ISOLATION OF *ALLIUM SATIVUM AND MENTHA PIPERITA* EXTRACTS; EVALUATION OF THEIR ANTIOXIDANT AND ANTI-TYROSINASE ACTIVITIES IN-VITRO

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Abstract

Present study was conducted at the Department of zoology at Mirpur University of Science and Technology (MUST), during 2018-2019 to isolate extracts from selected plants (Garlic and Peppermint) to explore their tyrosinase inhibition activity. Tyrosinase is a key enzyme of melanogenesis which determines the mammalian skin, hair and eye colour. Hyper-pigmentation leads to various skin disorders like melasma, sunspots, age spots and freckles. Moreover, abnormal skin pigmentation is a serious aesthetic concern which leads to psychosocial problems. Thus to achieve melanin inhibition, inhibition of tyrosinase might be an effective approach. To this end we prepared methanolic (MeOH) extracts from leaves and roots of Garlic *Allium sativum* (AS) and Peppermint *Mentha piperita* (MP), which were further processed for 1:1 fractional distillation to prepare methanolic n-hexane (MeOH_n-Hx), methanolic ethyl acetate (MeOH_EA) and methanolic chloroform (MeOH_$CHCl_3$) extracts, aiming to evaluate tyrosinase and anti-oxidant activities *in-vitro*. Our results confirmed that all MeOH-crude AS and MP extracts showed significant anti-oxidant activity with IC_{50} values ranging from 0.05 ± 0.2 mg/ml to 4.3 ± 2.3mg/ml. Moreover, AS and MP all 16 extracts have significant anti-tyrosinase activity with IC_{50} range from 0.014 ± 0mg/ml to 1.205 ± 0.07mg/ml. Interestingly, AS leaf MetOH_EA, AS leaf MetOH_$CHCl_3$, AS root MetOH_EA and MP leaf MetOH_$CHCl_3$ showed significant anti-tyrosinase activity even higher than positive control kojic acid. AS leaf MetOH_$CHCl_3$ extract being the most potent among all tested extracts is proposed as potential candidate to treat tyrosinase rooted hyper-pigmentation in future.

Key Words: *Allium sativum, Mentha piperita*, Extracts, Anti-tyrosinase, Antioxidant, Kojic acid

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INTRODUCTION

Melanogenesis is synthesis of melanin pigment responsible for skin, hair and eye color. Melanin absorbs free radicals and protects our skin from ultraviolet (UV) radiations (Souza *et al.,* 2012). Despite its protective role, its abnormal accumulation may result in increased pigmentation causing unwanted aesthetic problems such as darkening of skin, aging, acne and wrinkles (Singhal *et al.,* 2013). Thus, to resolve such aesthetic problems melanin inhibition is desirable.

Melanogenesis is controlled by various factors including tyrosinase enzyme and anti-oxidant agents. Tyrosinase being key enzyme in melanin synthesis is an effective target to control pigmentation (Lai *et al.,*2019*).* Tyrosinase enzyme is involved in (a) hydroxylation of L-tyrosine to L-dopa (l-3,4-dihydroxyphenylalanine) and (b) oxidation of L-dopa to dopaquinone ultimately effecting melanin production (Solano *et al.,*2006). Anti-oxidants, anot her factor effecting melanogenesis can s cavenge free radicals and reactive specie s (RS) important for melanin synthesis. Thus, to control hyper-pigmentation the use of tyrosinase inhibitor with anti-oxid ant properties is desirable.

Tyrosinase inhibitors can be obtai ned from synthetic sources easily. Synth etic agents such as pearl powder, sebum REG, candelilla wax, butylated hydroxyl t oluene, beeswax, lanolin alcohol, ethylen e diamine tetra acetate, triethanolamine and ozokeritewax are easily available an d frequently used in cosmetics (Singhal *et al.,*2013). Many synthetic tyrosinase inhibitors have given promising results however their long use have shown side effects which encouraged us to find new safer sources such as from edible stuff for the isolation of tyrosinase inhibitors.

Thus, in present study we selected *Allium sativum* (AS) or garlic and *Mentha piperita* (MP) or peppermint which is common ingredient of Asian food. AS is cultivated for food and medicinal purposes (Gangadhar *et al*., 2012).Previous studies have reported AS as strong antibacterial source (Uchida *et al*., 1975; Ncir *et al*.,2018). It is also an effective antioxidant that can protect from oxidative stress induced by reactive species (RS). Moreover, it helps in controlling blood pressure; this characteristic is linked to its allicin content and hydrogen sulphide producing property (Londh *et al.,*2011). Throughout the world, plants are considered imperative source of safe and effective medication. Many studies have been conducted using local plants to explore their biological applications such as antioxidant, anticancer and antibacterial potential (Rafiq 2020; Akhtar *et al* 2-18; Ahmed *et al* 2016). Our second study plant, *Mentha piperita* (MP) belongs to Lamiaceae family, commonly found in damp and wet places. It's important species i.e. *M.* x *piperita* L. (peppermint oil), *M. spicata* L. (spearmint oil), and *M. arvensis* L. (cornmint oil) have been cultivated around the globe and are frequently been used in beverage, confectionary, food, cosmetics and pharmaceutical industries (M. Gulluce *et al.,* 2007). These essential oils have multiple applications such as, astringent, anti-septic, anti-microbial and anti-pruritic (G. Iscan *et al.,*2002). However, in present study we prepared AS and MP methanolic extracts aiming to evaluate their anti-oxidant and anti-tyrosinase potential, *in-vitro*.

MATERIALS AND METHODS

PLANT MATERIAL AND CHEMICALS

Allium sativum (AS) and *Mentha piperita* (MP) were collected from Mirpur city, AJK, Pakistan. Mushroom tyrosinase, l-3, 4-dihydroxyphenylalanine (l-DOPA) and kojic acid were purchased from sigma chemicals. Sodium phosphate monobasic monohydrate and sodium phosphate dibasic dihydrate were obtained from duksan reagents, Korea.

PREPARATION OF PLANT EXTRACT

Allium sativum (AS) and *Mentha piperita* (MP) leaves and roots were selected for current study. Selected parts were washed with water, dried in shade and fine powder was prepared.

Figure 1: Extraction steps for *Allium sativum* (AS). AS leaves (a) fresh (b) dried powder(c) filtrate for extraction and AS roots (d) fresh (e) dried powder (f) filtrate for extraction are shown.

Samples were dipped in methanol 1: 10 (g: ml) ratio for 15 days, gentle shaking was ensured twice per day. Later, sample was filtered; filtrate was evaporated by rotary evaporator at 36 ^{o}C (Figure 1 and Figure 2). Obtained MetOH_crude extracts were further processed for fractional distillation to prepare methanolic n-hexane (MeOH_n-Hx), methanolic ethyl acetate (MeOH_EA) and methanolic chloroform (MeOH_$CHCl_3$) extr acts for the evaluation of tyrosinas activity.

Figure 2: Extraction steps for *Mentha piperita* (MP). MP leaves (a) fresh (b) dried powder (c) filtrate for extraction and MP roots (d) fresh (e) dried powder (f) filtrate for extraction are shown.

TYROSINASE ASSAY

Tyrosinase assay was performed as reported previously (Wang *et al.,* 2015, Ali *et al*, 2019) with slight modifications. Briefly, 140 µl of 20 mM phosphate buffer (pH 6.6), 20 µl mushroom tyrosinase enzyme and 20 µl extract solutions were mixed. Ten min later, 20 µl L-DOPA (0.85 mM) was added and sample was incubated again for 20 min at 25 °C. Later, dopachrome as measure of tyrosinase activity was monitored at 450 nm and percentage of inhibition was calculated as below.

$$\text{Inhibition}(\%) = \left[\frac{1 - (\text{Sample absorbance} - \text{Control1 absorbance})}{\text{Control 2} - \text{Control 3}}\right] \text{x}100$$

Sample = Test sample (serial dilutio ns) along with buffer, tyrosinase and L-DOPA
$Control_1$= Test sample along with bu ffer, solvent (methanol) and L-DOPA
$Control_2$= Tyrosinase along with buff er, solvent (methanol) and L-DOPA
$Control_3$= Buffer, solvent (methanol) and L-DOPA
Finally, IC_{50} value was calculated fro m inhibition (%) to compare inhibito ry potential of tested extracts.

ANTIOXIDANT ACTIVITY TEST

To test antioxidant activity DPPH assay (2,2-diphenyl-1-picrylhydrazyl) wa s performed following Rabia *et al.,* 2015 with modifications. Briefly, DPPH stock solution prepared by dissolving 24mg DPPH powder in 100 ml methanol. Stock solution was further diluted with methanol to get OD 0.98± 0.02 at 475nm. Our selected plant extract was mixed with DPPH working solution in 1:1 ratio and incubated at 20° C in dark. Later, absorbance was tracked at 475nm and percentage was calculated as below.

$$\text{Scavenging}(\%) = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \text{ x } 100$$

Sample = Test sample (serial dilutio n) along with DPPH working solutio n,
Control= DPPH working solution alon g with methanol
Finally, $IC_{50}$was calculated from scav enging (%) to compare scavenging potential of test extracts.

RESULTS

In present study, *Allium sativum* (AS) and *Mentha piperita* (MP) plant leaves and roots were used to prepare various methanolic (MeOH) extracts for the screening of anti-tyrosinase and anti-oxidant potential. All the tested extracts showed significant anti-tyrosinase and anti-oxidant activities.

The AS leaves MeOH_crude, MeOH_n-Hx, MeOH_EA and MeOH_$CHCl_3$ showed tyrosinase 50% inhibitory concentration (IC_{50}) values as 0.068 mg/ml, 1.205 mg/ml, 0.024 mg/ml, 0.014 mg/ml, respectively (Table 1). MetOH_$CHCl_3$ extract showed lowest IC_{50} values among other AS leaves MeOH extracts however, reference kojic acid showed IC_{50} value as 0.05 mg/ml (Table 1). In comparison, MetOH_EA and MetOH_$CHCl_3$ AS leaf extracts showed better inhibition than reference. However, the activity of remaining extracts is also comparable which make them important candidate to explore further.

Table 1: Anti-tyrosinase activity of *Allium sativum* (AS) leaf extracts.

AS leaf extract type	**Tyrosinase inhibition IC_{50}± SEM (mg/ml)**
MetOH_crude	0.068 ± 0.001
MetOH_n-Hex	1.205 ± 0.07
MetOH_EA	0.024 ± 0.002

MetOH_$CHCl_3$	0.014 ± 0.03
Kojic acid	0.05± 0.01

Similarly, AS root MeOH extracts were also tested and root MetOH_crude, MetOH_n-Hx, MetOH_EA and MetOH_$CHCl_3$ showed IC_{50} values for anti-tyrosinase activity as 0.157 mg/ml, 0.13 mg/ml, 0.03 mg/ml and 1.15 mg/ml, respectively (Table 2). Interestingly, MetOH_EA AS root extract showed 0.03 mg/ml IC_{50} value which is better than reference kojic acid (0.05 mg/ml).

Table 2: Anti-tyrosinase activity of *Allium sativum* (AS) root extracts.

AS root extract type	**Tyrosinase inhibition IC_{50}± SEM (mg/ml)**
MetOH_crude	0.15 ± 0.01
MetOH_n-Hex	0.13 ± 0.07
MetOH_EA	0.03 ± 0.01
MetOH_$CHCl_3$	1.15 ± 0.03
Kojic acid	0.05 ± 0.01

Our second study plant *Mentha piperita* (MP) was also evaluated for possible anti-tyrosinase activity. MP leaf MetOH_crude, MetOH_n-Hx, MetOH_EA, MetOH_ $CHCl_3$ showed anti-tyrosinase activity with IC_{50} values as 1.16 mg/ml, 0.36 mg/ml, 0.6 mg/ml, 0.015 mg/ml, respectively (Table 3). In comparison, MetOH_$CHCl_3$ MP leaf extract showed better inhibition than reference. However, the activity of remaining extracts is also comparable which make them important candidate to explore further.

Table 3: Anti-tyrosinase activity of *Mentha piperita* (MP) leaf extracts.

MP leaf Extract type	**Tyrosinaseinhibition IC_{50}± SEM (mg/ml)**
MetOH_Crude	1.16 ± 0.170
MetOH_n-Hex	0.36 ± 0.01
MetOH_EA	0.6 ± 0.13
MetOH_$CHCl_3$	0.015 ± 0.03
Kojic acid	0.05 ± 0.01

Similarly, MP root MetOH_crude, MetOH_n-Hx, and MetOH _EA and MetOH_ $CHCl_3$showed IC_{50} values for anti-tyrosinase activity as 0.23 mg/ml, 0.21 mg/ml, 0.61 mg/ml and 0.16 mg/ml, respectively (Table 4).

Table 4: Anti-tyrosinase activity of *Mentha piperita* (MP) root extracts.

MP root extract type	**Tyrosinase inhibition IC_{50}± SEM (mg/ml)**
MetOH_crude	0.23 ± 0.04
MetOH_n-Hex	0.21 ± 0.03
MetOH_EA	0.61 ± 0.001
MetOH_$CHCl_3$	0.16 ± 0.014
Kojic acid	0.05 ± 0.01

Interestingly, AS leaves showed highest anti-tyrosinase activity among all MeOH-crude, MeOH_EA and MeOH_$CHCl_3$ extracts and, AS roots showed highest anti-tyrosinase activity among all MeOH_n-Hex extracts isolated from both

study plants (Table 5). In general, AS showed better anti-tyrosinase activity than MP extracts.

Table 5: Anti-tyrosinase activity trend with respect to extract type.

Extract type	Trend in anti-tyrosinase activity
MeOH_crude	AS leaf > AS root > MP root > MP leaf
MeOH_n-Hx	AS root > MP root > MP leaf > AS leaf
MeOH_EA	AS leaf > AS root > MP leaf > MP root
MeOH_$CHCl_3$	AS leaf > MP leaf > MP root > AS root

ANTIOXIDANT ACTIVITY

To test anti-oxidant activity DPPH assay was performed. Results confirmed antioxidant activity of all MeOH extracts with different level of IC_{50} values. AS leaf, AS root, MP leaf and MP root MeOH-crude extracts showed anti-oxidant IC_{50} values as 0.49 mg/ml, 0.05 mg/ml, 0.09 mg/ml and 0.16 mg/ml, respectively (Table 6).The antioxidant activity trend was observed as AS root> MP leaf> MP root> AS leaf MeOH-crude extracts.

Table 6. Anti-oxidant activity of AS and MP MeOH_crude extracts.

MeOH_crude extracts	DPPH inhibition IC_{50}± SEM (mg/ml)
AS leaf	0.49 ± 0.07
AS root	0.05 ± 0.2
MP leaf	0.09 ± 0.04
MP root	0.16 ± 0.02
Ascorbic acid	4.3 ± 2.3

DISCUSSION

In present study, leaves and roots of *Allium sativum* (AS) and *Mentha piperita* (MP) plants were selected to prepare MeOH_crude extracts which were further processed for 1:1 fractional distillation to prepare MeOH_n-Hx, MeOH_EA and MeOH_$CHCl_3$ extracts aim ing to evaluate their anti-tyrosinase activity *in-vitro*. Later, anti-oxidant potential of all MeOH-crude extracts was determined by DPPH assay.

Our results confirmed that AS and MP all MeOH-crude extracts showed significant anti-oxidant activity with IC_{50} values ranging from 0.05 ± 0.2 mg/ml to 4.3 ± 2.3mg/ml. Interestingly, previous reports confirmed a close association among antioxidants and tyrosinase rooted melanin inhibition. N-acetyl cysteine, an anti-oxidant abolish UVB-induced α-melanocyte stimulating hormone (α-MSH) and has been used to study the correlation of reactive oxygen species (ROS) with melanin synthesis (Funasaka *et al*., 2000). The up-regulation of endogenous antioxidants is reported to suppress melanin production (Maekawa *et al*., 1996, Chakraborty *et al*., 1996). Other studies also showed ROS direct association with melanogenesis (Chou,2010, Yanase *et al,.* 2001). They found that decrease in ROS level is associated with the suppression of melanogenic signaling molecules such as cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), mitogen-activated protein kinase (MAPK) and melanocortin 1 receptor (MC1R) which subsequently reduces tyrosinase and melanin content.

Interestingly, AS and MP all 16 test extracts have shown significant anti-tyrosinase activity with IC_{50} valuesrangingfrom0.014 ± 0 to 1.205 ± 0.07mg/ml. The trend in anti-tyrosinase activity of AS leaf and root was observed as leaf MetOH_$CHCl_3$>leaf

MetOH_EA>root MetOH_EA>leaf MetOH_crude>root MetOH_n-Hx>root MetOH_crude> root MetOH_$CHCl_3$>leaf MetOH_n-Hex. However, The trend in anti-tyrosinase activity of MP leaf and root extracts was noted as leaf MetOH_$CHCl_3$>leaf MetOH_crude=root MetOH_$CHCl_3$>root MetOH_n-Hx>root MetOH_crude>leaf MetOH_n-Hx>root MetOH_EA>leaf MetOH_EA. Previously, *Allium sativum* is reported as being widely used throughout the world and is considered as a herbal medicine for the prevention and treatment of various diseases from infections to heart disease (Rivlin, 2001). Similarly, *Mentha piperita* (oils) are extensively used in pharmaceutical and food industry to prepare cough drops, dental care creams, flavored chewing gums, and as a flavor in toothpastes, mouth refreshers (H. Hajlaoui *et al.,* 2009). However, in present study extracts isolated from both plants have shown good commitment for anti-tyrosinase activity.

For successful melanin inhibition, potent anti-oxidant and effective tyrosinase inhibitors are desirable. They can be obtained from diverse synthetic sources however; safety concerns including cytotoxicity and other side effects prevent the commercialization of many of these inhibitors. Therefore, natural sources such as edible plants are the center of interest because of their already proven safety. Thus, we selected *Allium sativum* (AS) and *Mentha piperita* (MP) common edible sources for the isolation of test extracts. Extracts from selected plants showed both anti-oxidant and anti-tyrosinase activity. Interestingly, AS leaf MetOH_EA, AS leaf MetOH_$CHCl_3$, AS root MetOH_EA and MP leaf MetOH_$CHCl_3$ showed significant anti-tyrosinase activity even higher than positive control kojic acid. AS leaf MetOH_$CHCl_3$ extract showed highest tyrosinase activity with lowest IC_{50} value (0.014 mg/ml) among all tested extracts proving an effective potential alternative to control tyrosinase.

CONCLUSION

Present study confirmed that all AS and MP tested extracts have significant anti-tyrosinase and anti-oxidant activities. The AS leaf MetOH_$CHCl_3$ extract with lowest IC_{50} value among all tested extracts and kojic acid is proposed as potent tyrosinase inhibitor for further evaluation to treat tyrosinase rooted hyper pigmentation disorders in future.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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