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Inhibitory activities of mushroom tyrosine and DOPA oxidation by plant extracts

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Abstract

Pigmentation has become an important phenotypical characteristic, in the pharmaceutical, medicinal as well as in the cosmetic field. Plants and their extracts are inexpensive and rich resources of active compounds that can be utilized to inhibit tyrosinase activity as well as melanin production. Natural and synthetic chemical agents can frequently modulate the metabolism of pigmentation produced. Methanol extracts of seven plants were evaluated for their inhibitory effect on the monophenolase and diphenolase activated forms of tyrosinase *in vitro*. Active extracts were also investigated for their inhibitory effect on melanogenesis in B16 melanoma cells. Extracts of *Hyaenanche globosa* and *Myrsine africana* showed 92% and 83% inhibition of tyrosinase activity and 42% and 62% inhibition of DOPA auto-oxidation at 500 µg/ml respectively. Rooibos tea which is known for its antioxidant activity did not show any inhibition of tyrosinase or DOPA oxidation activity. *M. africana* demonstrated 16% and 18% inhibition of melanin production at 6.25 and 12.50 µg/ml respectively.
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Keywords: Tyrosinase inhibition; DOPA auto-oxidation; Plant extracts; Melanin; Melanocyte cell

1. Introduction

Skin is an important component of body image and has immense physiological importance for both women and men. Skin pigmentation can be a source of significant emotional distress in individuals (Stratigos and Katsambas, 2004). Along human history, people have been struggling with numerous skin diseases, especially skin pigmentation (hyper/hypo-pigmentation). It is well documented that tyrosinase (E:C: 1.14.18.1) is an essential enzyme, which contributes towards pigment formation in mammalian's body as well as in plants, microorganisms and fungi. Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin-whitening effects. To approach this desire, many products have been already produced and tested to overcome melanogenesis problems.

As known, melanin is the root cause for blackening of the skin. Its formation beneath the skin proceeds through free-radical mechanism. UV-radiations facilitate this chain reaction and it could be disrupted by selective use of compounds, potent enough to inhibit this reaction. It is well known that tyrosinase (monophenol monooxygenase) or polyphenol oxidase (PPO), is a copper-containing monooxygenase. Tyrosinase is able to catalyze two reactions involving molecular oxygen: the hydroxylation of monophenols to *O*-diphenols (monophenolase activity) and the oxidation of the *O*-diphenols to *O*-quinones (diphenolase activity). Quinones are highly reactive compounds and can polymerize spontaneously to form high-molecular-weight compounds or brown pigments (eumelanin or pheomelanin) or react with amino acids and proteins that enhance the brown colour produced (Nerya et al., 2003).

Melanin is one of the most widely distributed pigments and is found in bacteria, fungi, plants and animals. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis (Kim and Uyama, 2005). Melanocytes are neural crest-derived cells that migrate to the epidermis during embryogenesis and subsequently synthesize and distribute melanin to

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surrounding keratinocytes (Yaar et al., 2006). Melanocytes have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin (Tiedtke et al., 2004). Melanin plays an important role in protecting human skin from the harmful effects of ultraviolet (UV) radiations by absorbing UV sunlight, removing reactive oxygen species (ROS) and scavenging toxic drugs and chemicals. Melanin also induces the expression and synthesis of a variety of cytokines, primarily of keratinocyte origin, that act in a paracrine fashion to further induce melanogenesis (Yaar et al., 2006).

The accumulation of an abnormal melanin amount in specific parts of the skin as more pigmented patches (melasma, freckles, ephelide, senile lentigines etc.) might become an aesthetic problem (Solano et al., 2006). The type and amount of melanin synthesized by the melanocytes and its distribution in the surrounding keratinocytes determine the actual colour of the skin (Kim and Uyama, 2005).

A number of skin lightening agents which are derived from natural resources particularly plants, are already used in cosmetic products such as; Hydroquinone (isolated from *Cystoseria jabokae* and *C. adriatica*), Azelaic acid (isolated from *Pitirosporum ovale*), Kojic acid (fungal metabolic product) and arbutin (a glycosylated hydroquinone found in certain plants). Despite the extensive researches on lightening agents and hyperpigmentation, the existing agents have got limitations in term of high toxicity, low stability, poor skin-penetration, and insufficient activity. Tyrosinase inhibition is the most common approach to achieve skin hypo-pigmentation as this enzyme catalyses the rate-limiting step of pigmentation (Solano et al., 2006). Therefore, in this study we investigated the potential of a few plants for skin hypo-pigmentation.

Undoubtedly, South Africa is one of the world's major sources of useful plant resources. The South African flora consists of over 25 000 species of higher plants, of which 8600 are endemic (Taylor et al., 2001). Due to the rich plant diversity existing in South Africa; it is very encouraging to explore the potential of South African plants for cosmaceutical purposes. A number of South African plants are known to have various phenolic and triterpenoid compounds such as catechin, chalcone, flavones, quercetin, saponins etc. (Arramon et al., 2002).

Plants [*Aspalathus linearis* N.L. Burm. (Fabaceae), *Hyaenanche globosa* Lamb. (Euphorbiaceae), *Myrsine africana* L. (Myrsinaceae), *Quisqualis indica* L. (Combretaceae), *Salvia africana lutea* L. (Lamiaceae), *Quercus robur* L. (Fagaceae), and *Olea europaea* L. (Oleaceae)] were selected for the present study based on their chemical constituents specially those which are known to be rich in the phenolic and triterpenoid group of compounds. They are being used frequently for skin-hyperpigmentation and medicinal purposes. *A. linearis* is used in various cosmetic products ranging from shampoos, tonics, creams and skin care products. It is used frequently to treat skin problems such as eczema, nappy rash and acne, so when applied topically it has a soothing effect on the skin. An aqueous suspension of powdered seeds of *M. africana* is used as a body tonic for young men (Githihiori, 2004). Belcour-Castro et al. (2007) reported the usage of the extract of *M. africana* for dyeing keratin fibers. The Nama (a Namibian tribe) people from South Africa use a decoction of the leaves of *S. africana lutea* for coughs, colds and female ailments (Nerya et al., 2003). More than 20 compounds have been isolated from the bark of *Q. robur* such as epicatechin, catechin, catechin gallat, saponins etc (Arramon et al., 2002) and (Kuliev et al., 1997). These compounds are known to have anti-tyrosinase activity. Two flavonoids "diosmin" and "rutin" which are widely used as pharmaceuticals have been isolated from the leaves of *O. europaea*. It is well known that the flavonoids show good inhibition of tyrosinase activity (Kuliev et al., 1997).

Methanol extracts of selected plants were evaluated for their inhibitory effect on the monophenolase and diphenolase activated forms of tyrosinase *in vitro*. The active extracts were also investigated for their inhibitory effect in melanogenesis in B16 melanoma cells.

2. Material and methods

2.1. Materials

L-Tyrosine, L-DOPA, tyrosinase, arbutin and kojic acid were obtained from Sigma-Aldrich (Kempton Park, South Africa). Cell culture reagents and equipment were purchased from Highveld Biological (Sandringham, South Africa), LASEC (Randburg, South Africa) and The Scientific Group (Midrand,

Table 1
Inhibitory activities of mushroom tyrosinase and DOPA auto-oxidation by plant extracts

Plant name	Plant part used	Inhibition of DOPA auto-oxidation (%) at 500 μg/ml	Inhibition of tyrosinase (%) at 500 μg/ml	Voucher herbarium specimen number
Aspalathus linearis N.L. Burm. (Rooibos)	Tea bag	17	7	_
Hyaenanche globosa Lamb.	Aerial parts	42	92	S.M. 95499
Myrsine africana L.	Aerial parts	62	83	S.M. 95503
Olea europaea L.	Bark	49	74	S.M. 95501
Quercus robur L.	Aerial parts	29	25	S.M. 95500
Quercus robur L.	Bark	24	20	S.M. 95500
Quiscualis indica L.	Aerial parts	34	39	S.M. 95504
Salvia africana lutea L.	Aerial parts	36	48	S.M. 95505

South Africa). The B16-F10 mouse melanocyte cell line was obtained from Highveld Biological (Sandringham, South Africa).

2.2. Preparation of plant extracts

Six plants (aerial parts and bark) were collected from the Botanical Garden of the University of Pretoria during September 2005 (Table 1). The plants were identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria. Rooibos (fermented) tea bags were also used in the present study (Glen Rooibos, Manufactured by, Unilever Bestfood Robertstons (Pty) Ltd. 15 Nollsworth Crescent, La Lucia, 4051, South Africa). Forty grams of each powdered plant (shade dried) was soaked in 200 ml of methanol for 4 h and after filtration the solvent was removed under vacuum (BUCHI, Rotavapor, R-200) to yield dry extracts.

2.3. Inhibition of tyrosinase activity and DOPA auto-oxidation

This assay was performed using methods as described earlier (Curto et al., 1999; Nerya et al., 2003). Extracts were dissolved in DMSO (dimethyl sulphoxide) to a final concentration of 20 mg/ml. This extract stock solution was then diluted to 600 μg/ml in 50 mM potassium phosphate buffer (pH 6.5). Firstly, the extracts were tested only at one concentration 500 μg/ml for their inhibitory effect on the monophenolase and diphenolase activated forms of tyrosinase *in vitro*. Thereafter the best two samples were selected for dose response study and the concentrations ranged from 3.125 to 400 μg/ml. Arbutin and Kojic acid, which were used as positive controls were also tested at concentrations 1.562 to 200 μg/ml (Curto et al., 1999). In 96-well plate, 70 μl of each extract dilution was combined with 30 μl of tyrosinase (333 Units/ml in phosphate buffer) in triplicate. After incubation at room temperature for 5 min, 110 μl of substrates (2 mM L-tyrosine or 12 mM L-DOPA) was added to each well. Incubation commenced for 30 min at room temperature. Optical densities of the wells were then determined at 492 nm with the BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa). The concentration of plant extract at which half the original tyrosinase activity is inhibited (IC_{50}), was determined for each plant extract.

The percent inhibition of tyrosinase activity was calculated as follows:

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

Where A = absorbance at 492 nm without test sample, and B = absorbance at 492 nm with test sample.

2.4. Cell culture

Best extracts; *H. globosa*, *M. africana* and positive controls (Kojic acid and arbutin) were also investigated for their inhibitory effect on melanogenesis in B16 melanoma cells at concentrations ranging from 1.5 to 100 μg/ml. This assay was carried out using method as described earlier (Curto et al., 1999; Nerya et al., 2003). The murine melanocyte cell line, 'B16-F10' was cultured in complete basal medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM L-glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin and 0.25 μg/ml fungizone at 37 °C with 5% CO_2 in a humidified atmosphere. Cells were sub-cultured in a ratio of 1:3 on every third or fourth day. For *in vitro* experiments, B16-F10 cells were resuspended in complete DMEM medium containing 10% fetal bovine serum, 1.5 g/L $NaHCO_3$, 2 mM L-glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml fungizone.

2.5. Toxicity test

The toxicity of the best extracts and positive controls (arbutin and Kojic acid) on the B16-F10 cells was assayed by making use of an XTT (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro benzene sulfonic acid hydrate), labelling reagent) cytotoxicity assay. Stock solution of the extracts and positive controls (Kojic acid and arbutin) were prepared to a concentration of 20 mg/ml in DMSO. The samples (extracts and positive controls) were diluted with medium and 100 μl of each concentration was added to the wells to give the final concentrations ranging from 1.5 to 100 μg/ml. Fifty microlitres of XTT reagent (1 mg/ml XTT was mixed with 0.383 mg/ml PMS) was added to the wells and incubated for 1 h at 37 °C. The optical densities of the wells were then measured at 450 nm (690 nm reference wavelength). By referring to the control (medium with DMSO), cell survival was assessed (Zheng et al., 2001).

2.6. Inhibition of melanin production in melanocyte cells

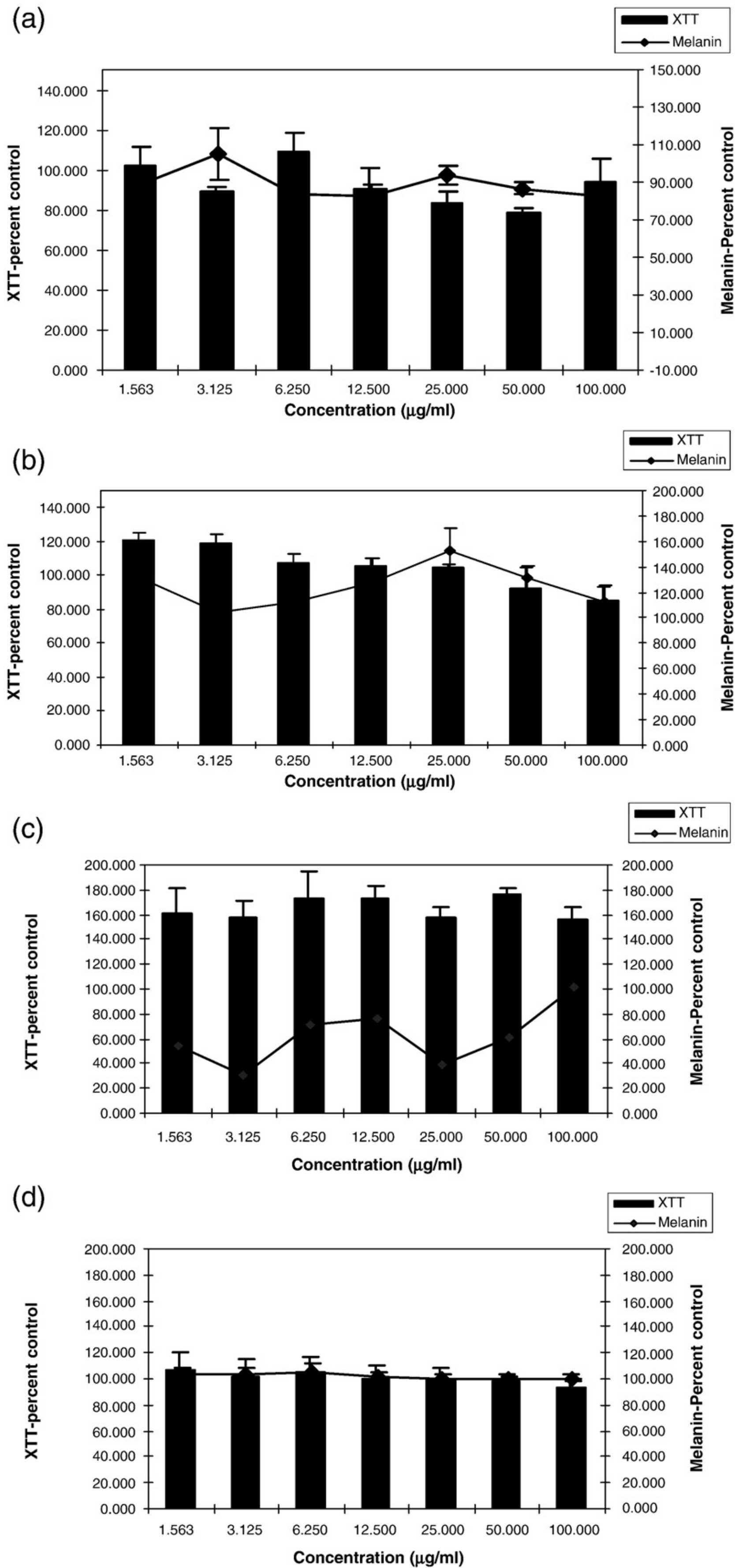
The effect of the extracts on melanin synthesis was determined by washing the cells in the 24-well plate with PBS and lysing with 200 μl of sterile distilled water. Optical densities were determined at 405 nm. The effect on melanin production was determined by referring to the control sample (medium with DMSO) (Curto et al., 1999) and (Nerya et al., 2003).

2.7. Statistical analysis

The final results are expressed as the mean (mean ± standard deviation). The group means were compared using the ANOVA

Table 2
The IC_{50} (concentration of plant extract at which half the original tyrosinase activity is inhibited) values of *Hyaenanche globosa* and *Myrsine africana* for monophenolase (L-tyrosine) and diphenolase (L-DOPA) inhibitory activity

Sample	IC_{50} (tyrosinase) μg/ml	IC_{50} (DOPA) μg/ml
Hyaenanche globosa	27.1 ± 0.078	>200
Myrsine africana	22.51 ± 0.420	200
Arbutin	149	>200
Kojic acid	2.145 ± 0.082	26.66 ± 0.104


(a)
XTT
Melanin
XTT-percent control
Melanin-Percent control
Concentration (µg/ml)
1.563
3.125
6.250
12.500
25.000
50.000
100.000
(b)
XTT
Melanin
XTT-percent control
Melanin-Percent control
Concentration (µg/ml)
(c)
XTT
Melanin
XTT-percent control
Melanin-Percent control
Concentration (µg/ml)
(d)
XTT
Melanin
XTT-percent control
Melanin-Percent control
Concentration (µg/ml)

test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple Range Test was applied to compare the means. Values were determined to be significant when p was less than 0.01 ($p<0.01$).

4. Results and discussion

The extracts of *H. globosa*, and *M. africana* showed 92% and 83% inhibition of tyrosinase activity respectively at 500 µg/ml ($p<0.01$). They also demonstrated 42% and 62% inhibition of DOPA auto-oxidation respectively at 500 µg/ml ($p<0.01$). Despite it is well known that *A. linearis* (Rooibos) is rich in antioxidant compounds in our experimental condition the extract of Rooibos tea did not show any tyrosinase or DOPA inhibitory activity (Table 1). Other extracts showed marginal inhibition of tyrosinase and DOPA auto-oxidation activity. The "GraphPad Prism 4" program was used to calculate the IC_{50} values. The IC_{50} values of *H. globosa* and *M. africana* were determined as 27.1 and 22.51 µg/ml respectively, whereas the IC_{50} of the reference compounds, Kojic acid and arbutin were 2.145 µg/ml and 149 µg/ml respectively for monophenolase inhibitory activity. The IC_{50} values of *H. globosa* and *M. africana* were found to be >200 and 200 µg/ml respectively, as compared to the reference compounds, Kojic acid (26.66 µg/ml) and arbutin (>200 µg/ml) for DOPA inhibitory activity (Table 2).

Our results compare well with other reported results of the effect of plant extracts for inhibition of tyrosinase activity. It has been reported that *Glycyrrhiza glabra*, *Morus alba* and *Gastrodia ellata* (80% ethanol extract) showed 65%, 68% and 85% tyrosinase inhibition at the concentration of 333 µg/ml respectively (Lee et al., 1997). *Entada africana* [10% propylene glycol/deionized water (%, 50/50)], *Portulaca pilosa* [10% propylene glycol/deionized water (%, 50/50)], *Prosopis africana* [10% propylene glycol/deionized water (%, 50/50)] and *Cariniana brasiliensis* [10% propylene glycol/deionized water (%, 50/50)] exhibited 94%, 93%, 91% and 90% inhibition of tyrosinase activity respectively *in vitro* (Baurin et al., 2002).

M. africana and *H. globosa* did not show any toxicity on the melanocyte cells even at the highest concentration tested (100 µg/ml). *M. africana* demonstrated 16% and 18% inhibition of melanin production at 6.25 and 12.50 µg/ml respectively ($p<0.01$). *H. globosa* did not show significant inhibition of melanin production (Fig. 1a,b). Kojic acid (positive control) showed no significant toxicity to B16-F10 cells at the highest concentration tested and exhibited 60% reduction in melanin content at 3.1 µg/ml (Fig. 1c). Similar results have been obtained from the other plant extracts. Seventy percent ethanol extract of *M. alba* shows 7% and 10% inhibition of melanin production at concentrations 5 and 10 µg/ml respectively (Kim et al., 2006). It has been reported that Glyasperin C; an isolated compound from *Glycyrrhiza uralensis* showed 17.65% inhibition of melanin production at 5 µg/ml (Kim et al., 2005). Arbutin exhibited 2% inhibition of melanin production at 50 µg/ml and 100 µg/ml, while more than 90% of murine melanocyte cells were viable (Kim et al., 2006).

It can be concluded that *M. africana* and *H. globosa* showed good tyrosinase inhibitory activity and can be considered as effective tyrosinase inhibitors alone or in combination with the other plant extracts. These extracts exhibited more inhibition of tyrosinase activities and anti-melanogenesis activities than arbutin but less than Kojic acid. Identification and isolation principles from these plants are underway.

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Fig. 1. The effect of samples on cell viability/proliferation and melanin production by mouse melanocyte cells, (a) *Myrsine africana*, (b) *Hyaenanche globosa*, (c) Kojic acid and (d) arbutin.

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