedure described in the TOX-7 cytotoxicity assay kit. To evaluate the effect of *R. sativus* extracts, after 48 hr-seeding, 200 µl of various concentrations of the extracts in DMEM was added to each well (n = 3 wells per concentration) and the cells were incubated for another 24 hr followed by similar LDH analysis. The extent of LDH release was calculated as the ratio of the absorbance at 490 nm of the extract-treated cells to that of the untreated, 100% lysed cells.

Results and Discussion

Determination of total phenolic, flavonoid and L-ascorbic acid contents

The yields of the dried aqueous and methanolic *R. sativus* root extracts were 4.21 and 2.59% w/w, respectively, based on the weight of the fresh radish root. The freeze-dried juice was a pale yellow powder whereas the methanolic extract had a brown powder appearance. The two extracts were subsequently analyzed for the contents of total phenolics, total flavonoids and L-ascorbic acid. The results are given in Table 1. The amount of total phenolics was expressed as μg of sinapic acid equivalent per milligram dried extract. The assay is based on a redox reaction in which the phenolate ion of the phenolic compounds becomes oxidized whilst the phosphomolybdic-phosphotungstic acid (Folin-Denis) reagent is reduced in the presence of an alkali (sodium bicarbonate) turning into a blue-colored solution. The total phenolic content in the extract sample was obtained from the standard calibration curve of sinapic acid using linear regression equation. The amount of total phenolics in the freeze-dried juice and the methanolic extract was found to be 10.09 ± 0.07 μg and 6.59 ± 0.05 μg of sinapic acid equivalent per 1 mg sample, respectively.

The content of total flavonoids in the two extracts was also analyzed by colorimetry based on the principle that aluminium chloride forms acid stable complexes with C-4 keto group and either the C-3 or the C-5 hydroxyl group of flavones and flavonols. Aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the flavonoid rings [26]. From the quercetin standard curve, the amount of total flavonoids in the freeze-dried juice and the methanolic extract was found to be 0.51 ± 0.007 μg and 0.33 ± 0.004 μg of quercetin equivalent per 1 mg of the dried sample, respectively.

Phenolic compounds or polyphenols are a group of chemicals which are ubiquitous in plants. Their basic skeleton is an aromatic ring bearing one or more hydroxyl substituents. They are secondary plant metabolites having a wide range of structure ranging from simple molecules such as phenolic acids, stilbenes and flavonoids to highly polymerized compounds such as lignins, melanins and tannins. Polyphenols exhibit a wide range of biological effects including antibacterial, anti-inflammatory, anti-allergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions, many of which have been attributed to their reactive species scavenging and antioxidant activity [26].

Among the low molecular weight polyphenols, flavonoids are the most common and widely distributed in plants [27]. Their basic structure is C6-C3-C6, with the two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. Depending on the structural variations within these rings as well as the number and nature of the substitute groups, flavonoids can be subdivided into flavonols (e.g. quercetin and kaempferol), flavones (e.g. apigenin and luteolin), flavanols (e.g. catechins), isoflavones (e.g. genistein), flavanones (e.g. naringenin) and anthocyanidins (e.g. pelargonidin and cyanidin) [27,28]. Many of these flavonoids such as catechins had been shown to be powerful antioxidants that can directly scavenge several free radicals like super oxide anion [29]. Therefore, quantitation of both the phenolic and flavonoid contents in the plant extracts is a key indicator of their antioxidative properties and potential use in health-related products.

Data in Table 1 reveal that the contents of both total phenolics and total flavonoids in the methanolic extract were less than the freeze-dried juice. This was due to the fact that the latter was a juice squeezed from the fresh root of *R. sativus* in which all the aqueous phenolic and flavonoid contents were expected to remain in the sample whereas the former contained only

Table 1 Total phenolic, flavonoid and L-ascorbic acid contents in *R. sativus* root extracts

1 mg of sample	Total phenolic content (μg of sinapic acid equivalent)	Total flavonoid content (μg of quercetin equivalent)	L-ascorbic acid content (μg)
Freeze-dried juice	10.09 ± 0.07	0.51 ± 0.007	24.11 ± 0.01
Methanolic extract	6.59 ± 0.05	0.33 ± 0.004	8.28 ± 0.20

substances that were extractable by methanol. Only about 65% w/w of either the phenolic or flavonoid compounds was found in methanolic extract as compared to the freeze-dried juice. However, the individual compositions in each extract might be different because the methanolic extract contained substances that were extracted from both the juice and the solid part of the root. It should also be noted that the content of total flavonoids was about 5% w/w of the total phenolics regardless of the extract type. This suggested that the majority of the phenolic compounds in both extracts are non-flavonoids.

Table 1 also shows the content of L-ascorbic acid in the two extracts. L-ascorbic acid is oxidized to dehydroascorbic acid upon direct titration with iodine using starch as an indicator. The L-ascorbic acid content (mean of five determinations) was 24.11 ± 0.01 and 8.28 ± 0.20 µg per 1 mg of freeze-dried juice and methanolic extract, respectively. L-ascorbic acid is a reduced form of vitamin C commonly present in fresh fruits and vegetables. It involves in wound healing and tyrosinase metabolism. It is a strong antioxidant capable of scavenging several reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, aqueous peroxyl radicals and singlet oxygen and seems to have a protective effect for many kinds of cancer [30-32]. The data indicated that the freeze-dried juice contained 3 times higher L-ascorbic acid than the methanolic extract. This could be due to the lower solubility of L-ascorbic acid in methanol compared to the aqueous juice, resulting in lower extractability. In addition, the lower contents of total phenolics, flavonoids and L-ascorbic acid in the methanolic extract might as well be a result of their decomposition after a long extraction period at a relatively high temperature.

Determination of tyrosinase inhibitory activity

Percent tyrosinase inhibition of the two *R. sativus* extracts was plotted as a function of concentration in comparison with L-ascorbic acid and licorice extract as shown in Figure 1. The two extracts inhibited tyrosinase activity in a concentration-dependent manner and the effect reached plateau at some concentration, indicating a nearly complete enzyme inhibition similar to the reference inhibitors.

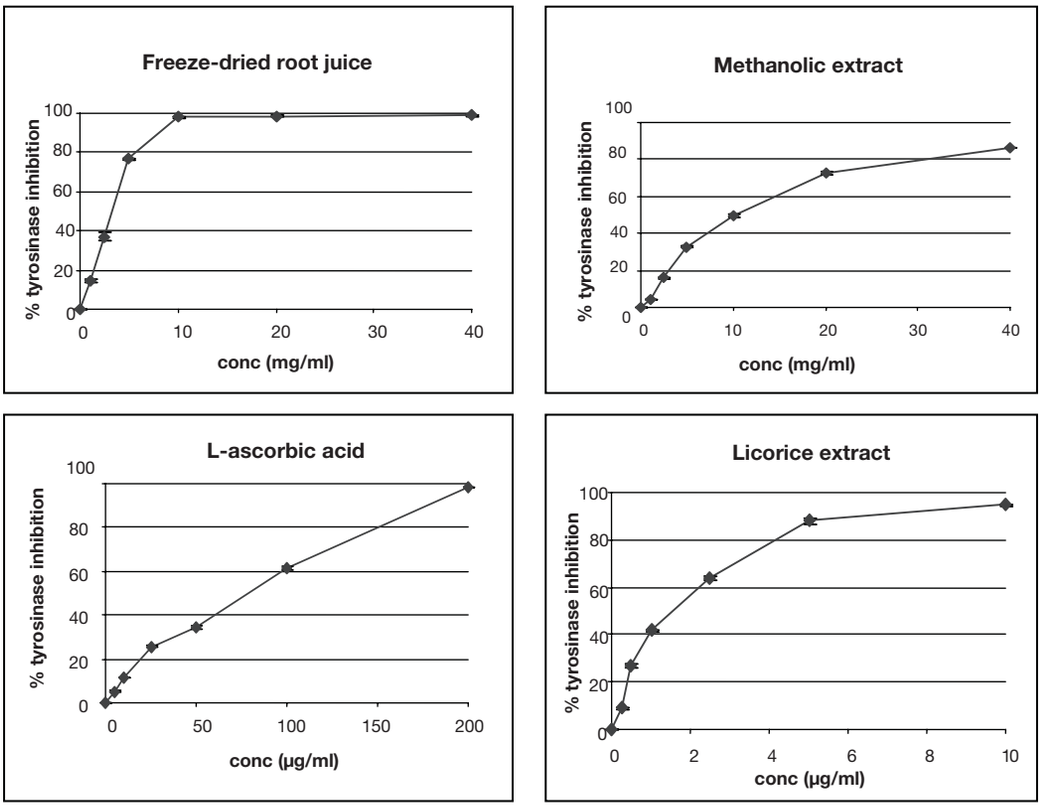

Figure 1 The relationship between % tyrosinase inhibition and the concentration of freeze-dried root juice, methanolic extract and reference tyrosinase inhibitors, L-ascorbic acid and licorice extract (mean ± SD, n = 3)

The concentration at which 50% inhibition of enzyme activity occurred (IC_{50}) was determined for each extract from these plots and shown in Table 2. The IC_{50} of the freeze-dried juice was 3-times smaller than the methanolic extract (3.09 mg/ml versus 9.62 mg/ml), indicating that the dried juice was a stronger tyrosinase inhibitor than the methanolic extract. However, both extracts still exhibited much lower antityrosinase activity than L-ascorbic acid (IC_{50} 73.57 μg/ml) and licorice extract (IC_{50} 1.23 μg/ml), which are potent tyrosinase inhibitors commonly used in skin-whitening cosmetic products [33].

By multiplying the IC_{50} value of each extract (3.09 mg/ml for the freeze-dried juice and 9.62 mg/ml for the methanolic extract) with their corresponding value of L-ascorbic acid content (24.11 and 8.28 μg per 1 mg extract), the new IC_{50} value of the two extracts in terms of L-ascorbic acid equivalent was calculated to be 74.75 μg/ml and 79.66 μg/ml, respectively. These transformed IC_{50} values are nearly identical to that of pure L-ascorbic acid (73.57 μg/ml). The data thus suggested that the antityrosinase activity of the two extracts was mainly due to the presence of L-ascorbic acid.

Although licorice extract is the most potent tyrosinase inhibitor in this study, it is also extremely expensive and has to be imported. Due to its mild antityrosinase activity, the extract of the *R. sativus* root may have implication as a secondary skin whitening agent in cosmetic products provided that more studies on the *in vivo* safety and efficacy are established.

Scavenging effect on DPPH, superoxide anion and singlet oxygen

The scavenging extent of DPPH, superoxide anion radical and singlet oxygen exerted by *R. sativus* root extracts was determined at various concentrations. Both the freeze-dried juice and methanolic extract were capable of scavenging the three reactive species in a concentration dependent manner similar to antityrosinase activity (individual data not shown). The IC_{50} values (concentration of 50% scavenging) were subsequently determined and tabulated in Table 3 in comparison with that of Trolox© and L-ascorbic acid, which were used as the reference antioxidants.

Comparison of the IC_{50} values between the freeze-dried root juice and the methanolic extract revealed that the former was more active in scavenging the three species than the latter. This was in line with the greater contents of total phenolic compounds and L-ascorbic acid found in the root juice since these

Table 2 Inhibitory effect of *R. sativus* root extracts on tyrosinase activity, expressed as concentration of 50% inhibition (IC_{50}), in comparison with L-ascorbic acid (mean ± S.D., n = 3)

Sample	IC_{50} (mean ± S.D.)
Freeze-dried root juice	3.09 ± 0.05 mg/ml
Methanolic extract	9.62 ± 0.08 mg/ml
Licorice extract	1.23 ± 0.02 μg/ml
L-ascorbic acid	73.57 ± 1.26 μg/ml

Table 3 Scavenging effect of radish root extracts, expressed as concentration of 50% inhibition (IC_{50}), on DPPH, superoxide anion and singlet oxygen (mean ± S.D., n = 3)

Sample	DPPH	Superoxide anion	Singlet oxygen
Freeze-dried root juice	0.64 ± 0.01 mg/ml	4.20 ± 0.14 mg/ml	1.42 ± 0.04 mg/ml
Methanolic extract	1.25 ± 0.01 mg/ml	6.28 ± 0.11 mg/ml	2.40 ± 0.02 mg/ml
Trolox®	6.92 ± 0.29 μg/ml	52.08 ± 0.32 μg/ml	79.59 ± 0.66 μg/ml
L-ascorbic acid	3.60 ± 0.19 μg/ml	78.96 ± 1.04 μg/ml	86.11 ± 1.55 μg/ml

compounds are known to be effective antioxidants, the major role of which is to remove reactive oxygen species (ROS) involved in the chain initiation and propagation.

One mechanism of an antioxidant is by donating hydrogen to a free radical which is reduced to a non-reactive species. The general tendency of a compound to donate hydrogen can be determined by assessing its ability to scavenge 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable, synthetic free radical. The results obtained here clearly demonstrated that both the freeze-dried juice and the methanolic extract were capable of donating hydrogen to DPPH.

Superoxide anion is a natural ROS which is a reduced form of molecular oxygen created by receiving one electron. It is an initial free radical formed from mitochondrial electron transport systems and plays an important role in the formation of other ROS [34]. Many of its toxic effects on cells and DNA can be attributed to its metal-catalyzed interaction with hydrogen peroxide to produce hydroxyl radical, the most highly reactive free radical that can react with most biomolecules [35]. Singlet oxygen ($^1O_2$), on the other hand, is not a free radical (no unpaired electrons) but is also highly reactive due to its photo-excited state. It can be obtained by irradiation of normal oxygen in the presence of a photosensitizer such as porphyrin [36]. Singlet oxygen reacts with several compounds containing carbon-carbon bond such as polyunsaturated fatty acids in membranes. Production of $^1O_2$ has been shown to cause lipid peroxidation, collagen cross-linking and matrix metalloproteinase production in human dermal fibroblast [37,38]. Thus, compounds capable of scavenging these reactive species may have implications in preventing oxidative stress-induced cellular damages and may possess cosmeceutical benefits.

It is also interesting to note that the IC_{50} values of the two extracts were much higher than the pure antioxidants (Trolox® and L-ascorbic acid). This indicated that the root of *R. sativus* exhibited weaker antioxidant/ROS-scavenging activities than the two vitamins. It is possible that not all the phenolic compounds present in the two extracts possessed antioxidant activity. The presence of relatively small contents of total flavonoids (5% of the total phenolics) may also be another reason responsible for the moderate antioxidant activity of the two extracts. Further refinement of the extraction process appeared to be necessary in order to obtain a more concentrated or purified extract with extensive clarification of its chemical composition. The data obtained here thus suggested that the *R. sativus* root extracts were capable of inhibiting tyrosinase enzyme activity and scavenging several types of ROS. The two extracts also maintained their antioxidant activities for at least 3 months during storage at -20 °C based on the reassessment of their IC_{50} on DPPH scavenging, which was unchanged.

Cytotoxicity of Raphanus sativus extracts in human dermal fibroblast cells

Table 4 shows the extent of lactate dehydrogenase (LDH) release from normal human fibroblasts after 24-hr exposure to various concentrations of *R. sativus* juice and methanolic extract. At 0.25 mg/ml and below, both extracts did not cause any detectable release of LDH into the cytoplasm whereas at 0.50 mg/ml, a small amount of LDH was detected (0.36 - 0.73%). As the concentration was further increased, the extent of LDH release also increased, reaching 5.0 and 3.0% at 10 mg/ml for freeze-dried juice and methanolic extract, respectively. For comparison, the cytotoxicity of pure antioxidants L-ascorbic acid and sinapic acid was also evaluated from 0.025 to 0.25 mg/ml concentration. Their patterns show similar concentration-dependent LDH release. However, the effect was more intense. At 0.10 mg/ml, the LDH release was 1.6 and 1.0% for L-ascorbic acid and sinapic acid, respectively, and further increased to 2.8 and 1.8% at 0.25 mg/ml. On the other hand, both *R. sativus* root extracts did not cause any LDH release during this concentration range. Thus, the freeze-dried juice and the methanolic extract prepared in this study appeared to have relatively lower cytotoxicity than the more potent, pure antioxidants like L-ascorbic acid and sinapic acid, at least in the fibroblast cell systems.

Table 4 Percentage LDH release (relative to 100% cell lysis) from normal human dermal fibroblast after 24-hr exposure to various concentrations of freeze-dried juice and methanolic extract of *R. sativus* root in comparison with L-ascorbic acid and sinapic acid (mean ± S.D., n = 3)

Concentration (mg/ml)	Freeze-dried juice (%)	Methanolic extract (%)	L-ascorbic acid (%)	Sinapic acid (%)
0.025	0.00	0.00	0.91 ± 0.16	0.36 ± 0.16
0.10	0.00	0.00	1.64 ± 0.27	1.00 ± 0.17
0.25	0.00	0.00	2.82 ± 0.42	1.82 ± 0.20
0.50	0.73 ± 0.16	0.36 ± 0.20	-	-
2.50	1.37 ± 0.28	0.64 ± 0.16	-	-
5.00	3.37 ± 0.16	1.73 ± 0.17	-	-
10.00	5.01 ± 0.64	3.01 ± 0.28	-	-

Conclusion

The results from this study revealed that the freeze-dried juice and the methanolic extract of *R. sativus* fresh root, were capable of inhibiting tyrosinase activity and scavenging DPPH, superoxide anion and singlet oxygen. The antityrosinase and antioxidant activities appeared to depend on their content of total phenolic compounds and L-ascorbic acid since the freeze-dried juice, which contained greater amount of the above components, was more active than the methanolic extract. The two extracts also exhibited mild toxicity in the dermal fibroblast system when compared to pure antioxidants L-ascorbic acid and sinapic acid. Thus, judging from the multiple activities and low toxicity, the inexpensive and easily available *R. sativus* root extract may have potential for use as an antioxidant and antityrosinase agent for cosmetic applications provided that further purification or a new extraction procedure is developed to achieve extracts with higher potencies.

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