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Anti-Tyrosinase Activity Of *Stachytarpheta Cayennensis* in Vitro

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ABSTRACT: Tyrosinase, also known as polyphenol oxidase, is a key enzyme that catalyzes synthesis of melanin in plants, microorganisms and mammalian cells. Melanin biosynthesis inhibitory compounds are useful for skin whitening agents used in cosmetics and also as a remedy for disturbances in pigmentation. Contributing to their roles in tissue remodeling in health and disease, several studies have reported investigations on plant extracts as inhibitors of proteinases and as anti-oxidants.

The correlation between the radical scavenging activity and tyrosinase inhibitory activity designate the ability of the compound in preventing free radical caused skin cells damage, anti-wrinkle and reduce hyperpigmentation. Phenolic-like structure in their compounds and were reported to possess tyrosinase inhibitory effect. Arbutin, kojic acid and kojic derivative were used as whitening cosmetic and medicines, but clinical effects of these compounds are unsatisfactory. The polyphenolic compounds found in many plants were gained interest for cosmetic product development as they might possess less toxicity than the synthetic compounds. The usage of pure chemical in the cosmetic product such as hydroquinone as whitening agent was reported to be toxic, induce severe skin irritation and skin cancer in long-term used.

The anti-tyrosinase activity of the different plant extracts was determined by using mushroom tyrosinase as a suitable model system. The antioxidant based on DPPH scavenging activity and tyrosinase inhibitory
activities in vitro were performed and determined for the correlation of both activities. The results showed that anti-tyrosinase activity was not in dose dependent manner with the plant extract, which have shown 10.8% inhibition at the concentration of 20µg/ml. This result suggests that the plant extract might be used as skin whitening and anti- ageing agents.

KEYWORDS: Tyrosinase, Melanin, Anti-tyrosinase activity, antioxidant, skin whitening, anti-ageing properties

1. INTRODUCTION

Skin ageing is time dependent and environmental factor exert and influence. Dyschromic skin changes occur in aged skin where slower turnover causes thinning of epidermis that makes the skin look translucent [1]. Skin wrinkling and loss of elasticity follows the decreases of fibroblast cells and affects the decrease of collagen and elastic fibre synthesis, in aged skin. The photoageing skin damage is caused by reactive oxygen species (ROS) which may reduce the strength of skin cell walls, as well as degrading collagen and elastic fibers, resulting in loss of skin humidity and elasticity leading to skin wrinkling [2.3.4] However, sunlight can induce skin tanning by ROS which may activate tyrosinase enzyme.

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It is proposed that oxidative stress plays a central role in initiating and driving the signaling events that lead to cellular mutations that cause chronic disease (and skin ageing). When there is an escalation of endogenous and exogenous oxidative stressors, the surplus of reactive oxygen species (ROS) may have a degenerative effect on the body (and skin). At the cellular level, ROS may denature proteins, alter cell cycles, and influence the release of pro-inflammatory mediators (i.e.cytokines), which may trigger the induction of some inflammatory skin diseases.

Hyper-pigmentation of the skin is a common problem in middle aged and elderly people. It is caused due to over production of melanin pigment, which is responsible for the colour of hair and skin in humans. The overproduction of melanin may be due to the chronic exposure to sun, melasma, or other hyper pigmentation diseases. Tyrosinase is the key enzyme in melanin production [5, 6.7]

The enzyme tyrosinase (EC 1.14.18.1, syn. polyphenol oxidase, PPO; monophenol; dihydroxy-L-phenylalanin; oxidoreductase) is known to be a multifunctional copper-containing enzyme from the oxidase superfamily. This is the key enzyme which is involved in the biosynthesis of the large biological pigment, melanin. This enzyme catalyzes two types of reactions of melanin biosynthesis, the hydroxylation of L-tyrosine to 3-4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to o-dopaquinone. This o-quinone is a highly reactive compound and can polymerize spontaneously to form the pigment melanin, which causes a serious aesthetic problem in human beings [8, 9.10]

Biological systems need ROS for metabolic pathways and thus the body is capable of forming reactive species such as superoxide ($O2^-$) and nitric oxide (NO) [11]. When ROS are overproduced, redox-active transition metal ions such as iron (II) or copper (II) can cause severe oxidative stress and thus damage tissues and the cellular DNA, protein, lipid and carbohydrate constituents within [12]. Superoxide dismutase (SOD) which naturally breaks down $O2^-$ into H2O2and O2 has a short plasma half-life and thus novel SOD mimetics are being developed [13]. Flavonoids derived from plants can form complexes with metal ions which mean they have the potential to bind with metalloenzymes thus altering or inhibiting metabolic pathways [14] and flavonoid-metal complexes have shown potential to be SOD mimetics [15]. In terms of anti-ageing, finding inhibitors of elastase enzymes can be useful to prevent loss of skin elasticity and thus skin sagging.

The tyrosinase inhibitors are not only useful for the medicinal purposes, but also they can be used for improving food quality and nutritional value, controlling insect, pests, etc. So it is very important to discover novel and potent inhibitors of the enzyme tyrosinase from the plant extract materials.

II. MATERIALS AND METHODS

2.1 Preparation of plant extracts

The plant *Stachitarpheta cayennensis* is dried in an open environment. It was then crushed in grinder to get the powder (50g). The powder was packed in the filter paper by making the pocket and was then placed in the soxhlet apparatus and the 500ml of methanol was added on the first day and the very next day additional 500ml of methanol was added. The methanolic extract was collected filtered using filter paper and the filtrate was evaporated at 40°c in hot air oven. The required material was stored at -20°c.

2.2 Antiradical Activity Test –

The antiradical activity of the extracts was estimated according to the procedure by using quercetin. 0.3mM solution of DPPH radical solution in ethanol 90% was prepared and then 1ml of this solution was mixed with 2.5ml of different concentrations of each extract. After 30 min incubation in dark and at room temperature, absorbance (A) was measured at 518nm in a spectrophotometer. The percentage of the radical scavenging activity (RSA) was calculated by the following equation.

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$$RSA\% = [A_{control} - (A_{sample} - A_{blank})] / A_{control} \times 100$$

A_c-Absorbance of control (DPPH)
A_s- Absorbance of sample and DPPH
A_o- Absorbance of sample or standard without DPPH

Ethanol 90% (1ml) plus each sample solution (2.5ml) was used as a blank. DPPH solution (1ml) plus ethanol 90% (2.5ml) was used as a negative control, rutin solution (at the concentrations of used 100µg/ml, 50µg/ml, 25µg/ml, 10µg/ml, 5µg/ml, and 2.5µg/ml) as a positive control.
The IC_{50} value for each sample defined as the concentration of the test sample leading to 50% reduction of the initial DPPH concentration was calculated from the nonlinear regression curve Log concentration of the test extract (µg/ml) against the mean percentage of the radical scavenging activity.

2.3 Reagents
Enzyme: Tyrosinase (from mushroom, 25000units, Sigma, USA, store at -20°c). [16, 17]
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°c
Other chemicals were of the highest grade commercially available.

2.4 Preparation of working solutions
Enzyme: Stock1 (25000U/250ul)Working solution (5600units/ml): 50ul of stock1 is made up to 1ml with 50mM Potassium phosphate buffer, pH6.5.
Substrate: 10mM (1mg/ml) L-DOPA is Prepared in 0.1Mphosphate buffer.
Positive control (1mg/ml): 5mg of Kojic acid is dissolved in 5ml of 50mM Potassium phosphate buffer, pH6.5.
Test Sample: (1mg/ml) test sample is dissolved in 0.1M phosphate buffer.

2.5 Principle

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

Quantification of Dopachrome done at 475 nm spectrophotometrically

2.6 Method
The assay was carried out by following steps

- Tyrosinase enzyme (80µl) is mixed with 80µl of kojic acid or sample in 96 well plate and incubated at 37°c for 15 min.
- L-DOPA (40µl) is added to the mixture and incubated at 37°c for 30 min.
- Concentration of L-DOPA in the final reaction mixture is 1mM
- The amount of Dopachrome formation is read at 475nm.
- Well without any inhibitor /samples serve as control percentage of tyrosinase inhibition (% inhibition) is calculated using the formula.

% inhibition = 100 - {(A_{475} of sample / A_{475} of control) * 100}

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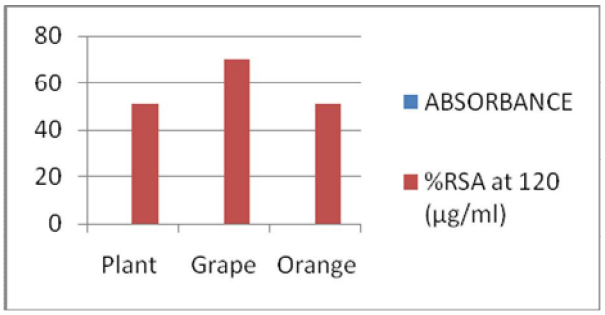
III. RESULTS AND DISCUSSION

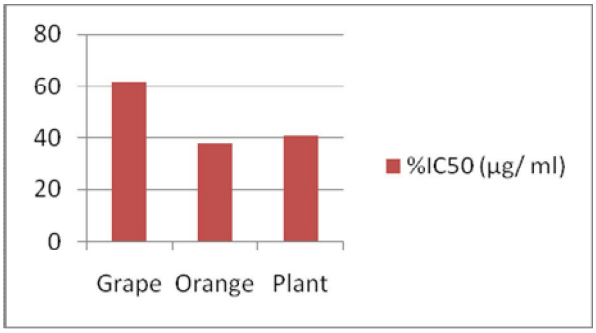
Hence from the above results it can be said that the grape seed extract shows the highest percentage of radical scavenging activity while the plant and the orange peel extract shows the equal amount of radical scavenging at the given maximum concentration i.e. (120 ug/ml).



Graph: 1 ---- % RSA

SAMPLE	%IC_{50} (µg/ ml)
Grape	61.76
Orange	38.23
Plant	41.17

Table: 1 -- IC_{50} Values



Graph: 2 ---- The IC_{50} value of the grape seed extract was found to be of the highest value and the least was found to be of orange peel extract.

The anti-tyrosinase assay was performed by taking kojic acid as standard. It was found out that all the three samples have potential of skin whitening but it was not in dose dependent fashion. Grape seed extract have shown 7% inhibition at the concentration 20µg/ml, while the plant extract have shown10.8% inhibition at the concentration of 20µg/ml and 7.7% inhibition was found at the concentration of 20µg/ml (Table 2). Among the three, plant extract is more effective and the least have been shown by grape seed extracts.

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Name of compound	Concentration	A_{485} nm	% Inhibition
Control	0.000	0.600	0.000
Kojic Acid	40	0.549	8.39
	80	0.538	10.24
	100	0.534	10.87
	200	0.495	17.47
	400	0.464	22.63
Grape seed	10.000	0.577	3.82
	20.000	0.556	7.27
Plant	10.000	0.549	8.54
	20.000	0.535	10.82
Orange peel	10.000	0.564	6.00
	20.000	0.554	7.72

Table 2 Anti-tyrosinase results

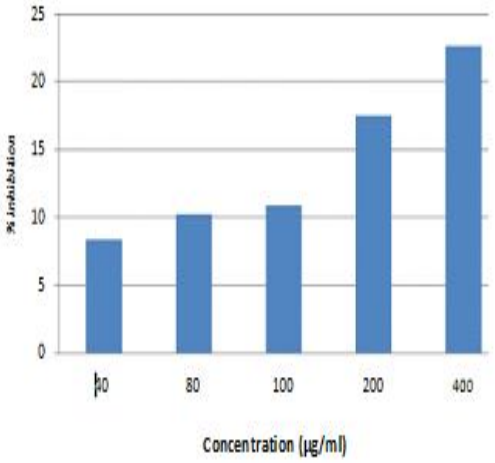

Fig. 1 Standard graph for Kojic acid

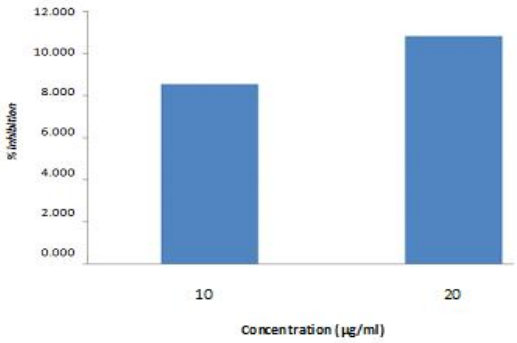

Fig. 2 Inhibition of tyrosinase by Plant extracts

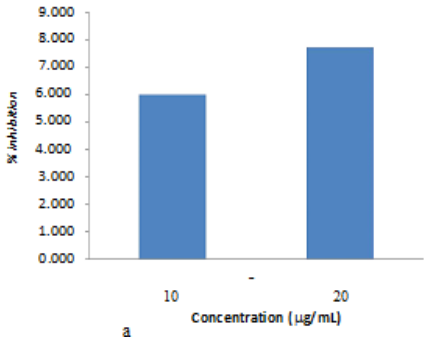

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Fig. 3 Inhibition of tyrosinase by Orange peel extract

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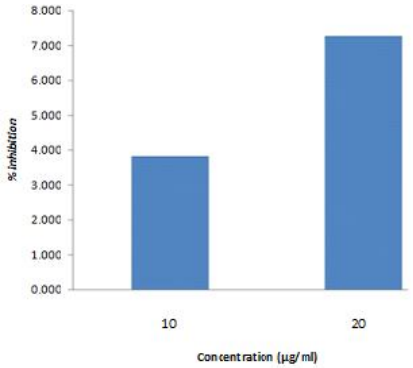

Fig. 4 Inhibition of tyrosinase by Grape seed extract HPLC Data

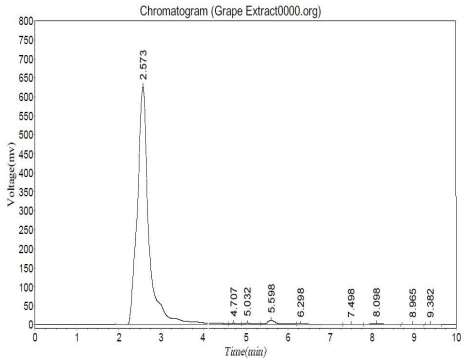

Fig. 5 The chromatogram of the grape seed extract

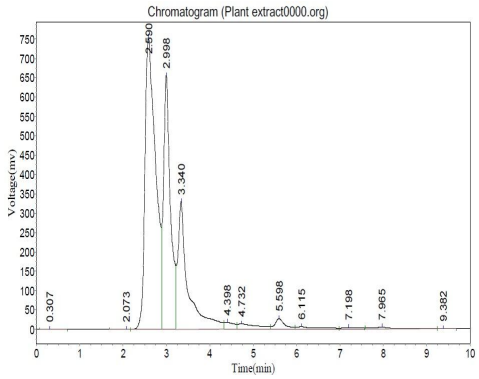

Fig. 6 The chromatogram of the *Stachytarpheta cayennensis* plant extract

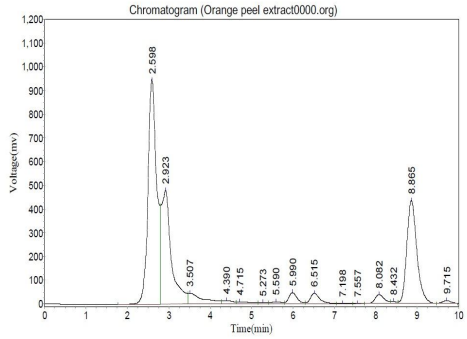

Fig. 7 The chromatogram of the orange peel extract

The phenolic concentration of the extract is determined with the standard gallic acid in HPLC. It was found out that the percentage content of phenolic compound in the given sample of Orange peel extract, *Stachytarpheta cayennensis* plant extract and grape extract was found to be equivalent to0.23 %, 0.22 % and 0.12 % of gallic acid respectively**.**

In one the study total phenolic content varied between 0.05 and 0.26 mg gallic acid equivalents (GAE)/mL with the exception of white tea (0.77 mg GAE/mL). For anti-oxidant assessment, the Trolox equivalent antioxidant capacity (TEAC) assay revealed activity for all extracts. White tea had the highest activity equivalent to ~21 µM Trolox for a 6.25 µg aliquot. High activity for white tea was also found in the superoxide dismutase (SOD) assay in which it exhibited ~88% inhibition of reduction of nitroblue tetrazolium. High activities were also observed for green tea (86.41%) (18).

Investigations on the anti-elastase, anticollagenase and anti-hyaluronidase activity of C. glabrum, P. capensis, P. africanum and S. brachypetala.
The free radical scavenging activity and enzyme inhibitory activity of the plant extracts suggests that they can help restore skin elasticity and thereby slow the wrinkling process. *P. africanum* was the plant with the most promising activity and will be subjected to further testing and isolation of the active compound/s [19]

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In another research study on the herbal plants a potential effectiveness as skin-whitening agents and in maintaining skin health were observed in the extract of *Glycyrrhiza glabra (rhizome)* was shown to be potent tyrosinase inhibitors in human skin [20]

All the parts of *Ixora coccinea*, when exerted showed good anti-tyrosinase and antioxidant activities in methanol when compared to other solvent extracts of respective parts. This indicates that the compounds responsible for the above said activities are highly extractable in methanol. It can be inferred from the results of this study that *Ixora coccinea* is a potential candidate as the active ingredient of products for topical application as skin whitening and anti-ageing in cosmetic formulations [21].

IV. CONCLUSION

Stachytarpheta cayennensis Plant we have selected might have the immense potential to be used as an ingredient in medicine for skin whitening and cosmetics. The plant is available throughout the year therefore it is very suitable and economical for medicinal use. The mixture of these three materials could be an ideal formulation on the (tyrosinase inhibitory) skin whitening and anti ageing properties. Since these obtained results *in vitro* can be validated *in vivo* before brought in use

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