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Research Article

Rapid screening and tentative identification of tyrosinase inhibitor from *Aspergillus Ornatus*

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ABSTRACT

A.niger-1 and *A.ornatus* showed 98.4 and 98.3 percent inhibition which were highest as compared to other fungi. Plate assay method appears to be the best and rapid method for the screening of tyrosinase inhibitors, as all isolates screened by plate assay has shown inhibition to partially purified enzyme. *A.ornatus* showed a strong positive reaction with $FeCl_3$ test indicating that the compound is kojic acid which was separated by TLC with a standard kojic acid and the separated compound again showed 98.5% tyrosinase inhibition. *A.niger -1* didn't show any of the compounds tested and require a detail study. When the percent inhibition of synthetic kojic acid was compared with naturally screened and identified kojic acid, the percent inhibition was 91% with synthetic IC_{50} 1mM, where as it was 98.5% inhibition which is appreciable with natural kojic acid irrespective of the concentration. Thus, use of such natural products in cosmetics from microbial sources play a significant role as they can be produced in an economical way with no side effects.

Keywords: *Aspergillus ornatus;* kojicacid; tyrosinase inhibition; Tyrosinase.

INTRODUCTION

Tyrosinase catalyzes the oxidation of phenolic compounds to the corresponding quinones and is responsible for the enzymatic browning of fruits and vegetables. It is an important enzyme in controlling the quality and economics of fruits and vegetables in food industry (Friedman M.1996). In addition to the undesirable color and flavor, the quinone compounds produced in the browning reaction may irreversibly react with the amino and sulfhydryl groups of proteins. The quinone-protein reaction decreases the digestibility of the protein and bioavailability of essential amino acids, including lysine and cysteine. The unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional value in mushrooms, sea foods and has been of great concern (Friedman M.1996; Ogawa, J. and Shimizu, S 1999).

Tyrosinase plays an important role in the developmental and defensive functions of insects. Tyrosinase is involved in melanogenesis, wound healing, parasite encapsulation and sclerotization in insects (Sugumaran M.1991). The development of tyrosinase inhibitors have become an active alternative approach in controlling insect pests. In addition, tyrosinase inhibitors have become increasingly important in cosmetic and medicinal products in relation to hyper pigmentation (Maeda K and Fukuda M.1991).

Melanin plays an important role in protecting human skin from the harmful effects of UV radiation from the sun, also determines phenotypic appearance. Various dermatological disorders such as hyper pigmented lentigenes include melasma, age spots and sites of actinic damage result in the accumulation of an excessive level of epidermal pigmentation Therefore, development of high-performance tyrosinase inhibitors is necessary for the applications in agricultural, cosmetic, pharmaceutical and food industries.

Since most of the tyrosinase inhibitors were studied are synthetic inhibitors such as sulfiting agents, formulations of ascorbic and citric acids, hexylresorcinol and few natural inhibitors especially plant origin. However Most of the synthetic inhibitors are mutagenic, carcinogenic having side effects. Hence there is a need to search alternative compounds with no harmul effects with low cost and easy availability. Our study was mainly focused on rapid screening, of fungal metabolites as antityrosinase compounds where no reports are existing in this area. Hence screening of tyrosinase producing fungus *Acremonium rutilum* were isolated and tyrosinase was purified. *A.rutilum* was used as in-

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dicator organism and the anti tyrosinase compounds were screened by plate assay and spectrometric inhibitory assay.

MATERIALS AND METHODS

Checking the effect of Synthetic tyrosinase Inhibitors

Plate Assay Technique

Sterile Enriched Czapek Dox Agar and broth were amended with synthetic inhibitors- benzoic acid, kojic acid, EDTA, L-cysteine, L-ascorbic acid, PVP resorcinol and L-phenylalanine were inoculated with *A.rutilum* and were incubated for 5-8days at 30°C.

On Partially Purified Tyrosinase

Purification of tyrosinase from *A.rutilum* was done as per Krishnaveni et.al (2016).

Ascorbic acid, Benzoic acid, kojic acid and EDTA with different concentrations (0.025, 0.1, 0.5, 1mM) were added to the standard reaction mixture containing 8mM L-dopa, 0.1M sodium acetate buffer (pH5.5) and 0.5 ml purified tyrosinase and rate of inhibition was checked at 475nm using UV-Vis spectrophotometer. The % inhibition of the tyrosinase activity was calculated by the equation

$$\left\{\frac{(A-B)-(C-D)}{(A-B)}\right\}\times 100$$

Screening of Tyr Inhibitors from Fungus by Rapid Plate Assay Method

Based on the previous experiments with synthetic inhibitors, fungal inhibitors were screened. Isolation of fungi was done by collecting soil samples in Gulbarga region. The tyrosinase producing *A.rutilum* was used as indicator and was inoculated on Enriched Czapekdox agar. The isolated colonies were inoculated near to the indicator at 48hrs and again incubated for 4 days at 30°C.

Screening of Tyr Inhibitors from Fungus by Inhibiting purified Cytosolic tyrosinase from *A.rutilum*

Twenty five ml of sterile potato broth media amended with 0.2% L-Tyrosine, 2% starch, 1% glucose, 1%sucrose, 0.5%yeast extract, and 0.5% peptone was inoculated with a loopful of the selected fungi by plate assay. The flasks were incubated for 8 days on orbital shaker incubator at 180rpm, 30 °C.

0.5 ml of samples were tested against 0.5 ml purified cytosolic tyrosinase catalysed L-DOPA oxidation at 475nm. The % inhibition of the tyrosinase activity was calculated by the equation

$$\left\{\frac{(A-B)-(C-D)}{(A-B)}\right\}\times 100$$

Identification of the Compound

A.niger -1 and *A.ornatus* were grown in 100ml of the broth and the samples were checked for the presence of L-DOPA (Arnow, L.E.,1937), alkaloids (Gururaj C 2001),flavonoids (Upadhya and Upadhya 2001), kojic acid (Parrish F. W.et al 1966, citric acid, ascorbic acid and oligosaccharides by qualitative analysis and TLC experiments.

Absorption Spectrum of kojic acid by *A.ornatus*.

The UV spectrum of the 0.5ml broth sample in 0.1M sodium acetate buffer (pH 5.5) was determined by double beam UV- Vis spectrophotometer.

RESULTS AND DISCUSSIONS

Effect of Synthetic Tyrosinase Inhibitors

Plate assay technique

No brown pigmentation on 5th and 8 th day with EDTA and resorcinol indicating that they are strong inhibitors of both laccase and tyrosinases. No brown pigmentation on 5th day and appearance of brown pigmentation on 8th day indicates only tyrosinase inhibition but not Laccase. With cysteine and phenylalanine no brown pigmentation at the earlier stage but turned to reddish pigmentation and reddish purple at the later stages indicates formation of pheomelanins. With kojic acid, ascorbic and benzoic acid light brown pigmentation and slight tyrosine hydrolysis was seen indicating they are strong inhibitors of monophenolase activity of tyrosinase (Fig.1.1&2). The same observations were shown in the broth samples with all the inhibitors.

On Purified Tyrosinase

Synthetic inhibitors selected for inhibitory studies were Ascorbic acid, Benzoic acid, Kojic acid and EDTA. Benzoic acid has shown 100% inhibition with IC 50 value of 1mM. Next to it was EDTA with 98% inhibition whereas Ascorbic acid and kojic acid have shown 96 and 91% at the same concentration. However, EDTA with 0.5mM, 99% of inhibition was observed. As per the experimental data it is proved that benzoic acid and EDTA are good inhibitors of tyrosinase of *A.rutilum* (Table -1.1). This indicates that tyrosinase isolated from *A.rutilum* were metallo enzymes.

Duckworth and Coleman (1970) reported that benzoic acid inhibited diphenolase activity of tyrosinase and this inhibition was competitive with catechol and irreversible and bound to Cu (I) which was associated with the deoxy form of tyrosinase. Mushroom tyrosinase showed 70% inhibition with 100µM benzoic acid (Streffer (2002). Khan and Andrawis reported an Ic 50 value of 300 µM using L-tyrosine as the substrate and detection of dopachrome. Benzoic acid with Ic 50 value of 0.64mM showed a mixed type of inhibition with L-DOPA (Kahn, V. & Andrawis, A. 1985). Gutteridge and Robb (1975) showed with *Neurospora* tyrosinase that the nature of the inhibition of benzoic acid was competitive with respect to o-diphenol and catechol but that it was a mixed inhibitor of oxygen. EDTA was reported to be a metal chelator and a strong inhibitor of metalloenzymes especially laccase and tyrosinase (Maurice R et al 1980). Cilliers, J.J.L., & Singleton, V.L.,

(1990) reported that cysteine is an effective inhibitor of enzymatic browning.

L-ascorbic acid and its various neutral salts and other derivatives have been the leading GRAS antioxidants for use on fruits and vegetables and in fruit juices, for the prevention of browning and other oxidative reactions (Bauernfeind, J.C., & Pinkert, D.M., 1970). Hulme *et al.* (1964) suggested that PVP inhibits by combing with the catechol oxidase substrate complex by attachment to the phenolic substrate moiety. Harel *et al.,* (1964) observed that PVP and its monomer, N-vinyl-2-pyrrolidone inhibited the enzyme irreversibly and was able to act in the absence of added substrate. Our result correlates with the assumption of Loomis *et al.,* (1966).Although resorcinol is a poor tyrosinse inhibitor, substitution in the 4-position yields increased inhibitory activity. 4-substituted resorcinol showed a competitive inhibition on mushroom tyrosinase with L-DOPA as a substrate.

Screening of Tyrosinase inhibitors From Fungus

By Rapid Plate assay method

The results of screening of antityrosinase compounds from fungi by plate assay method with *A.rutilum* as indicator were tabulated (Table-1.2 and Fig 1.3). According to the results 2 factors are observed. 1) The appearance of tyrosine hydrolysis, reddish brown pigmentation, simultaneous disappearance of brown pigmentation indicates diphenolase reaction gets inhibited 2) no tyrosine hydrolysis, and absence of brown/reddish pink pigmentation of the media indicates inhibition of monophenolase activity. The fungus which will show the above characteristics may be considered as inhibitor producers.

Screening of Antityrosinase Compounds from Fungi by Inhibiting the Purified Cytosolic Tyrosinase from *A.rutilum*

According to the results of screening of antityrosinase compounds producing fungus by inhibiting the purified Cytosolic tyrosinase from *A.rutilum, A.niger-1* and *A.ornatus* showed 98.4 and 98.3 % inhibition which was highest as compared to other fungi (Table-1.3).Plate assay method appears to be the best and rapid method for tyrosinase inhibitors, as all isolates screened by plate assay have shown inhibition to partially purified enzyme.

Besides higher plants, compounds from fungal sources have also been identified and reported by Nazzro-Porro M et al (1979) by for their inhibitory activity on tyrosinase. Schallreuter K. U. and Wood J. W. (1990) reported azelaic acid a naturally occurring straight-chain, saturated dicarboxylic acid produced by, *Pityrosporum ovale,* has a definite cytotoxic effect on malignant melanocytes of primary cutaneous melanoma, though normal melanocytes appeared not to be affected. Kojic acid a fungal metabolite produced by many species of *Aspergillus niger* (Vasantha K.Y et al 2014) and *Penicillium* (Parrish F. W., et al 1966), are good chelators of transition metal ions and good scavengers of free radicals Niwa Y. and Akamatsu H. (1991). Khan V (1995) reported kojic acid effectively inhibited the formation of pigmented products and oxygen uptake when DL-DOPA, nor epinephrine and dopamine were oxidized by tyrosinase, which means that kojic acid is able to reduce o-quinone to o-diphenol to prevent the final pigment forming and be oxidized to a yellow product by chemical interaction with o-quinone. Kojic acid with Ic 50 value of 0.014mM showed a mixed type of inhibition with L-DOPA (Kobayashi T et al 1995). Saruno *et al.,* (1979) demonstrated that kojic acid from *Aspergillus albus* inhibited mushroom PPO activity. The yeast metallothioneins are ubiquitous cytosolic proteins, usually characterized by selective binding of a large amount of heavy metal ions (Zn2+, Cu2+ and Cd2+) and high cysteine content (Byrd J et al 1988). *N. crassa* copper-metallothionein was reported as a metal donor for apotyrosinase. Lerch K (1980) reported Metallothionein from *A. niger* was also found to be an inhibitor exhibited a higher inhibitory effect on the oxidation of catechin compared with that of chlorogenicacid. Other fungal extracts such as agaritine and inhibitors (Ia and Ib) from *Agaricus* species were also isolated, purified and characterized. Agaritine, b-N-(g-L (+)-glutamyl)-4-hydroxymethylphenylhydrazine, showed a depigmenting effect that prevented melanin formation.

From microbial metabolites inhibitors were screened and identified the active molecule, terrein, from *Penicillium spp*.which inhibits melanogenesis in Mel-Ab cells by down regulating microphthalmia-associated transcription factor (Mitf) via extra cellular signal-regulated kinase (ERK) activation leading to the inhibition of tyrosinase production (Park, S. H et al 2004). The majority of aspochalasins have been isolated from the species of *Aspergillus* and inhibits tyrosinases (Rochfort, S., J. et al 2005).

Identification of the Compound

Detection of the compound by TLC

A.ornatus showed a strong positive reaction with $FeCl_3$ test indicating that the compound is kojic acid which was separated by TLC with a standard kojic acid and the separated compound again showed 98.5% tyrosinases inhibition (Fig.1.4 &1.5). When the percent inhibition of synthetic kojic acid was compared with naturally screened and identified kojic acid, the percent inhibition was 91% with synthetic Ic 50 1mM, where as it was 98.5% inhibition which is appreciable with natural kojic acid irrespective of the concentration.

Absorption spectrum of kojic acid from *A.ornatus*

The absorption spectrum from 200-800nm of *A.ornatus* sample showed a sharp peak with absorbance of 0.219 -0.524 at 280-290 nm which states that the proteins with tryptophan residues are more than tyrosine

Figure 1.1: Effect of PVP, Resorcinol, EDTA, Phenylalanine and cysteine on Melanin Pigmentation of A.rutilum on 5th day at 30°C

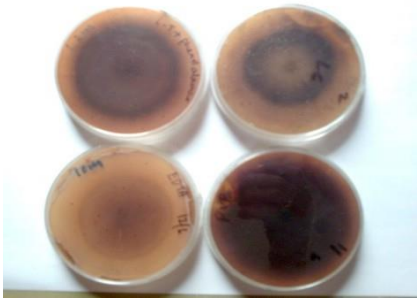

Figure 2.2: Effect of PVP, Resorcinol, EDTA, Phenylalanine on Melanin pigmentation of A.rutilum on 8th day at 30°C

Table 1: Effect of inhibitors on partially purified Cyt tyr

Inhibitor	Concentration (mM)	%inhibition
Ascorbic acid	0.025mM	72
	0.1mM	88.1
	0.5mM	96
	1mM	96
Benzoic acid	0.025mM	79.8
	0.1mM	94
	0.5mM	96
	1mM	100
Kojic acid	0.025mM	70.3
	0.1mM	96
	0.5mM	92
	1mM	91
EDTA	0.025mM	51
	0.1mM	90.5
	0.5mM	99
	1mM	98

residues. At 300nm the O.D was 0.55 which was highest. Again at 423 nm sharp peak with 0.D 0.188 was seen which may be due to some other metabolites. According to the literature kojic acid showed kojic acid in aqueous solutions is colorless with high peak at 217and lowest peak at 270nm with a λ-max at 340nm (Parrish F. W et al 1966)

CONCLUSION

The present study concludes that the tyrosinase inhibitor, kojic acid was tentatively identified by biochemical assay which is a major secondary metabolite of *Aspergillus* sps. The isolate *Aspergillus Ornatus* proved to be a potent tyrosinase inhibitor and produces kojic acid. Kojic acid chelates the metal ions and hence inhibits tyrosinase. Because of its inhibitory effect on tyrosinase, kojic acid is commonly used in cosmetic industry as whitening agent and as effective compound of creams protecting the skin against UV radiation. Although kojic acid is a good inhibitor of polyphenol oxidase, its toxicity is of concern. Thus, attention has recently been focused on the novel derivatives of kojic acid products in cosmetics. Not only in cosmetic sector is it extensively used in medicine as the anti-inflammatory and analgesic drug. In the food industry, kojic acid is used as flavor enhancer and as food

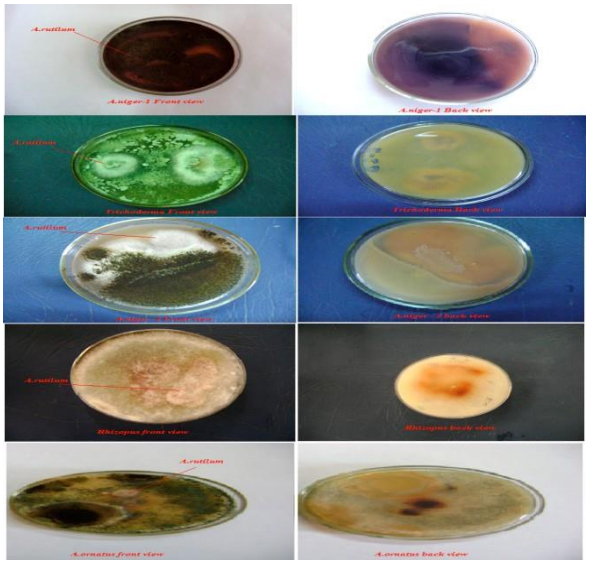

Figure 1.3: Effect of Fungal Inhibitors of a) A.niger -1, b) Trichoderma.,c) A.niger-2, d) Rhizopus and e) A. ornatus by Plate Assay

Table 2: Screening of Antityrosinase Compounds- Plate Assay method

Fungus	Pigmentation of the media	Pigmentation of the fungus	L-Tyrosine hydrolysis
A.niger -1	Dark reddish pink	brown pigment absent	++++
A.niger -2	Light pink	-do-	++++
Aspergillus spp. (Light brown)	Yellowish	Brown at backside	++
A.flavus	colorless	brown pigment absent	+++
Aspergillus.spp (pecock green)	colorless	-do-	++
A. terrus	-	-do-	++
Trichoderma spp.	Florescent greenish yellow	-do-	-ve
Rhizopus spp.	Reddish	-do-	-ve
A.clavatus	colorless	-do-	+++
A.niger -4	colorless	-do-	+++
Unidentified	Magenta	-do-	+ve (very less)
A.ornatus	colorless	-do-	+ve (very less)
A.niger -3	Yellowish	-do-	++

Table 3: Screening of Antityrosinase compounds - by inhibiting the Partially Purified cyt Tyr from A.rutilum

Sl.no	Fungus	Color of the broth	O.D at 475 nm (8mML-DOPA)	Percent inhibition (A-B/A*100)
1	*A .niger -1*	Straw	0.007	98.3
2	*A.ornatus*	Yellow	0.008	98.1
3	*A.flavus*	yellow	0.022	94.7
4	*Rhizopus spp.*	Straw	0.025	94.9
5	*A.clavatus*	Light orange	0.029	93.1
6	*Aspergillus spp.* (Light brown)	Dark yellow	0.102	75.7
7	*Trichoderma spp.*	Dark Flouresecnt yellow	0.108	74.3
8	*A.niger -3*	Pale yellow	0.117	72.1
9	*Aspergillus spp* (pecock green)	Dark Flouresecnt yellow	0.137	67.4
10	*A .niger -2.*	Pale yellow	0.148	64.8
11	Control(Without inhibitor)		233.8	

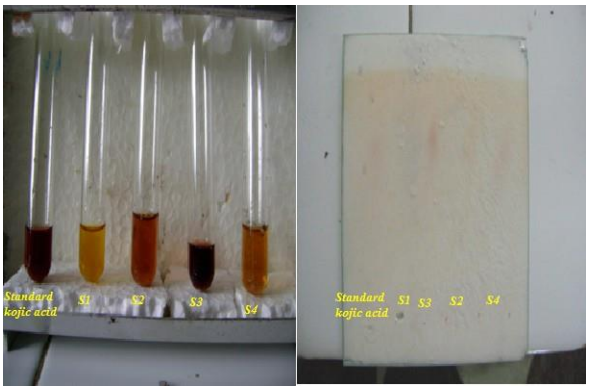

Figure 1.4: a) A.niger–1(S1) (-ve for kojic acid), A.flavus(S2), A.ornatus(S3) (blood red +ve for kojic acid) and A.terrus(S4) samples were compared with standard for kojic acid test. B) A.ornatus (blood red +ve for kojic acid) compared with Standard kojic acid on

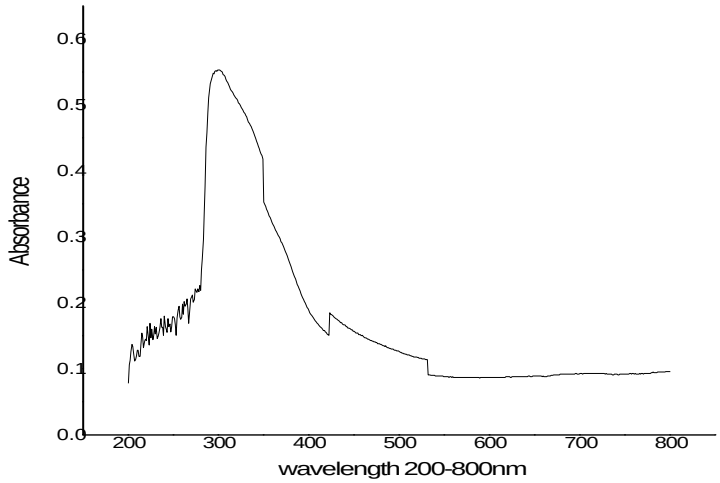

Figure 1.5: Absorption spectrum of the antityrosinase compound from A.ornatus

antioxidant improving i.e. stability of edible fats and oils. Besides, kojic acid and its derivatives tend to have antifungal, insecticidal, anticancer and bacteriostatic properties, its application in the above industries is significantly spreading. Hence the research in this direction is encouraging.

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