International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 4 | OCT-DEC | 2018 | 495-501
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

SCREENING ANTI-OXIDANT AND ANTI-TYROSINASE POTENTIAL OF PLANTS AND EARTHWORM EXTRACTS

Usha Rani. J [1], Siva Prasad. B [4], Vutukuru. S.S. [3], Kamala. G [2] and Chand Pasha[2]*

[1]Department of Microbiology, Little Flower Degree College, Uppal, Hyderabad, India
[2]Department of Microbiology, Nizam College, Osmania University, Hyderabad, India
[3]Department of Biotechnology, Sreenidhi Institute of Science and Technology, Hyderabad, India
[4]Biotechnology Division, Environment Protection Training and Research Institute, Hyderabad, India

*Corresponding Author Email: cpasha21@gmail.com

ABSTRACT

Plant and invertebrate animal such as earthworm extracts containing antityrosinase activity can be used as skin whitening agents. Application of such extracts in pharmaceutical and personal care products aids in protecting human skin from various diseases. Exploitation of these natural extracts for isolating antioxidant compounds and skin whitening agents is paramount. In this context, research pertaining to the antioxidant potential and tyrosinase inhibition of plant and earthworm extracts is gaining prominence. Moreover, reports inferring the occurrence of both antioxidant potential and tyrosinase inhibition in plant extracts such as flowers, herbs, vegetables, fruits, and earthworm are meager. Therefore, present study is intended to evaluate the total antioxidant potential thereby tyrosinase inhibition of plant and earthworm extracts by phosphomolybdate method. This study compared the total antioxidant potential and tyrosinase inhibition of plant extracts in flowers, vegetables, fruits. The results of the study revealed that extracts of flower- Rosa rugosa and fruits- Punica granatum Prunus armeniaca possess both antioxidant activity and anti-tyrosinase inhibition properties. Interestingly, earthworm extracts showed more antioxidant and antityrosinase activities when compared to plant extracts. Outcomes of the study assist in characterization of natural products including peptides that can serve as an antioxidant and anti-tyrosinase compounds. Thus, the study contributes to the existing knowledge on screening and identification of medicinally important peptides or compounds from plants and earthworm to treat skin disorders.

KEY WORDS

Antioxidants, Plant extract, Earthworm, Tyrosinase inhibition

INTRODUCTION

Hyper pigmentation is the most common pigmentary disorder observed in skin, a local increase in melanin synthesis or uneven distribution of melanin can cause local hyper pigmentation or spots. Tyrosinase is the ubiquitous enzyme that initiates synthesis of melanin. Chemicals including Hydroquinone, arbutin and Koijic acid are conventionally employed as skin whitening agents. In recent times, peptides that have potential to treat hyperpigmentation were reported from plants and earthworms and other natural sources [1]. Plant and animal extracts are the chief sources for identification of antityrosinase peptides with lower toxicity. Hence there is a need to screen and identify peptides from various plant materials. Further, free radicals are produced during the pathogenesis of many diseases including cancer, arthritis, atherosclerosis, Alzheimer's

disease, and diabetes [2, 3]. Application of various synthetic antioxidants in pharmaceuticals and other personal care products in mitigating the adverse effects of free radicals result in side effects. Hence, application of formulations that are developed from animal and plant extracts in personal care products is emphasized [4]. These natural extracts provide an important and unexplored avenue for developing potential novel antityrosinase compounds. Hence use of plant extracts from fruit, vegetables, spices, leaves, roots, and bark for identification of natural antioxidants has gained prominence in pharma and personal care products. Plant and animal extracts do possess nutritional value thereby contributing to the protection of vital organs of living systems from damage caused by free radicals [5, 6]. Thus phytochemicals have potential to replace artificial antioxidants (tert-butylhydroxytoluene, tert-butylhydroxyanisole, and tert-butyl hydroquinone) and tyrosinase inhibitors (hydroquinone and Kojic acid).

Tropical and subtropical regions across the globe are rich in plant biodiversity. These plants can be a source of natural products. They have the potential to be developed into new drugs for treating various diseases [7]. Biodiversity of India offers a rich and relatively unexploited source of a variety of plants for formulating new drugs from plant extracts. The antioxidative phytochemicals in vegetables and fruits have received increasing attention for their potential role in the prevention of human disease [8]. Information on antioxidant and antityrosinase activities of plant extracts will contribute to additional knowledge about their bioactivities [9]. Moving forward, invertebrates such as earthworm (*Eisenia fetida*) also are rich in antioxidants those protect the human body from various diseases. Recent body of literature affirms that phytochelatins present in higher plants and earthworm play an important role in binding to heavy metal complexing peptides via cysteine thiol residues [10]. However, the occurrence of antioxidant potential and tyrosinase inhibition potential varies among various species, developmental stages and part of a given plant. Therefore, present study is aimed to understand antioxidant and antityrosinase potential of plant and earthworm extracts. It was carried out by screening plant and earthworm extracts (table 1) which are used in traditional medicine.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used in the present study are of analytical grade and used without any further purification.

Collection and preparation of Plants' part and earthworms

Fresh and clean plant materials were collected in sterile bags from local markets across Hyderabad. Earthworms (*Eisenia fetida*), were purchased from the Vermiculture Project at Hayathnagar and Mallapur, Hyderabad, India. Worms were carefully brought to the laboratory within an hour along with the moist soil in a perforated jute bag. Prior to the experiments, earthworms were kept on a moist filter paper for three hours for depuration to clear their gut contents. Subsequently, they were subjected to extraction.

Extraction of plant proteins

Fresh plant parts from flowers, fruits and vegetables were dried, ground to fine powder and extracted with Phosphate buffer. Plant parts were dried to 10% moisture at 60 °C, and used for liquid nitrogen treatment with precooled mortar and pestle. To 1gm of powder add 3 ml of extraction buffer in a 15-mL Falcon tube, vortexed, and incubated with shaking for 10 min on ice. These samples were subjected to centrifugation for 30 min at 1000×g at 4 °C in a cooling centrifuge (KEMI, 2011).

Plant extracts (1g) were grounded in liquid nitrogen and then mixed with 3 volumes of ice cooled extraction buffer consisting 25 mM phosphate buffer(pH 7.0), 225 mM NaCl, 10 mM EDTA, 5 mM DTT, 1.5%polyvinylpyrrolididone (PVP) (w/v), and 1 mM PMSF. The mixture was homogenized, filtered and centrifuged at 10,000 rpm for 20 min at 0 °C. The protein content in the crude extract was determined according to Lowry method [11] Phosphomolybdate antioxidant assayed as described by Huda-Faujan et al. [12]

Tissue preparation and protein extraction from earthworm

Live earthworms (*Eisenia fetida*) that were subjected to depuration on filter paper for 3 hours were homogenized in 0.1 M phosphate buffer (pH 7.5) (10 % w/v) using Potter-Elvehjam homogenizer which is equipped with a Teflon pestle. The homogenate was centrifuged at 5000×g for 10 min in Beckman (TLX-361544) centrifuge and the supernatant was further centrifuged at 5000×g for 10 minutes. Subsequently,

supernatant from the second round of centrifugation was used to estimate the activity of antioxidant enzymes, acetylcholinesterase and lipid peroxidation. All enzyme preparations were carried out at 4°C. Protein was estimated by the method of Lowry method [11].

Ammonium sulfate fractionation

The prepared crude extracts of plants and earthworm were subjected to ammonium sulphate fractionation [13] at 20%, 40%, 60%, and 80%. Pellets obtained after incubation at 0 ºC for 1h were collected, resuspended in extraction buffer, and dialyzed it.

Evaluation of total antioxidant capacity by phosphomolybdate method

Evaluation of the total antioxidant capacity of the plant extract/fraction was performed by measuring the absorbance at 695 nm [12]. 0.3 mL (0.5 mg/mL) of the plant extract was mixed with 3.0 mL of the reagent (600 mM sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After incubation of the reaction mixture at 95 °C for 60 minutes, the absorbance was measured at 695 nm against a blank contained 3 mL of reagent solution. The total antioxidant activity of a given plant extract was expressed as equivalent to ascorbic acid represented in % activity.

Estimation of tyrosinase inhibition of plant and earthworm extracts

Tyrosinase inhibition potential of plant and extract was carried out [14]. L-3, 4-dihydroxyphenylalanine (L-DOPA) was used as a substrate in the assay. Samples extracted were diluted in potassium phosphate buffer (10 mM, pH 6.8) to 600 µg/ml. Assays were carried out using a spectrophotometer (ELICO, 2011). All experimental steps of the assay were conducted at room temperature. Triplicates of a plant extract were run by preparing mixing (70 µl) sample with 30 µl of mushroom tyrosinase (333 Units/ml in phosphate buffer, pH 6.5). After 5 min incubation, 110 µl of substrate (12 mM L-DOPA) was added to the reaction mixtures and incubated further for 30 min. The final concentration of the extract was between 2.6 – 333.3 µg/ml. Kojic acid (1.04 – 133.33 µg/ml) was used as a positive control. A blank for the test was prepared by adding all the components except L-DOPA. Results were compared with a control consisting of Phosphate buffer. Absorbance values of the wells were then determined at 495nm. The percentages of tyrosinase inhibition of plant and earthworm extracts were calculated as compared to the controls.

STATISTICAL ANALYSIS

The statistical analysis of minimum three independent preparations of each plant and earthworm extract was performed by ANOVA at $p<0.05$.

RESULTS AND DISCUSSION

Description of plant and earthworm species

Plant and earthworm extracts containing antioxidants and tyrosinase inhibiting compounds from indigenous plant species of India were considered in this study. Colour and aroma of flowers, bark, leaves, fruit or any other part of the plants one of the valid scientific inquiries in natural products research. These attributes are important indicators of their ornamental and hence they are used in culinary purpose as well as in medicine. Many ornamental plants, vegetables contain high levels of antioxidant compounds and tyrosinase inhibition properties. Screening of these plant and earthworm extracts for antioxidants and tyrosinase inhibitors add value to the existing repository of natural products. In order to investigate these important attributes in various parts of plant and earthworm extracts (Table 1) are considered in our study. The results of total protein content (Figure 1), total antioxidant activity (Figure 2) and tyrosinase inhibition (Figure 3) of these extracts are discussed below.

Table 1 List of various plants and their corresponding parts used in tyrosinase inhibition

S. No.	Common name of the plant/ invertebrate	Scientific name of the plant/ invertebrate	Part of the plant/ earthworm
1.	Hibiscus	*Hibiscus rosa-sinensis*	Flower
2.	Jasmine	*Jasminum officinale*	Flower
3.	Rose	*Rosa damascena*	Flower
4.	Saffron	*Crocus sativus*	Flower
5.	Aamla	*Phyllanthus emblica*	Fruit
6.	Apricot	*Prunus armeniaca*	Fruit
7.	Beetlenut	*Areca catechu*	Fruit
8.	Bitter guard	*Momordica charantia*	Fruit
9.	Clove	*Syzygium aromaticum*	Fruit
10.	Grape (Black)	*Vitis vinifera*	Fruit
11.	Grape (White)	*Vitis vinifera*	Fruit
12.	Mango kernel	*Mangifera indica*	Fruit
13.	Olive	*Olea europaea*	Fruit
14.	Orange	*Citrus sinensis*	Fruit
15.	Papaya	*Carica papaya*	Fruit
16.	Pomegranate	*Punica granatum*	Fruit
17.	Sapindus	*Sapindus saponaria*	Fruit
18.	Tomato	*Solanum lycopersicum*	Fruit
19.	Cabbage	*Brassica oleracea*	Leaf
20.	Cauliflower	*Brassica oleracea*	Leaf
21.	Green tea	*Camellia sinensis*	Leaf
22.	Henna	*Lawsonia inermis*	Leaf
23.	Mango leaf	*Mangifera indica*	Leaf
24.	Aloevera	*Aloe vera*	Stem
25.	Sandal wood	*Santalum album*	Stem
26.	Turmeric	*Curcuma longa*	Stem
		Earthworm	
	Earthworm	***Eisenia fetida***	**Whole body extract**

Estimating total protein content of plant and earthworm extracts

Results (Figure 1) indicated presence of more amount of protein in earthworm and apricot. It is evident from the results that dry fruits, leaves, and herbs are rich sources of plant proteins. However, these differences of total protein content of plant extracts can be imparted to their phylogenetic relations among them. Analysis is needed to distinguish species and cultivars of the plants for describing their similarity total protein content [15]. Presence of more amounts of proteins in leaves might be attributed to the protein factors that are involved in photosynthesis reactivation and in the maintenance of the chloroplast functionality. Our results are in fine tune with findings of [16] who investigated the changes in protein patterns in leaf and root extracts of Zea mays plant. Earthworm extract is one of the major sources for isolating pharma ingredients including special active proteins or peptides [17, 18]. Earthworm whole body extract contains proteins (9.34%) and free amino acids (78.73 mg) in a given liter of raw fluid [19]. Present study is in agreement with that earthworm body extract is rich in proteins. However, exploitation of these proteins for isolating peptides and proteins is warranted.

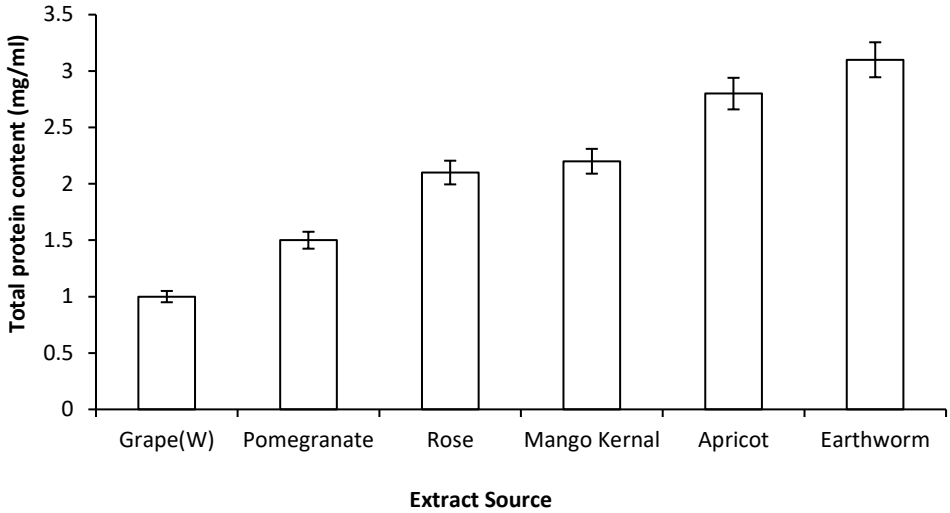

Figure 1 Total protein content in plant and earthworm extracts. The data is represented as the concentration of protein ± S.E (*p<0.05*) of three independent preparations of a given extract. (Grape (w=White)

Total antioxidant activity of plant and earthworm extracts

Percent of antioxidant activity of plant and earthworm extracts is presented in Figure 2. Results indicate that Grape, Rose and earthworm extracts showed more antioxidant potential. Other samples showed antioxidant activity <50%. The differences in antioxidant activity as exhibited by different extracts might be attributed to the varying amounts of antioxidant substances present in them. While the higher antioxidant activity of the extracts of leaves, rhizomes, and fruits maybe attributed to higher levels of the constituent flavonoids, ferulic and gallic acid derivatives, avenanthramides, and other phenolics [20]. However, further analysis of extracts of these plants can be subjected to screening for identifying various antioxidant compounds present in them. Earlier studies proved that earthworm extract exhibit antioxidant potential [21]. Present study reveals the antioxidant potential of earthworm extracts. It will help in furthering the concept of antioxidants analysis from earthworms.

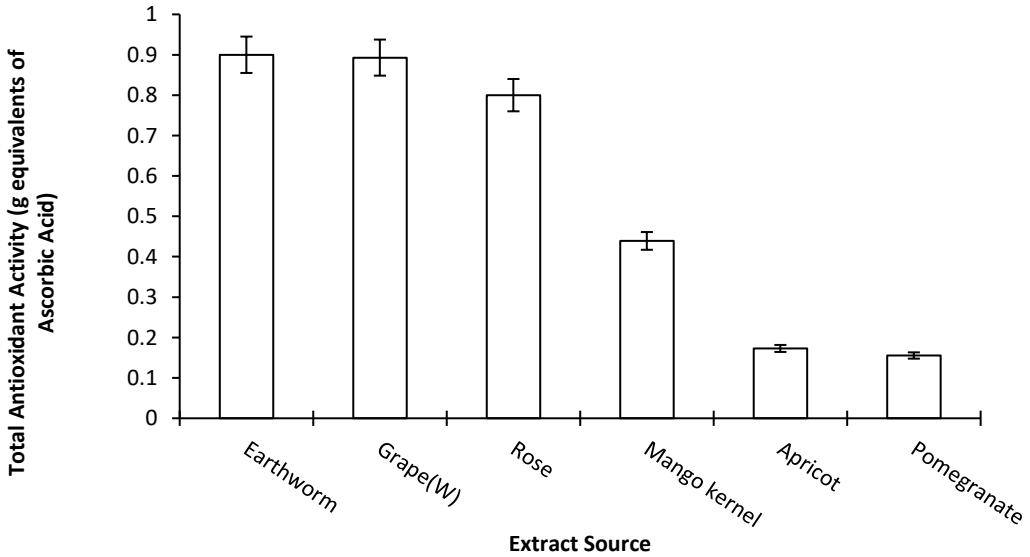

Figure 2 Percent antioxidant activity of plant and earthworm extracts. The data is represented as percent of antioxidant activity ± S.E (p<0.05) of three independent preparations of a given extract. (Grape (w=White)

Tyrosinase inhibition of plant and earthworm extracts

Tyrosinase inhibition potential of earthworm, and plant flower (Rose) and fruits (apricot, grapes, mango kernel, and pomegranate) are presented in Figure 3. Percent of mushroom tyrosinase inhibition of the plant extracts were represented as equivalents of L-ascorbic acid. Among the plant and earthworm extracts assayed for tyrosinase inhibition, Earthworm extract showed highest (65%) tyrosinase inhibition. Flower extract of rose and fruits extract of Apricot, Grapes, Mango kernel as well as pomegranate showed 27% and 58%, 45%, 31%, 19% of tyrosinase inhibition respectively.

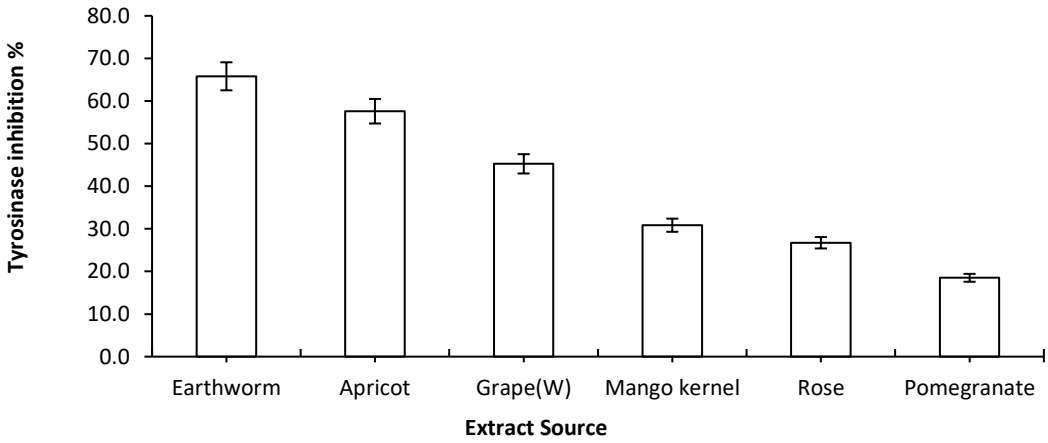

Figure 3 Percent tyrosinase inhibition capacity of plant and earthworm extracts. The data is represented as percent activity ± S.E (*p<0.05*) of three independent preparations of a given extract. (Grape (w=White).

Tyrosine based peptides have potential to tyrosinase activity via oxidation of L-3, 4-dihydroxyphenylalanine. It leads to the decomposition of melanin (pigment) thereby facilitating whitening of skin [22]. Hence it was surmised that earthworm extract dos possess tyrosinase-binding agents/ peptides that play a vital role in tyrosinase inhibition. Thus, earthworm extract is considered as one of the prime sources for isolating tyrosinase inhibiting peptides.

Outcomes of the study substantiate that plants extracts consist of compounds which can inhibit the activity of tyrosinase [23]. The expected natural products include phytochelatin peptides, flavonoids, aromatic acids, polyphenols, or aromatic aldehydes as these compounds can act as effective competitive inhibitors of melanin synthesis of melanin [24]. Variation in the activity of mushroom tyrosinase can be attributed to the presence of tannins in extracts which have the ability to precipitate proteins thereby limiting the tyrosinase inhibition [25]. In summary, the plant extracts from apricot rose, and pomegranate possesses both tyrosinase inhibition ability and high antioxidant activity.

CONCLUSION

The findings from the current study postulate the basis to promote the use of plant and earthworm extracts to identify natural products that are useful as an antioxidant and antityrosinase pharmaceutical ingredients. Earthworm extract is considered as the prime source for isolating antityrosinase peptides/ proteins. However, further studies on the characterization of bioactive small peptides followed by an evaluation of their potential to act as antioxidants and tyrosinase inhibitors are imperative. Hence present study aids in identification of and screening of peptides from plants and earthworm extracts.

ACKNOWLEDGEMENT

Authors thank Little Flower Degree College, Uppal, Hyderabad, and Department of Microbiology, Nizam College, Hyderabad for providing facilities for research.

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Received:08.08.18, Accepted: 09.09.18, Published:01.10.2018

***Corresponding Author:**
Chand Pasha*
Email: **cpasha21@gmail.com**