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Effect of different extraction methods on total phenolic content and antioxidant activities of *Raphanus sativus*

Jamuna K.S.[1], Ramesh C.K.[1]*, Riaz Mahmood[2], Pallavi M.[1], Aditya Rao S.J.[1]

[1]Molecular Biomedicine Laboratory, PG Dept. of Studies and Research in Biotechnology, Sahyadri Science College, Kuvempu University, Shimoga - 577203, Karnataka, India.

[2]Postgraduate Department of Studies and Research in Biotechnology and Bioinformatics, Jnana Sahyadri, Kuvempu University, Shimoga 577451, Karnataka, India.

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Abstract: Extraction is the first and crucial step in preparation of plant formulations. The use of bioactive compounds needs the most appropriate and standard method to extract the active components from plant material. Thus standardization of the extraction process is an important step for the establishment of a consistent biological activity, a consistent chemical profile or for quality assurance in production and manufacturing of herbal drugs or any herbal formulations. Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency and wide applicability. In the present study, different extraction methods are employed in order to assess the effect on total phenolic content and antioxidant activities in *Raphanus sativus*. Among the extraction methods applied, fresh material cold extraction was found to be superior followed by soxhlet and dried material cold extraction in terms of antioxidant activities tested. The results were discussed.

Key words Antioxidant; Extraction; *In Vitro* Scavenging Activities; Phenolics; *Raphanus Sativus;*

INTRODUCTION

Screening the plant extracts for the desired bioactivity is among the most important operations in medicinal plant research and extraction is the first crucial step of the process. Extraction is the technique used for separation of therapeutically desired active constituent(s) and elimination of unwanted insoluble material by treatment with selective solvents (Anonymous, 2006 and Kothari, 2011). To have a complete idea of bioactivity of plant extracts, it becomes necessary to optimize the extraction methodology, so as to achieve maximum possible extraction efficiency (Kothari *et al.,* 2009). Present study aimed at comparing different extraction methods with respect to their ability to extract antioxidant components from *Raphanus sativus L, a* commonly consumed vegetable. *Raphanus sativus* L. (Radish) is a member of the Brassicaceae/ Cruciferae family. Epidemiological studies have shown that a diet rich in cruciferous vegetables can lower the risk of various cancers (Yuan *et al*., 1998 and Cohen *et al*., 2000). The major active compounds in cruciferous vegetables, the derivatives of glucosinolates *viz.* indole-3-carbinol, sulforaphane, isothiocyanates *etc.* exhibit promising cancer protective properties *in vitro* and *in vivo* (Wattenberg and Loub, 1978 and Cover *et al*., 1998).

Plants are sources of natural antioxidants, and some of the compounds have significant antioxidative properties and health benefits (Exarchou *et al.,* 2002, Jamuna *et al.,* 2011 and Ramesh *et al.,* 2011). The need for selection of most appropriate extraction methodology is evident from the fact that when different methods are applied on same plant material with same solvent, extraction efficiency can vary significantly. In the present investigation, different extraction methods *viz.* cold extraction involving fresh and dried plant material and soxhlet extraction were tested for their efficiency in terms of total phenolic content, total antioxidant capacity, reducing power assay and i*n vitro* free radical scavenging activities utilizing Radish root as plant material.

***Corresponding Author:**
Dr. Ramesh C.K,
P.G. Department of Studies and Research in Biotechnology,
Sahyadri Science College, Kuvempu University,
Shimoga - 577 203, Karnataka, India.

MATERIALS AND METHODS

Collection of plant material

Raphanus sativus L (Radish) of tropical India was procured from a local market of Shimoga, Karnataka and was authenticated Mr. Avinash K, Assistant Horticulture Officer, Kadur, Chikmagalur District, Karnataka, India. The collected material was processed for extraction procedures.

Preparation of plant material

After selection, vegetable was washed under running tap water followed by washing with distilled water to remove the surface debris. The leaf portion and the outer most root skin layer of *Raphanus sativus* were removed and the remaining edible root portion was used for the analysis.

Fresh material cold extraction

Exactly 150g of vegetable was weighed. Finally, the above prepared vegetable sample was chopped into small pieces using cutter and later minced using a mixer grinder and finely macerated. After homogenization, it was extracted in 70% ethanol for 7 days in dark at room temperature with intermittent shaking. After 7 days, the whole extracts are filtered using muslin cloth at first and then through Whatman No. 1 filter paper and the filtrate is concentrated in air. The yield of crude extract obtained was noted, stored in desiccators for maximum of 3 days; later preserved in deep freezer (-20^{o}C) for further use.

Dried material cold extraction

The plant material was chopped into small pieces using cutter and then spread over on a blotting paper and then dried in hot air oven at 40-50^{o}C for 48 hrs. Exactly 150g of dried vegetable was weighed and grinded into a fine powder. The powdered plants were extracted in 70% ethanol for 7 days in dark in the room temperature with intermittent shaking. Further processing was done as explained previously under fresh material cold extraction.

Soxhlet Extraction

The plant material was chopped into small pieces using cutter and then spread over on a blotting paper and then dried in hot air oven at 40-50°C for 48 hrs. Exactly 150g of dried vegetable was weighed and grinded into a fine powder. The powdered plants were extracted in 70% ethanol using soxhlet apparatus and concentrated in a rotary evaporator. The yield of crude extract obtained was noted and stored as above.

Yield calculation

The yield of the extract was calculated by using the following equation:

Percentage of extraction yield = $m_1/m_0 \times 100$

Where m_1 is mass of the extract in gram and m_0 is mass of sample in gram.

Qualitative phytochemical analysis

The qualitative phytochemical studies were performed for testing the different chemical groups present in ethanolic extracts of *Raphanus sativus* (Khandelwal, 2006).

Evaluation of in vitro antioxidant activities

General chemicals: All chemicals and solvents used in the study were of analytical grade. Folin–Ciocalteu reagent, Na_2CO_3, Catechol, H_2SO_4, sodium phosphate, ammonium molybdate, Ferric chloride, Potassium ferricyanide, TCA (Trichloro acetic acid), DPPH (1, 1-diphenyl, 2-picryl hydrazyl), sulphanilamide, naphthylethylenediamine dichloride, phosphoric acid, sodium nitroprusside, ferrous chloride, ferrozine, EDTA (Ethylene diamine tetra acetic acid) and ascorbic acid were obtained from Hi media Mumbai, India.

Total phenolic content: The total phenolic content was measured by the method of Chandler *et al.,* 1993. At different concentration ranges, extracts were prepared and made up to 5ml of distilled water and 0.5ml of 50% Folin Ciocalteu reagent. The mixture was allowed to react for 5 min and 1ml of 5% Na_2CO_3 was added. Thereafter, it was thoroughly mixed and placed in the dark for 1h and the absorbance was measured at 725nm using UV/Visible spectrophotometer. Using catechol, a standard curve was prepared. The total phenolic content was calculated and expressed as catechol equivalent in μg/mg of extract.

Total antioxidant capacity: The total antioxidant capacity was measured by spectrophotometeric method of Prieto *et al.,* 1999. At different concentration ranges, extracts were prepared and mixed with 1ml of reagent solution (0.6M H_2SO_4, 28mM sodium phosphate, 4mM ammonium molybdate mixture). The tubes were incubated for 90min at 95ºC. The mixture was cooled to room temperature and the absorbance was read at 695nm against blank. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid.

Reducing power assay: The reducing power of the extracts was evaluated according to Oyaizu, 1986. Different amounts of extracts were mixed with 2.5ml of 0.2M phosphate buffer (pH 6.6), and 2.5ml of 1% $K_3Fe(CN)_6$. This mixture was incubated at 50°C for 20 min, 2.5ml of 10% TCA was added to the blend and centrifuged at 3000rpm for 10 min. The upper layer of the solution (2.5ml) was assorted with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700nm. Increase in absorbance of the reaction mixture indicates increased reducing power.

DPPH radical scavenging activity: DPPH free radical scavenging assay was measured using DPPH free radical test, by employing the method of Wong *et al.,* 2006. The different concentrations of each of the extracts were prepared in ethanol and were added to 3ml of 0.1mM methanolic solution of DPPH. The tubes were shaken vigorously and allowed to stand for 30min at room temperature in dark. Changes in absorbance of samples were measured at 517nm. A control reading was obtained using ethanol instead of the extract. Ascorbic acid was used as the standard. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula and the results are expressed as IC_{50}, which is the amount of antioxidant necessary to decrease the initial concentration by 50%.

% Inhibition = $((A_0-A_1)/A_0 \times 100)$

Where, A_0 = absorbance of the control (without test samples) and A_1 = absorbance of test samples.

Nitric oxide radical scavenging activity: Nitric oxide radical scavenging activity was determined according to the method reported by Garrat, 1964. Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP (10mM) in phosphate buffer saline (PBS) was mixed with different concentration of extract (100-1000μg/ml) of the drug dissolved in ethanol and water and incubated at 25°C for 180 minutes. The samples from the above were reacted with Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 546nm and referred to the absorbance of ascorbic acid, used as a positive control treated in the same way with Griess reagent and the results are expressed as IC_{50}.

% Inhibition = $((A_0-A_1)/A_0 \times 100)$

Where, A_0 = absorbance of the control (without test samples) and A_1 = absorbance of test samples.

Fe^{2+} chelating activity: The chelation of ferrous ions by extracts was estimated by method of Dinis *et al.,* 1994. To 0.5ml of different concentrations of the extracts, 1.6ml of de-ionized water and 0.05ml of 2mM $FeCl_2$ was added. After 30 seconds, 0.1ml of 5 mM ferrozine was added. Ferrozine reacted with divalent iron to form stable magenta complex species that were very soluble in water. After 10 minutes at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562nm. EDTA was used as a standard metal chelating agent. The chelating activity of the extract for Fe^{2+} was calculated using the following formula and the results are expressed as IC_{50}.

% Inhibition = $((A_0-A_1)/A_0 \times 100)$

Where, A_0 = absorbance of the control (without test samples) and A_1 = absorbance of test samples.

Statistical analysis

All the experiments were carried out in triplicates. The result of the replicates was pooled and expressed as mean ± standard error (SE).

RESULTS

Extraction

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical and cosmetic products. The result revealed that soxhlet extract showed high percentage yield (19.37g) followed by fresh material cold extract (9.89g) and dried material cold extract (5.05g) for 150g of plant material.

Qualitative phytochemical analysis

The results of qualitative phytochemical analysis revealed that all the three ethanolic extracts of *Raphanus sativus* showed the presence for several bioactive compounds *viz.* polyphenols, flavonoids, terpenoids, steroids, glycosides, alkaloids and saponins.

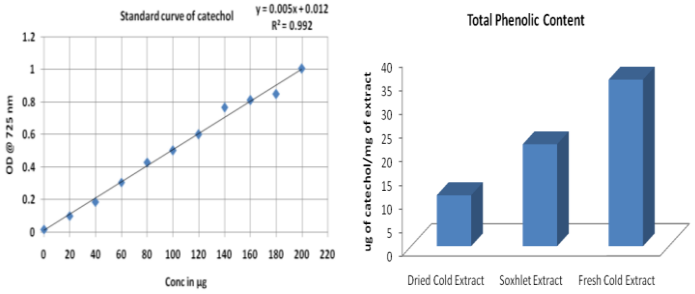
Table 1: Qualitative phytochemical analysis of different extraction methods of *Raphanus sativus*

Tests	Fresh Cold Extract	Dried Cold Extract	Soxhlet Extract
Steroids	+	+	+
Glycosides	+	+	+
Terpenoids	+	+	+
Saponins	+	+	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Tannins and Polyphenols	+	+	+

Total phenolic content

Results of the phenolic content in *Raphanus sativus* extracted with three different methods were presented in **Fig.1**. The *Raphanus sativus* extract using fresh cold extraction showed the highest content of phenolic compound (35.29±0.01 µg of catechol per mg of extract) followed by soxhlet extraction (21.6 ± 0.01 µg of catechol per mg of extract) and dried cold extraction (10.8 ± 0.01 µg of catechol per mg of extract).

Figure 1: Total Phenolic Content

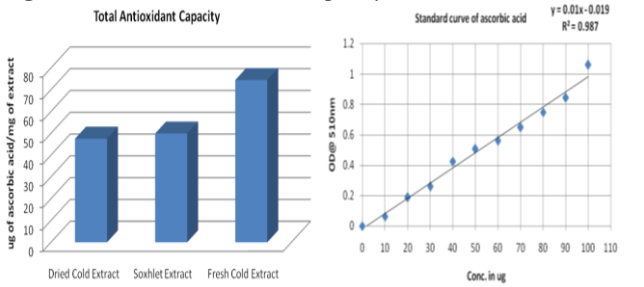


(a) Standard curve of Catechol **(b)** Catechol equivalence

Total antioxidant capacity

The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid (µg/mg of extract). The results were presented in the **Fig.2.** All the test extracts showed potent total antioxidant capacity. Among the three different test extracts, total antioxidant capacity was found to be high in fresh cold extract (74.44±0.02) followed by soxhlet extract (49.9±0.01) and dried cold extract (47.52±0.01) of *Raphanus sativus*.

Figure 2: Total Antioxidant Capacity

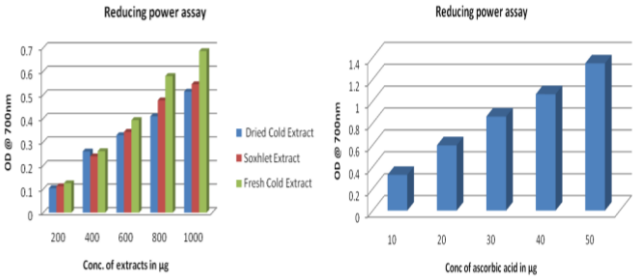


(a) Ascorbic acid equivalence **(b)** Standard curve of ascorbic acid

Reducing power assay

The reductive capabilities of three different extracts of *Raphanus sativus* were compared with standard ascorbic acid and the results were presented in **Fig.3.** The results revealed that, dose dependent reducing ability of test extracts. Wherein among the three different extraction methods, reducing power was found to be high in fresh cold extract followed by soxhlet and dried cold extracts at a conc. of 200-1000µg/ml.

Figure 3: Reducing power assay



(a) *Raphanus sativus* extracts **(b)** Standard ascorbic acid

DPPH radical scavenging activity

In this study, DPPH was used to determine the proton scavenging activity of all the three different extracts of *Raphanus sativus* at varying concentrations were measured along with standard ascorbic acid and their IC_{50} values are presented in **Fig.4.** was determined (IC_{50}). The IC_{50} values for test extracts were found to be highest in fresh cold extract of *Raphanus sativus* (920µg/ml) followed by soxhlet extract (1847µg/ml) and dried cold extract (3859.5µg/ml). Standard ascorbic acid has IC_{50} value of 3.9µg/ml.

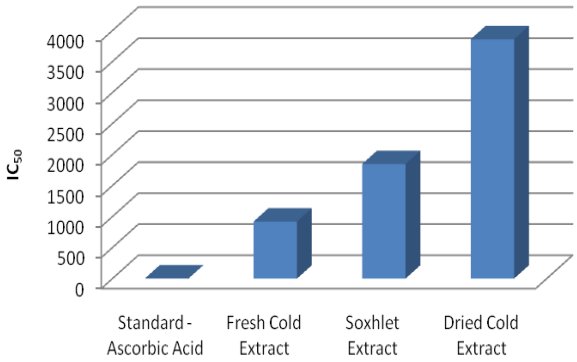


Figure 4: DPPH radical scavenging activity (IC_{50}) of *Raphanus sativus* extracts

Nitric oxide radical scavenging activity

All the three different extracts of *Raphanus sativus* showed a better activity in competing with oxygen to react with nitric oxide and thus the inhibition of anions. Test extracts inhibited nitric oxide by exhibiting the IC_{50} values 1343.36µg/ml (fresh cold extract), 1608.23µg/ml (soxhlet extract) and 1685.2µg/ml (dried cold extract); whereas standard ascorbic acid has recorded lower IC_{50} value of 38.38µg/ml (**Fig.5**).

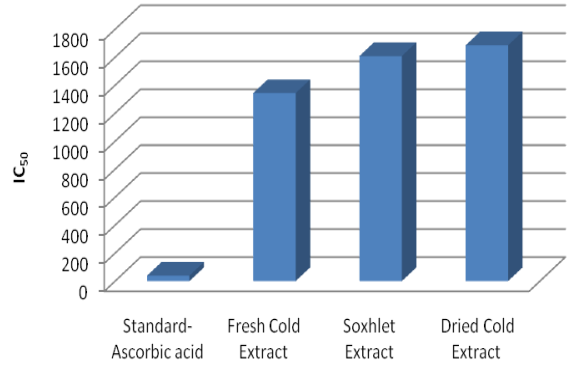


Figure 5: Nitric oxide radical scavenging activity (IC_{50}) of *Raphanus sativus* extracts

Fe^{2+} chelating activity

The results of Fe^{2+} chelating activity were expressed in terms of IC_{50} (**Fig.6**). Among the three different extracts of *Raphanus sativus,* fresh cold extract showed better activity in terms of IC_{50} 754.7µg/ml followed by soxhlet extract (889.9µg/ml) and dried cold extract (989.5µg/ml) whereas standard EDTA has IC_{50} of 10.9µg/ml.

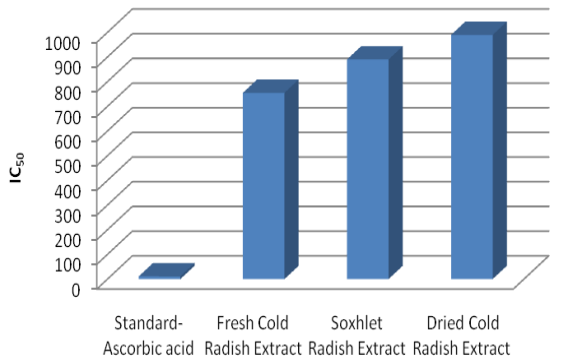


Figure 6: Iron chelating activity (IC_{50}) of *Raphanus sativus* extracts

DISCUSSION

The extraction of bioactive compounds from natural materials has gained many interests from researchers as the demand for functional ingredients obtained *via* natural processes keep increasing due to consumers are getting more interested in functional foods. Standardization of extraction procedures contributes significantly to the final quality of the herbal drug. Selective separation of the target components from the sample at maximum amount and/or interferences elimination are the main objectives of the extraction processes (Dobias *et al.,* 2010). To have a complete idea of the bioactivity of crude extracts and to obtain therapeutically desired portion, it becomes necessary to optimize the extraction methodology to achieve the broadest possible range of phytochemicals, besides eliminating the inert material by treatment with selective solvents and methods (Kothari *et al.,* 2009).

The purpose of the study was to evaluate the efficiency of different extraction materials and methods in *Raphanus sativus L.* on the antioxidant properties. The methods employed were fresh material cold extraction, dried material cold extraction and soxhlet extraction using 70% ethanol as a solvent. Solvent extraction offers good recovery of antioxidant phytochemicals from various samples, such as fruits and vegetables and is the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. The differences in the extract yields from the tested plant materials in the present analysis might be ascribed to the type of solvent, extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the plants (Hsu *et al.,* 2006). Even though the qualitative analysis was similar with different extraction materials, the quantitative estimation of total phenolics, total antioxidant capacity and evaluation of *in vitro* radical scavenging assays revealed significant differences.

The study revealed that *Raphanus sativus* possess the antioxidant potentials irrespective of different extraction methods. Fresh material cold extraction of *Raphanus sativus* showed better antioxidant activity when compared to soxhlet and dried material cold extraction methods. This may be due to the efficiency of the extracting solvent to dissolve bioactive compounds in fresh material cold extraction

whereas such compounds could be subjected to degradation in other two methods. Therefore, the present study shows that when different extraction methods were applied on the same plant with same solvent its extraction efficiency varies significantly in terms of parameters assessed. This could be helpful in selection of most appropriate extraction method for the further evaluation of biological activity.

CONCLUSION

The present study documents that for the studies of biological screening of vegetable, *Raphanus sativus,* fresh material cold extraction method could yield productive results.

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