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Research Article

Tyrosinase Enzyme Inhibitory Activity of selected Indian Herbs

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ABSTRACT

The screening of some Indian medicinal plants was done for tyrosinase-inhibitory activity. Tyrosinase (Phenol oxidase) is a key enzyme that catalyzes melanin synthesis in plants, microorganisms and mammalian cells. Melanin biosynthesis inhibitory compounds are useful not only as skin whitening agents used in cosmetics but also as a remedy for disturbances in pigmentation. Therefore, many tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way of preventing over production of melanin in epidermal layers. Methanolic and water extracts was prepared for screening tests, studied for those with high activity. *Glycyrrhiza glabra (rhizome), Azadiracta indica (bark), Aesculus indica(fruits), Camellia sinensis(leaves), Nelumbo nucifera(seed), Acasia catechu(bark), Mangifera indica(leaves)* were screened as highly inhibiting samples compare to others at 1000ug/ml. *Glycyrrhiza glabra* methanol extract shows more than 50% inhibition.

Key Words: Screenin, Tyrosinase, Inhibitory activity, IC_{50}.

INTRODUCTION

Melanin is the major pigment for color of human skin. It is secreted by melanocyte cells in basal layer of the epidermis[1]. Melanin may be overproduced with chronic sun exposure, melasma, or other hyper pigmentation diseases[2].Therefore, a number of depigmenting agents have been developed for cases of undesirable skin discoloration. Tyrosinase, a copper-containing monooxygenase, is a key enzyme that catalyzes melanin synthesis in melanocytes[3]. It catalyzes two major reactions, including hydroxylation of tyrosine oxidation of the o-diphenyl product, L-Dopa. Dopa oxidation produces a highly reactive intermediate that is further oxidized to form melanin by free radical-coupling pathway. If free radicals are inappropriately processed in melanin synthesis, hydrogen peroxide (H_2O_2) is generated, leading to production of hydroxyl radicals ($HO^{\cdot}$) and other reactive oxygen species (ROS)[4]. Melanin bio-synthesis can be inhibited by avoiding ultraviolet (UV) exposure, by inhibition of melano-cyte metabolism and proliferation[5] by inhibition of tyrosinase, or by removal of melanin by corneal ablation.

Tyrosinase inhibitors therefore can be clinically useful for the treatment of some dermatological disorders associated with melanin hyper pigmentation. They also find uses in cosmetics for whitening and depigmentation after sunburn. In addition, tyrosinase is known to be involved in the molting process of insects and adhesion of marine organisam[6].

Traditional herbal medicines provide an interesting, largely unexplored source for development of potential new drugs. The potential use of traditional herbal medicines for development of new skin-care cosmetics has been emphasized recently[7]. It is of great interest to know whether preparations used cosmetically in folk medicines have activities that might be useful in modern formulations. Hence, in the present study an attempt will be made to find tyrosinase inhibitors from the selected Indian medicinal plants extracts.

The aim of the work was to focus on development of strategies and in-vitro screening methods, extraction procedures, bioassay-directed isolation and characterization of natural products. The present study was under taken for screening and to evaluate the tyrosinase inhibitory (skin whitening) potential of selected Indian medicinal plants with various ethnobotanical uses aiming to discover or localize new tyrosinase inhibitors.

2. MATERIALS AND METHODS

2.1. Reagents

Enzyme: Tyrosinase (From mushroom, 25000units, Sigma, USA, store at -20°c); **Substrate:** *l*-3,4-dihydroxyphenylalanine (*l*-DOPA) Sigma,USA, store at RT;

Buffer: Potassim dihydrogen orthophosphate, Himedia, India, store at RT; Potassium hydroxide (KOH) Lenoid chemicals Pvt. Ltd. India store at RT;

Positive control: Kojic acid, store at 2-8 ^{o}c. Other chemicals were of the highest grade commercially available.

2.2. Materials

Indian herbal medicines were purchased from local medicinal markets and identity was confirmed by Natural Remedies Pvt. Ltd, Bangalore shown in **Table No-1.**

2.3. Preparation of extracts

Dried traditional Indian herbal medicines were pulverized in a grinder and extracted by refluxing with methanol, of 1:3 ratio and then filtered. The procedure was repeated two times. The filtrates were combined and were concentrated under reduced pressure, vaccum-dried, and stored in a closed container until use. Same procedure followed for water extraxtion.

2.4. Principle

L-Dopa $\xrightarrow{\text{tyrosinase}}$ Dopachrome

Spectrophotometric quantification at 475 nm.

2.5. Preparation of working solutions

Phosphate buffer potassium (50mM, pH 6.5 at25 ^{o}c):680.45mg of Potassium dihydrogen orthophosphate is dissolved in 100ml of deionized water by adjusting the pH to 6.5 with 1M KOH.

1M KOH : 5.611gm of potassium hydroxide is dissolved in 100ml of de-ionised water. **Enzyme :** Stock1(25000U/250ul)Working solution(5600units/ml): 50ul of stock1 is made up to 1ml with 50mM Potassium phosphate buffer, pH6.5.

Substrate (4.25mM) : 4.19mg L-DOPA is dissolved in 5ml of de-ionised water. **Positive control**(1mg/ml): 5mg of Kojic acid is dissolved in 5ml of 50mM Potassium phosphate buffer,pH6.5.

2.6. Method

The assay is carried out as per the procedure of with slight modifications[8]. In brief, add 112.5ul 50uM potassium phosphate buffer pH 6.5 / test sample / positive controle of various concentrations, 85ul of deionised water and 2.5ul of enzyme (5600 units/ml), mix and pre-incubation, add 50ul substrate (4.25mM) mix and incubate at 37 ^{o}c for 8 minutes. Following Pre-incubation, add 50ul substrate (4.25mM) mix and incubate at 37^{o}c for 10 minutes. The absorbance is measured at 475nm.A control reaction is carried out without the test sample.

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)} \quad \times 100}{\text{Absorbance (control)}}$$

Final assay concentration in 250ul reaction volume, the final concentration are 23.04mM of potassium phosphate buffer pH 6.5, 0.85mM of L-Dopa and 14 units of mushroom tyrosinase. All reaction is carried out in 96 microwell plate, Tarsons.

Assay quality control parameters: Control OD should be greter than 1.1. Blank value should be less than 0.05. IC_{50} positive control should be in between 20 to 45 ug/ml.

3. RESULTS AND DISCUSSION

In previous papers, inhibition of tyrosinase by a variety of compounds has been studied, with the result that several inhibitors are now used as cosmetic additives or as medicinal products for hyperpigmentation [9-10]. Recently, natural substances such as green-plant products have been in increased demand in the global market for new agents for depigmenting, cosmeceutical, and skin-lightening purposes[11]. Traditional Indian herbal medicines have been used in clinical practice for centuries; they are often used to maintain good health or used to treat various diseases. In the present study, these materials were selected based on compiled ethnobotanical data that revealed the agents are usually used clinically as skin applications (**Table-1**). Therefore, we evaluated their effects on tyrosinase enzyme as its percentage inhibition activities. The selected traditional Indian herbal medicines were extracted with methanol, with extract yields ranging from 2.1 to 33.5%.

In the present study, we used tyrosinase enzyme as an in vitro model because of the need to measure % inhibition. An assay for inhibition was employed before further in vitro testing in skin melanocytes was done to test melanin content. We used *l*-DOPA as the substrate to detect any tyrosinase inhibitory effect. Among the tested extracts, 7 extracts, *Glycyrrhiza glabra (rhizome), Azadiracta indica (bark), Aesculus indica (fruits), Camellia sinensis(leaves), Nelumbo nucifera(seed), Acasia catechu(bark), Mangifera indica(leaves),* showed relative % inhibition, with tyrosinase above 30% with

a concentration of 1000 µg/mL.Their % inhibition values 76.53, 43.59, 38.35, 32.76, 33.57, 44.40, 32.85 resp (see **Table-2**). Also none of the extracts screened are shows tyrosinase inhibition activity. *Glycyrrhiza glabra* methanol extract shows more than 75% inhibition. The IC_{50} value reference standard of kojic acid was obtained 25.35 (20.71-31.19) see **Table-3.** The IC_{50} value of Glycyrrhiza glabra was found to be within range **Table-4**. Thus, our tested extracts exhibited greater inhibitory activity.

Table 1: Selected traditional Indian herbal medicines used on the skin, and their recommended use

S. No.	Scientific name	Part used	Traditional importance
1.	*Acacia catechu* (Mimosaceae)	B	SD, L , LD (Varier,1993)[13]
2.	*Aesculus indica* (Hippocastanaceae)	Fr.	SB (Bunney, 1984)[14]
3.	*Aloe vera* (Liliaceae)	Lf.	SD (Varier,1993)[13] (Murthy,1998)[15]
4.	*Azadiracta indica* (Meliaceae)	Lf. B	SD, LD, L (Varier,1993)[13]
5.	*Bacopa monniri* (Scrophulariaceae)	WP	SD, LD, L (Varier,1993) [13] (Sharma et.al., 2000)[16]
6.	*Berberis aristata* (Berberidaceae)	S	SD (Sharma,1993)[17]
7.	*Bixa orellana* (Bixaceae)	Sd	SD (Varier,1993)[13]
8.	*Camelia sinensis* (Theaceae)	Lf.	T (Varier,1993) [13]
9.	*Centella asiatica* (Apiaceae)	WP	L (Murthy,1998)[15]
10.	*Cocos nucifera* (Arecaceae)	K	SD, L (Varier,1993) [13]
11.	*Curcuma longa* (Zingiberaceae)	Rhiz.	DS, SD, Scabies, Pruritus (Varier,1993) [13] (Murthy,1998)[15]
12.	*Curcuma zeodoria* (Zingiberaceae)	Rhiz.*	SD (Murthy,1998)[15]
13.	*Cyprus rotandus* (Cyperus rotandus)	Rhiz.	SD, L, Pruritus, Scabies (Varier,1993) [13]
14.	*Glycyrrhiza glabra* (Fabaceae)	Rhiz.	SD (Varier,1993)[13]
15.	*Hedychium spicatum*(Zingiberaceae)	Rhiz.	SD (Murthy,1998)[15]
16.	*Hemidesmus indicus* (Asclepiadaceae)	R	L, LD, SD, Pruritus (Varier,1993)[13] (Murthy,1998)[15]
17.	*Lawsonia inermis* (Lythraceae)	Lf.	SD, BS (Varier,1993)[13]
18.	*Mangifera indica* (Anacardiaceae)	Lf.	SD, BS (Varier,1993)[13]
19.	*Michelia champaka* (Magnoliaceae)	Flw.	SD (Varier,1993)[13]
20.	*Nelumbo nucifera* (Nymphaeaceae)	Flw. Sd.*	DP, LD, SD, L(Varier,1993) [13] (Murthy, 1998)[15]
21.	*Nyctanthus arbortistis* (Oleaceae)	Lf.	DP, SD, Pruritus (Varier,1993) [13] (Sharma et.al., 2000)[16]
22.	*Ocimum santum* (Lamiaceae)	Lf.	SD, L (Murthy,1998)[15] (Sharma, 1994)[12]
23.	*Prunus amugdalus* (Rosaceae)	Sd.*	SD (Varier,1993)[13]
24.	*Punica granathum* (Punicaceae)	Fr. P	SD (Varier,1993)[13] (Murthy,1998)[15]
25.	*Rosa alba* (Roseaceae)	Flw.	SD (Sharma,1993)[17]
26.	*Rubia cordifolia* (Rubiaceae)	R* S	SD, L, LD (Varier,1993)[13] (Sharma et.al., 2000)[16]
27.	*Symlocos racemosa* (Symplocaceae)	B	SD, L (Varier,1993)[13]
28.	*Tinospora cordifolia* (Menispermaceae)	S	SD, L (Varier,1993)[13]
29.	*Trigonella foenum graceum* (Fabaceae)	Sd Lf.	BS, T (Varier,1993)[13]
30.	*Vetivera zizconoides* (Poaceae)	R	LD (Sharma,1993)[17]
31.	*Vitis vinifera* (Vitaceae)	Fr.	SD (Varier,1993)[13] (Sharma et.al.,2000)[16]
32.	*Withania somnifera* (Solanaceae)	R	LD, T,BS, Psoriasis (Varier,1993)[13]

Abr :- B (Bark), Fr. (Fruits), Lf.(Leaf), R (Root), Rhiz. (Rhizome), S (Stem), Sd (Seed), WP (Whole plant), K (Kernel), Flw (Flower), P (Peel). SD:- Skin disorders; LD:- Leucoderma; DS:- Discoloration of skin; DP:- Dermatopathy; L:-Leprosy; BS:- Burning sensation; T:- Thermogenic; SB:- Sun burn * Plant parts subjected for extraction.

Table 2: Data showing tysonase inhibitory activity of methanolic and aqueous extracts of selected plant parts at a concentration of 1000µg/ml

S. No.	Scientific name	Part used	MI * (Methanol ext.)	MI * (Aqueous ext.)
1.	*Acacia catechu* (Mimosaceae)	B	44.40	12.78
2.	*Aesculus indica* (Hippocastanaceae)	Fr.	38.35	WI
3.	*Aloe vera* (Liliaceae)	Lf.	08.30	-
4.	*Azadiracta indica* (Meliaceae)	Lf. B	10.10 43.59	07.50 WI
5.	*Bacopa monniri* (Scrophulariaceae)	WP	09.50	04.60
6.	*Berberis aristata* (Berberidaceae)	S	20.80	WI
7.	*Bixa orellana* (Bixaceae)	Sd	08.20	WI
8.	*Camelia sinensis* (Theaceae)	Lf.	32.76	WI
9.	*Centella asiatica* (Apiaceae)	WP	11.28	-
10.	*Cocos nucifera* (Arecaceae)	K	23.82	WI
11.	*Curcuma longa* (Zingiberaceae)	Rhiz.	NA	14.34
12.	*Curcuma zeodoria* (Zingiberaceae)	Rhiz.	04.60	WI
13.	*Cyprus rotandus* (Cyperus rotandus)	Rhiz.	11.82	WI
14.	***Glycyrrhiza glabra*** (Fabaceae)	Rhiz.	**76.53**	-
15.	*Hedychium spicatum* (Zingiberaceae)	Rhiz.	17.87	07.00
16.	*Hemidesmus indicus* (Asclepiadaceae)	R	14.80	03.20
17.	*Lawsonia inermis* (Lythraceae)	Lf.	14.62	-
18.	*Mangifera indica* (Anacardiaceae)	Lf.	32.85	24.43
19.	*Michelia champaka* (Magnoliaceae)	Flw.	07.00	-
20.	*Nelumbo nucifera* (Nymphaeaceae)	Flw. Sd.	11.91 33.57	03.40 WI
21.	*Nyctanthus arbortistis* (Oleaceae)	Lf.	16.33	01.80
22.	*Ocimum santum* (Lamiaceae)	Lf.	07.30	04.30
23.	*Prunus amugdalus* (Rosaceae)	Sd.	10.92	WI
24.	*Punica granathum* (Punicaceae)	Fr. P	14.16 34.11	WI WI
25.	*Rosa alba* (Roseaceae)	Flw.	19.67	WI
26.	*Rubia cordifolia* (Rubiaceae)	R S	14.80 09.20	- 05.30
27.	*Symlocos racemosa* (Symplocaceae)	B	06.60	03.60
28.	*Tinospora cordifolia* (Menispermaceae)	S	14.35	WI
29.	*Trigonella foenum graceum* (Fabaceae)	Sd Lf.	13.35 16.15	09.90 04.60
30.	*Vetivera zizconoides* (Poaceae)	R	11.28	05.70
31.	*Vitis vinifera* (Vitaceae)	Fr.	08.60	02.60
32.	*Withania somnifera* (Solanaceae)	R	14.89	-

Abb :- B (Bark), Fr. (Fruits), Lf.(Leaf), R (Root), Rhiz. (Rhizome), S (Stem), Sd (Seed), WP (Whole plant), K (Kernel), Flw (Flower), P (Peel).
MI:-Mean % inhibition, NA:-Not active, Note: The value of >50% inhibition is indicated in bold.
*Determinations were done in duplicate.

Table 3: Data showing IC_{50} of reference standard (Kojic acid) for all methanolic and aqueous extracts

S. No.	Concentration in Kojic acid µg/ml	% Inhibition
1.	5 µg/ml	14.55
2.	10 µg/ml	23.20
3.	25 µg/ml	42.85
4.	50 µg/ml	66.96
5.	100 µg/ml	79.52
	IC_{50} = 28.60 (23.78 – 34.71)	

Table 4: Data showing IC_{50} of the methanolic extract of *Glycyrrhiza glabra*

S. No.	Concentrations of *Glycyrrhiza glabra* methanolic extract	% Inhibition
1.	100 µg/ml	23.54
2.	500 µg/ml	63.58
3.	1000 µg/ml	82.05
	IC_{50} = 286.02 (222.91 - 356.74)	

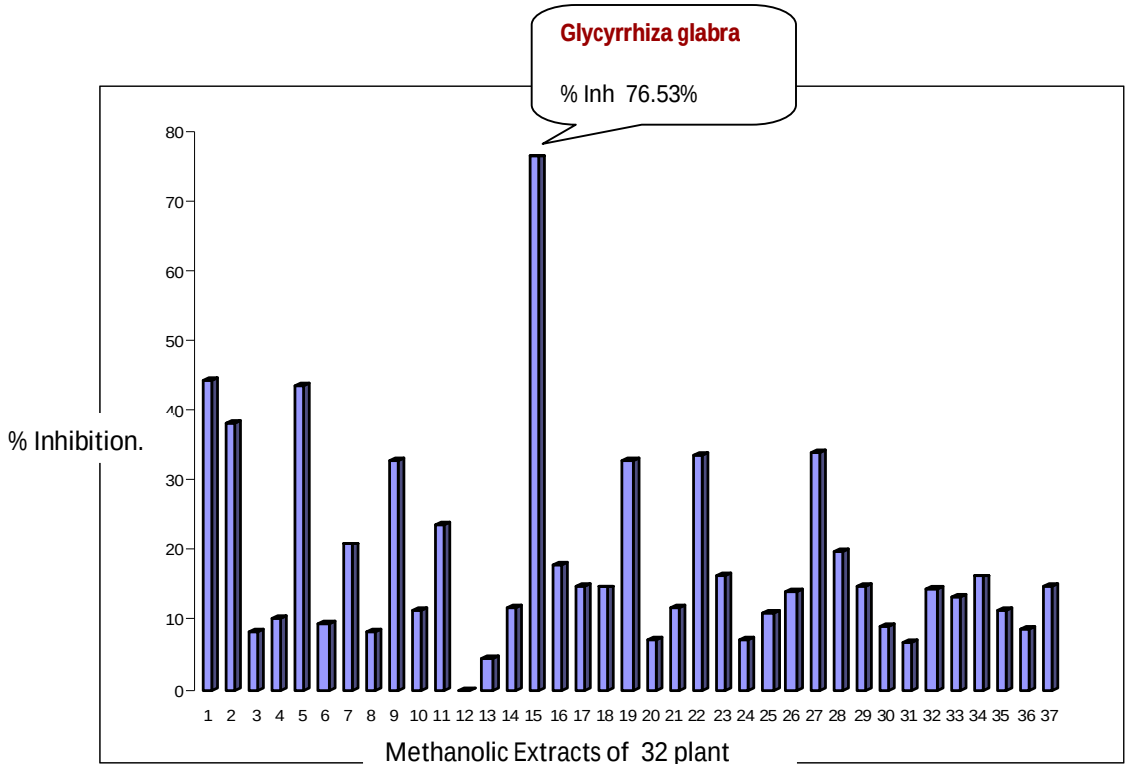

Fig. 1: Data showing % Inhibition of tyrosinase enzyme by 37 methanolic extracts of selected Indian medicinal plants

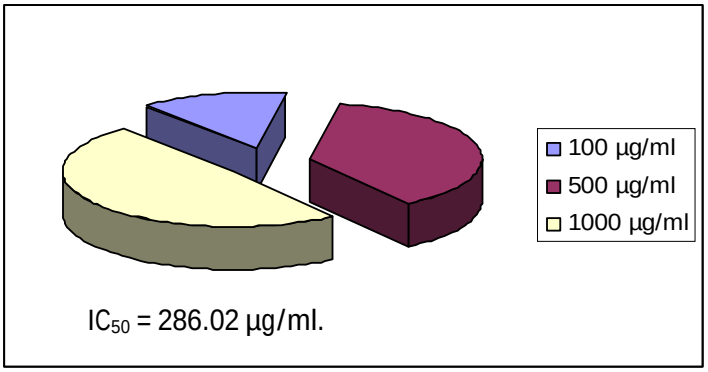

Fig 2: IC $_{50}$ value for *Glycyrrhiza glabra*

4. CONCLUSION

In the present study, 34 selected Indian herbal medicines were investigated for potential effectiveness as skin-whitening agents and in maintaining skin health. Extract of *Glycyrrhiza glabra (rhizome)* was shown to be potent tyrosinase inhibitors in human skin. In addition to extracts of *Azadiracta indica (bark), Aesculus indica (fruits), Camellia sinensis(leaves), Nelumbo nucifera(seed), Acasia catechu(bark), Mangifera indica(leaves)*, (see graph of **Fig. 1** & **2**) which are currently in use as cosmetic , results of this study indicate that exract of *Glycyrrhiza glabra (rhizome)* likely to be useful for cosmetic applications and products. Their bioactivity

guided isolated components may prove to have considerable value as cosmetics additives in the future.

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