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Isolation and Characterisation of Mushroom Tyrosinase and Screening of Herbal Extracts for Anti Tyrosinase Activity

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Abstract : Objective : The present study focus on synthesizing and characterizing tyrosinase from Indian edible mushroom *Agaricus bisporus* and analysis for its inhibition by selected herbal plants.

Methods : Crude extract were prepared by homogenization in 100mM cold phosphate buffer (pH 5.8). After centrifugation, the supernatant was applied to ammonium sulphate precipitation. Different precipitation steps were carried out for tyrosinase enzyme precipitation(45%-80%) and precipitate were collected. The precipitate was dialyzed against 100mM potassium phosphate buffer (pH 7.0). The dialyzed fraction was used for tyrosinase activity and protein content. The dialyzed ammonium sulfate fraction was applied to a sephadex G-100 column that was obtained after ammonium sulphate precipitation and sephadex G-100 column was subjected to ion exchange chromatography using DEAE cellulose column. The dialyzed enzyme preparation was loaded on DEAE-cellulose column which was preequilibrated with potassium phosphate buffer (100mM pH7.0).The fractions were collected and assayed for tyrosinase activity and those showing high activity were pooled and used for protein concentration. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin(BSA) standard. After protein estimation the purified mushroom sample is subjected to SDS-PAGE analysis. Powdered samples were extracted with 500 ml of methanol each using Soxhlet apparatus for ten hours of time. The extracts were condensed using rotary evaporator.

Result : The Methanolic extracts of *Glycyrrhiza glabra*, *Vetiveria zizanioides*, *Rosa indica* exhibited mushroom tyrosinase inhibition. The maximal toxic free concentration of plant extract was evaluated on vero cell line and found to be non toxic at the concentrations tested.

Conclusion : Tyrosinase inhibition is one major strategy used to treat hyperpigmentation. The results obtained from biological assays showed that *Glycyrrhiza glabra*, *Vetiveria zizanioides*, *Rosa indica* possessed anti-tyrosinase properties, which exhibited potential for application in medical cosmetology.

INTRODUCTION

Melanocytes are melanin producing cells located in the bottom layer of the skin's epidermis. Melanin is the pigment primarily responsible for skin colour. Melanin is contained in a special organelle called melanosomes.. Tyrosinase is an oxidase that is a rate limiting enzyme for controlling the production of melanin. The tyrosinase is usually extracted from Indian edible mushroom. If excess of melanin pigment is produced, it leads to *Hyperpigmentation*, *Melasma*, *Freckles* and etc. The abnormal pigment is a serious esthetic problem in human beings.

Mushrooms are considered a cheap source of tyrosinase. Mushroom tyrosinase from *Agaricus bisporus* is a tetramer of about 120kdal with monomeric isoform and with molecular masses of 30kdal. Furthermore microbial tyrosinase (Mushroom tyrosinase) like mammalian has an tetrameric structure and can be used for the clinical purposes.

Glycyrrhiza species are perennial plants belonging to the family Leguminosae. *Glycyrrhiza glabra* has proved to be powerful source of antioxidant and can replace chemical antioxidants [1]. Glycyhhrizin from *Glycyrrhiza glabra* reduces skin discoloration [2]. *G. glabra* are used in cosmetic preparations owing to their skin-whitening, anti-sensitizing, and antiinflammatory properties [3].

Vetiveria zizanioides (Vetiver grass), which is a perennial tussock grass of the Gramineae family, is famous as an eco friendly plant. This grass can prevent soil erosion and is used in the treatment of metalliferous-polluted ground due to its tolerance to heavy [4]. *Vetiveria zizanioides* has also been cultivated for many industrial applications, including the production of the commercially and medicinally valued volatile oil that can be distilled from its root [5]. *Vetiveria zizanioides* essential oil (VZ-EO) has been frequently used as a functional ingredients and fragrance in foods, aromatic products, and cosmetics. The *Vetiver* oil is an expensive edible oil in the Chinese market and which has also been used in India in many ways as a food additive, such as flavouring syrups, ice cream, and beverages and for food preservation. Moreover VZ-EO is commonly used as traditional medicine in Thailand and India for the treatment of numerous syndromes, such as *gastritis, fever, headache, mouth ulcers, toothache, and chronic inflammation* [6].

Rosa species have long been used for food and medicinal purposes. Chemical composition and bioactivity of their petals, fertilized flowers, unripe, ripening, and overripe hips were evaluated in order to valorize them as sources of important phytochemicals [7]. The key flavor compounds that contribute to the distinctive scent of rose oil are beta-damascenone, beta-damascone, beta-ionone, and oxide [8]. *R. damascena* is an ornamental plant and beside perfuming effect, several pharmacological properties including anti-HIV, antibacterial, antioxidant, antitussive, hypnotic, antidiabetic, and relaxant effect on tracheal chains have been reported for this plant. [9].

Kojic acid, produced by *Aspergillus* species and *penicillum* species is an intensively studied inhibitor of tyrosinase and this fungal metabolite is used as a cosmetic skin-whitening agent and as a food additive for preventing enzymatic browning. Basically, there has been research which suggests that if kojic acid is used repeatedly for extended periods of time, the user's skin may develop increased sensitivity which some say might become permanent to some degree. Technically known as dermatitis, this side effect be characterized by increased itchiness, red rash, irritation, etc. Tyrosinase inhibitors have become increasingly important in medicinal and cosmetic products in related to hyper pigmentation. Moreover, tyrosinase inhibitors have been used as depigmenting agents for the treatment or prevention of pigmentation disorders [10].

The present study, focus on synthesizing and characterization of the tyrosinase from Indian edible mushroom. The edible mushrooms are *Lactarius piperatus, Ganoderma lucidum, Lentinula edodes* and etc. These mushroom have tyrosinase, but *Agaricus bisporus* another edible mushroom shows very high similarities compared to human tyrosinase and it is the chiefly available mushroom. This study also evaluating the tyrosinase inhibition properties of *Vetiveria zizanioides, Glycyrrhiza glabra, Rosa indica*. using kojic acid as a standard.

Materials and Methods

Chemicals and solvent used in this study were of laboratory analytical grade and were obtained from SRL chemicals.

Collection of mushroom

The frozen mushroom (*Agaricus bisporus*) was obtained from native supermarket (fig 1).

Preparation of Mushroom Tyrosinase extract

Extraction of mushroom tyrosinase was performed as per protocol [11] with few modifications. The sliced mushrooms were homogenized in mortar and pestle. Enzyme extraction was done with 500mL of cold

phosphate buffer (pH 5.8) for 200 gram of mushroom. The homogenate was centrifuged at 10000 rpm for 15 mins and supernatant was collected. The sediments were mixed with cold phosphate buffer and were allowed to stand in cold condition with occasional shaking. The sediment containing buffer was subjected to centrifugation once again to collect supernatant. The supernatant was used as a source of enzyme.

Purification of the enzyme from the crude extract

Crude enzyme extract purified by ammonium sulphate precipitation, dialysis, gel filtration ion exchange chromatography, and so forth has been employed in series so as to obtain the enzyme in its purest form. The pure enzyme thus produced can be used for the further analysis. [11]

Ammonium sulphate precipitation and dialysis

Ammonium sulphate precipitation was done in an ice bath using the finely ground ammonium sulphate. The powder was weighed (200 ml of mushroom extract and 140 gram of ammonium sulphate) and added slowly to the extract by constant stirring to ensure complete solubility, and the solution was centrifuged at 10,000 rpm for 15 mins at 4°C. The precipitate was dialyzed against 100mM potassium phosphate buffer (pH 7.0) for 24 hours by changing the buffer thrice. The dialyzed fraction was used for tyrosinase activity and protein content.

Sephadex G-100 Gel Filtration

The dialyzed ammonium sulphate fraction was applied to a sephadex G-100 column that was pre equilibrated with a 100 mM phosphate buffer of pH 7.0. The protein elution was done with the same buffer at flow rate of 5mL/min. The active fractions were pooled, dialyzed against the 100 mM phosphate buffer of pH 7.0, and concentrated.

DEAE- cellulose column chromatography

Dialyzed enzyme preparation obtained after ammonium sulphate precipitation and sephadex G-100 column was subjected to ion exchange chromatography using DEAE-Cellulose column. The dialyzed enzyme preparation was loaded on DEAE-Cellulose column which was pre equilibrated with potassium phosphate buffer (100 mM, pH 7.0). The column was washed first with equilibrated buffer and then bound proteins were eluted using linear gradient of 7.5mM, 12.5mM, 25mM, 50mM, 100mM sodium chloride and 7.5mM, 12.5mM, 25mM, 50mM, 100mM potassium phosphate buffer at a flow rate of 1mL per min. The fractions (5.0 ml each) were collected and assayed for tyrosinase activity and those showing high activity were pooled and used for SDS-PAGE analysis.

Estimation of protein: Bradford Assay

Eight dilutions of a protein (using BSA) standard with a range of 0.15 mg to 10 mg protein were prepared. Unknown protein samples were diluted in 60 µl. 60 µl each of standard solution or unknown protein sample was added to an appropriately labeled test tube. Two blank tubes were set. For the standard curve, 60 µl H₂O was added instead of the standard solution. For the unknown protein samples, 60 µl protein preparation buffer was added instead. Protein solutions are normally assayed in duplicate or triplicate. 3 ml of Bradford reagent was added to each tube and mixed well. Tubes were incubated at room temperature (RT) for 5 min. Absorbance will increase over time; samples should incubate at RT for no more than 1 h. Absorbance was measured at 595 nm. A standard graph of concentration vs. absorbance was plotted. From this, by using absorbance of unknown samples, concentrations were estimated.

Sodium Dodecyl Sulfate – Polyacrylamide Gel (SDS-PAGE)

Separating gel was poured via the gap between the glass plates to $\frac{3}{4}$ of its length without air bubble. Sufficient amount of 1:1 ratio of toluene was added on top of the gel to prevent shrinkage of the gel. The gel was allowed to polymerize for 30 mins. After polymerization of the gel, the toluene was drained and the remaining toluene was dried by using filter paper. The separating gel was overlaid with the stacking gel upto the rim of the notched plate and immediately clean Teflon – comb was inserted into the stacking gel solution without air bubbles in between. The stacking gel was allowed to polymerize for 20 mins. After polymerization the comb was removed carefully. The wells were washed immediately with water. The water was removed by

using filter paper. The spacer was removed from the bottom and the glass plate was fixed in the electrophoresis apparatus filled with running gel buffer in lower and upper buffer tanks.

Sample preparation

3 microfuge tubes were taken and marked it as sample I, sample II and sample III. 10 µl of sample I, II, & III were added to the respective tubes and 10 µl sample solubilizing buffer was added to each tube. It was mixed and boiled at 100°C for 2 mins, by using water bath. The sample I, II and III were loaded to first three lanes and 10 µl of protein molecular weight marker was added to the next nearest lane. After loading, the power pack was switched on and the voltage was set at 50 V. It was run until dye reached the separating gel. Then the voltage was increased to 100 V and allowed to run until the dye reached the bottom of the gel. The gel plants were removed from the tank using spatula, the gel plates were separated carefully. The stacking gel was removed completely from the separating gel and the gel was placed in staining solution. The box was kept in the rocker for 30 mins at room temperature. 25 ml of destaining solution was added and allowed it for 1 hour with three changes.

Preparation of extracts

Twenty-five gram of *Vetiveria zizanioides*, *Glycyrrhiza glabra* and *Rosa indica* were air dried and powdered samples were extracted with 500 ml of methanol each using Soxhlet apparatus for ten hours of time. The extracts were condensed using rotary evaporator. After condensation the sample were reconstituted in their respective solvents to obtain a stock of 1mg/ml and were stored in a refrigerator.

Tyrosinase inhibitory activity

L-DOPA and mushroom tyrosinase were purchased from sigma chemical. 20 µL of extracted mushroom tyrosinase (1000 U/mL), 20 µL of 0.1 M phosphate buffer (pH 6.8) and 100 µL of the sample solution (20%) containing 20 µL of plant extracts, were mixed (called sample solution with enzyme). Sample solutions without enzyme were also prepared by repeating all previous steps but with no plant extracts added. Blank solutions with and without enzyme were also prepared with no test sample solution added. We also prepared positive controls of 0.5 mg/mL kojic acid solution (with water), with and without enzyme. 20 µL of 0.85 Mm L-DOPA solution as the substrate was added into every sample and blank. These mixture were incubated at 25°C for 10 min. The amount of dopachrome produced in the reaction mixture was measured at 475 nm ($\epsilon_{475} = 3600 \text{ M}^{-1}\text{cm}^{-1}$).

Percentage inhibition of tyrosinase activity was calculated as the following:

$$\% \text{ Tyrosinase inhibition} = \frac{(A-B) - (C-D)}{(A-B)} \times 100$$

A = absorbance of blank solution with enzyme

B = absorbance of blank solution without enzyme

C = absorbance of sample solution with enzyme

D = absorbance of sample solution without enzyme.

Toxicity assay

Assaying the toxicity of the isolated mushroom plant extracts on cell lines were carried out in tissue culture 96 well microtitre plates for evaluating the safety of the separated mushroom plant extracts and for determining the toxic free concentration of the test drugs. 0.1ml cellular suspension was transferred into each of the wells containing growth medium. The plates was incubated at 37°C in 5% CO₂ atmosphere in CO₂ incubator for 12 hours. The growth medium was removed after confluence obtained by micropipette. 1mg of the tyrosinase extracts to be dissolved (standard/ test) in 1ml 2% FBS MEM. The extracts mixed medium was serially diluted in two fold manner in 2% FBS MEM, from an initial concentration of 500µg/ml to a final concentration of 3.9µg/ml. 0.1ml of the serially diluted drug was added into the wells. The plates was incubated at 37°C in 5% CO₂ atmosphere for 72 hours and was observed under inverted phase contrast microscope for determination of toxic free concentration. [12]

Results

Mushroom contains a considerable amount of various phenolic compounds, which are readily oxidized during the homogenization process. Upon oxidation and successive polymerization of the phenolic contents of the mushroom extract, macromolecules of melanin are formed.

Extraction of *Agaricus bisporus*

The crude extracts were prepared by homogenization in 100mM phosphate buffer (pH 5.8). After centrifugation, the supernatant was applied to ammonium sulfate precipitation. Partial purification of tyrosinase using ammonium sulfate precipitation showed that the best fraction was 70% with respect to crude enzyme and other fraction. The pale color crushed mushroom was changed to brown colour after filtration. The colour change was due to the presence of melanin.

Purification of mushroom extract

Mushroom extract was purified by various methods which include dialysis, sephadex G-100 gel filtration, DEAE- cellulose column chromatography.

Sephadex G-100 Gel Filtration

The dialyzed ammonium sulphate precipitate that was subjected to sephadex G- 100 gel filtration and column chromatography showed tyrosinase activity. After dialysis process the brown color mushroom extract was applied to sephadex column and the protein was eluted as colourless form.

DEAE-Cellulose Column Chromatography

The colourless form of extract after gel filtration was applied to a DEAE –cellulose column and eluted at 7.5Mm to 100mM Sodium chloride. The eluted active fractions was chromatographed on the same column with a linear gradient of potassium phosphate buffer (7.5mM to 100Mm) passed through the column. This two-step purification scheme of ion exchange chromatography, resulted in a partially purified tyrosinase precipitate.

Protein Estimation

Protein concentration was determined by the method of Bar-ford (1976) using bovine serum albumin (BSA) standard.

SDS-PAGE

SDS-PAGE of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands progressively improved from the crude extract to the final step of the DEAE- cellulose column. It revealed only a single distinctive protein band for the pure precipitate of tyrosinase with an apparent molecular weight of 32KDa.

The protein was separated and bands were visualized under UV transilluminator. The molecular weight was found to be 29.0KDa.

Tyrosinase Inhibition Activity

Three samples *Vetiveria zizanioides*, *Glycyrrhiza glabra* and *Rosa indica* were analysed for its tyrosinase inhibition activity.

Percentage inhibition of tyrosinase activity was calculated as the following:

$$\% \text{ tyrosinase inhibition} = (A-B) - (C-D) \times 100 / (A-B)$$

Figure :1 *Agaricus bisporus*



Figure 2: Mushroom extract



Figure 3: Ammonium precipitate



Table -1 Estimation of potassium phosphate eluted protein sample

Estimation of Potassium Phosphate					
7.5mM	12.5mM	25mM	50mM	100mM	
0.113	0.142	0.142	0.089	0.128	

Table -2 Estimation of Sodium chloride eluted protein sample

Estimation of Sodium Chloride				
7.5mM	12.5mM	25mM	50mM	100mM
0.134	0.105	0.155	0.140	0.144

Table: 3 Tyrosinase Inhibitory Activity of Herbal Extract

S. No	Sample	Absorbance				Percentage of Inhibition
		A	B	C	D	
1.	<i>Vetiveria zizanioides</i>	0.423	0.061	0.294	0.167	64.91%
2.	<i>Glycyrrhiza glabra</i>	0.423	0.061	0.684	0.606	78.45%
3.	<i>Rosa indica</i>	0.423	0.061	0.687	0.531	56.90%

Figure 4: Sephadex column Gel filtration



Figure 5: DEAE-Cellulose column chromatography



Figure 6: *Inhibitory Activity of Tyrosinase by Herbal Extracts*

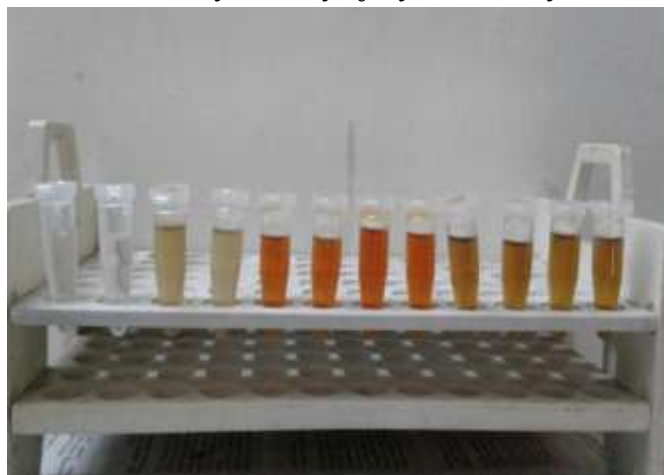


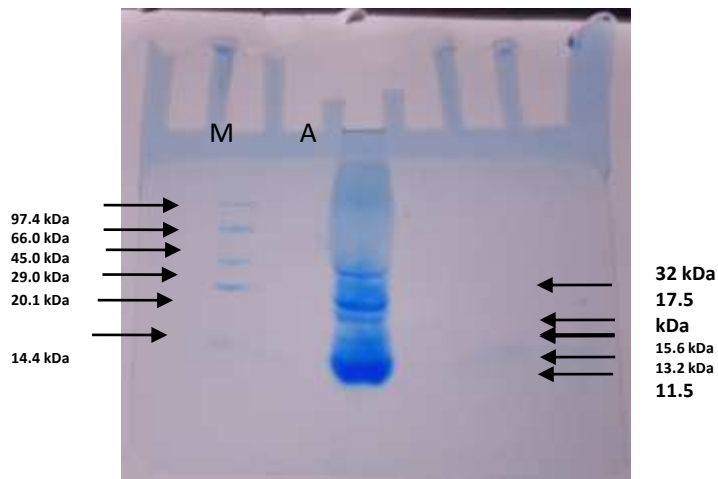
Figure 7: Potassium phosphate protein eluted sample



Figure 9: Sodium chloride protein eluted sample



Figure 11 : SDS PAGE Analysis Of Sample Tyrosinase



M: Protein Molecular weight Marker, A: Tyrosinase

Figure 8: Graphical representation of estimation of potassium phosphate eluted protein sample over the concentration of 7.5mM to 100mM

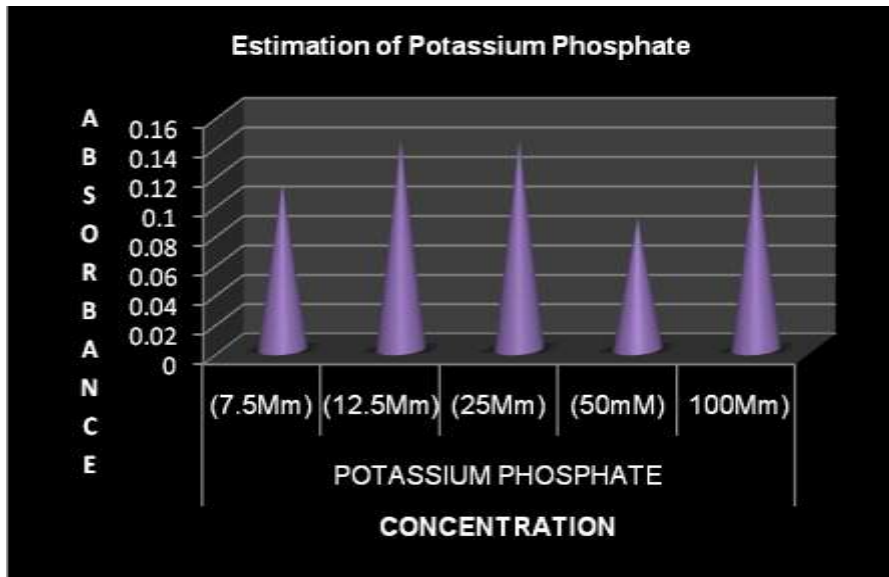


Figure 10: Graphical representation of estimation of sodium chloride eluted protein sample over the concentration of 7.5mM to 100mM

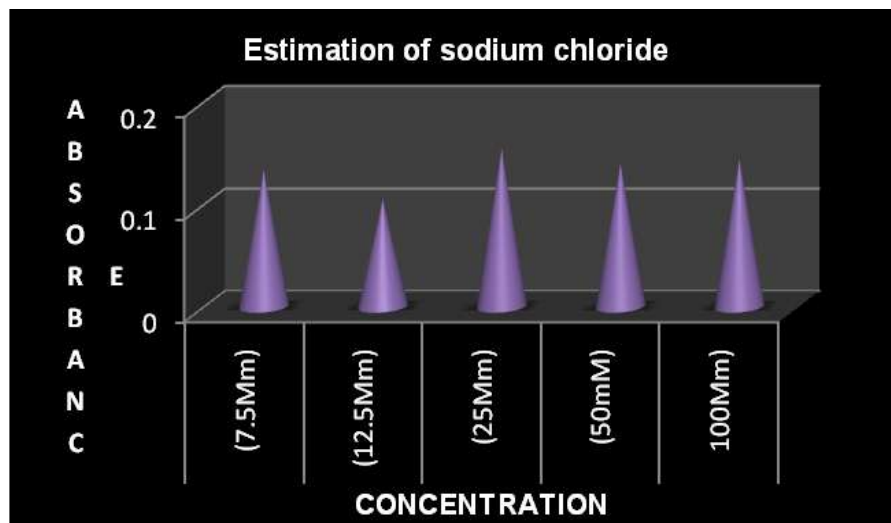
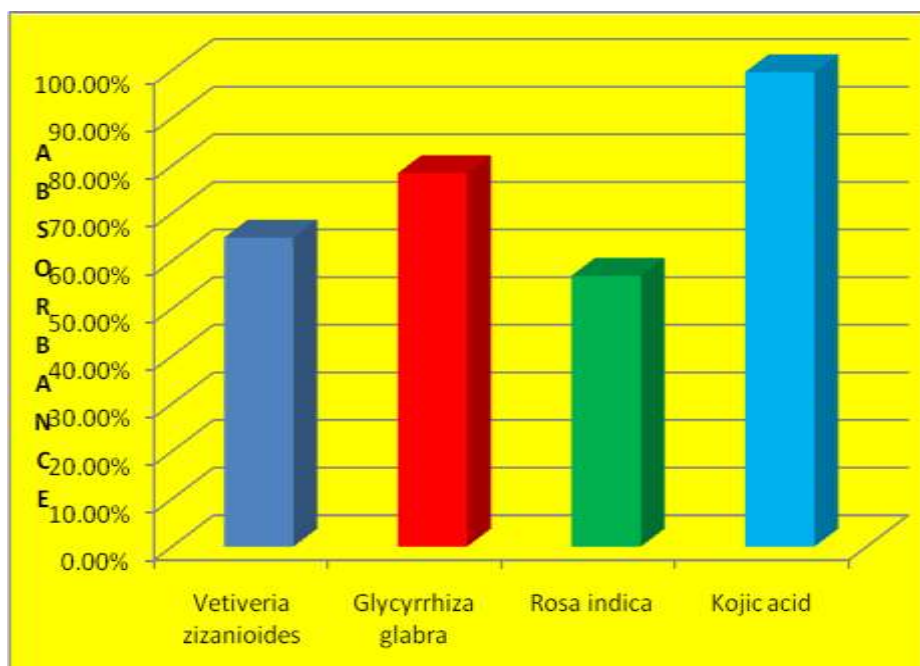


Figure 12 : Graphical representation of tyrosinase inhibitory activity of herbal extract and kojic acid

Cytotoxicity assay for plant extract

The *Glycyrrhiza glabra*) plant extract was evaluated for cytotoxic effects on Vero cell line. The morphology of the cells were inspected daily and observed for microscopically detectable alterations, *i.e.* loss of monolayer, granulation and vacuolization in the cytoplasm. Cell viability was monitored after 72 hrs and has been recorded.

Tyrosinase, also known as polyphenol oxidase, is a copper-containing enzyme, which is widely distributed in microorganisms, animals, and plants. Now a days mushroom tyrosinase has become popular because it is readily available and useful in a number of applications. *Agaricus bisporus* was known to contain tyrosinase and laccase enzymes and tyrosinase activity develops at the time of initiation and development of fruit bodies [13]. The discovery of its melanogenic properties, tyrosinase has been in prime focus and microbial sources of the enzyme are sought [11]. This work presents the extraction of tyrosinase enzymes from edible mushroom *Agaricus bisporus* and tyrosinase inhibition properties of *Vetiveria zizanioides*, *Glycyrrhiza glabra* and *Rosa indica*.

In this present study, initial extraction of enzymes was done by using cold phosphate buffer through homogenization and centrifugation of mushroom's (*Agaricus bisporus*) fruiting body. The extract obtained after centrifugation was dark in colour due to the occurrence of melanin. The extract was purified by ammonium sulphate salt precipitation, dialysis, gel filtration, ion exchange chromatography. After ammonium sulphate salt precipitation, dialysis and gel filtration purification steps, the extract was turned to colourless. Earlier studies stated that none of these methods were successful without causing a decrease in the recovery of enzyme activity or not being able to remove a substantial amount of colored material. In this present study, the change of colour indicates that the enzyme is successfully extracted and purified and enzyme activity recovered [11].

After purification, the extracted mushroom tyrosinase was analysed for its inhibition by selected herbal plants namely, *Vetiveria zizanioides*, *Glycyrrhiza glabra* and *Rosa indica*, Kojic acid was used as standard. The percentage of inhibition values were found to be 64.91%, 78.45%, 56.90% and 99.67% respectively. All selected herbs were found to have good tyrosinase inhibition activity.

When a fractionation with ammonium sulfate precedes ion-exchange chromatography in a purification procedure, it is often necessary to remove excess salt before application of the protein mixture to the column [14]. This is done to ensure that the ionic strength of the solution is sufficiently low for adsorption of at least

some of the proteins in the mixture. Diethylaminoethylcellulose (DEAE-cellulose) ion-exchange chromatography are widely used in protein purification are salting-out by high concentrations of ammonium sulfate. In this present study, active fraction of enzyme was finally purified by DEAE cellulose ion exchange chromatography to remove the ammonium sulfate. Sodium chloride and potassium phosphate were used as gradients. The enzyme was estimated by sodium chloride and potassium phosphate gradients. Upon estimation of purified enzyme, the sodium chloride eluted enzyme sample which gave maximum absorbance value 0.155 at 595nm was selected and confirmed by SDS PAGE technique. On SDS PAGE electrophoretic technique, a clear band of tyrosinase was observed with 32 kDa resides 29 kDa of protein molecular weight marker.

G. glabra are used in cosmetic preparations owing to their skin-whitening, anti-sensitizing, and anti-inflammatory properties [3]. In this study *Vetiveria zizanioides*, *Glycyrrhiza glabra*, *Rosa* sp. air dried powder sample were extracted using water in soxhlet apparatus. The extracts tyrosinase inhibitory activity were evaluated by using kojic acid as standard. Three extracts showed tyrosinase inhibition in L- dopa solution. Among three, the *Glycyrrhiza glabra* extract showing maximum tyrosinase inhibitory effect. It is proved to be that *Glycyrrhiza glabra* extract can inhibit tyrosinase activity upto 78.45% whereas kojic acid inhibited tyrosinase upto 99.67%. Kojic acid is currently used as tyrosinase inhibitors which are commercially available. Unfortunately, unstability during storage limits its use and new tyrosinase inhibitors of novel kojic acid derivatives are needed in cosmetics industry [15]

On compared with *Glycyrrhiza glabra*, though kojic acid has powerful inhibitory activity, but also contains lot of disadvantages on stability and on mammalian skin. *Glycyrrhiza glabra* on the other hand, does not possess any disadvantages or side effects while inhibiting tyrosinase. In this study it is concluded that *Glycyrrhiza glabra* can be used as melanin inhibiting hypopigmentation drugs and cosmetics incase of melasma, freckles, etc and as alternative of kojic acid.

In the study, Vero cell lines were used to check the cytotoxic effect of *Glycyrrhiza glabra*. It was found to be non toxic after cell line procedure. As reported in earlier studies Glycyhhrizin from *Glycyrrhiza glabra* reduces skin discoloration [2], the study proves that *Glycyrrhiza glabra* extract can inhibit tyrosinase is essential for melanin synthesis. The extract of *Glycyrrhiza glabra* proved to be safe and does not affect mammalian cells.

Conclusion

Tyrosinase enzymes constitute one of the most important group of commercial enzyme. These enzyme have ample utilization in industrial process, such as pharmaceuticals and cosmetic and food industries. There are considerable reports indicating the great potential of this enzyme in medicine, agricultural industries and analytical and environmental purposes. It is also used to produce synthetic melanin which provides protection against radiation and is used as cation exchangers, drug carriers, antioxidants, antiviral agents.

The present study, focus on synthesizing and characterizing of the tyrosinase from Indian edible mushroom. *Agaricus bisporus* was employed in the work in synthesizing tyrosinase, because its tyrosinase show very high similarities compared to human tyrosinase and it is the chiefly available mushroom.

In the study, Mushroom tyrosinase was extracted and purified. The selected plants namely *Vetiveria zizanioides*, *Glycyrrhiza glabra* and *Rosa indica* showed good tyrosinase inhibitory activity against extracted mushroom tyrosinase. The further studies on these herbs can lead to the new skin lightenig creams and can be used for hyperpigmentation like medicinal products in the future.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgement

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