ANTI-TYROSINASE AND ANTI-OXIDANT POTENTIAL OF METHANOLIC EXTRACTS OF SELECTED *Citrus bergamia* AND *Ficus carica* PARTS

Hina Ilyas[1], Umar Hanif[2], Anser Ali[1], Zahid Hassan Tarar[3],Hamza Javed[4], Tehreem Tahir[5], Muhammad Rafiq[6]*

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ABSTRACT

Tyrosinase is a key enzyme in melanogenesis. Its high activity is associated with increased pigmentation causing skin disorders like freckles, melasma and black spots. Therefore, search for new tyrosinase inhibitors is desirable.

In present study, methanolic (MeOH) extracts from leaves, fruit peel and pulp of *Citrus bergamia* (*C. bergamia*) and, leaves and fruit of *Ficus carica* (*F. carica*) were prepared which were further processed for fractional distillation using ethyl acetate (EA), n-hexane (*n*-Hx) and chloroform ($CHCl_3$) preparing total 20 extracts aiming to test their anti-tyrosinase potential, *in-vitro*. Our results confirmed that all *C. bergamia* and *F. carica* crude extracts showed significant anti-oxidant activity with IC_{50} range of 384.2 ± 19.1 to 77.3 ± 10.0 μgmL^{-1}, collectively. Moreover, significant anti-tyrosinase activity for *C. bergamia* (IC_{50} range = 4.1 ± 0.3 to 366.8 ± 36.5 $\mu g\ mL^{-1}$) and *F. carica* (IC_{50} range = 156.5 ± 12.4 to 15.1 ± 2.9 $\mu g\ mL^{-1}$) was found . Interestingly, *C. bergamia* MeOH-EA peel and *C. bergamia* MeOH-EA leaves extracts showed IC_{50} 4.1 ± 0.3 and 6.1 ± 1.2 $\mu g\ mL^{-1}$, respectively. Thus, *C. bergamia* MeOH-EA peel extract with lowest IC_{50} value among all the tested extracts, is proposed as potent candidate to control tyrosinase rooted hyperpigmentation in future.

Keywords: Anti-tyrosinase, Anti-oxidant, *C. bergamia*, *F. carica,* Extraction.

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[1]Department of Zoology, Mirpur University of Science and Technology (MUST), Mirpur-10250 (AJK)-Pakistan.
[2]Department of Medical Laboratory Technology, Riphah International University, Faisalabad, Pakistan..
[3]Soil and Water Testing Laboratory, Mandi Bahauddin, Pakistan.
[4]Department of Medical Laboratory Technology, Government College University, Faisalabad, Pakistan.
[5]Department of Biochemistry and Biotechnology, The Islamia University of Bahawalpur, Bahawalpur-63100, Pakistan.
[6]Department of Physiology & Biochemistry, Cholistan University of Veterinary & Animal Sciences, Bahawalpur-63100, Punjab, Pakistan.
*Corresponding author's email: rafiq@cuvas.edu.pk

INTRODUCTION

Melanin is an oligomeric pigment found in plants, fungi and humans (Surwase *et al.,* 2013; Riley, 1997). It contributes in the skin, eyes and hair pigmentation (Jimbow *et al.,*1976; Kim and Uyama, 2005). Moreover, melanin is also responsible for skin protection against ultra violet (UV) radiations. It absorbs UV rays and eliminates ROS (Costin *et al.,* 2007; Solano, 2020). Despite its advantages, its abnormal accumulation result in hyperpigmentation contributing to esthetic problems including age spots, melasma and freckles.

Tyrosinase is a key enzyme contributing in melanogenesis (Okun *et al.,* 1970). It involves L-3,4-dihydroxyphenylalanine (L-DOPA) synthesis from L-tyrosine followed by rapid oxidation of L-DOPA to L-DOPA quinine, a melanin precursor (Fitzpatrick *et al.,* 1979). Tyrosinase is also associated to certain neurodegenerative diseases i.e. Parkinson's disease (Asanuma *et al.,* 2003; Xu *et al.,* 1998). It is also reported to be involved in the browning of fruits and vegetables. Thus, to prevent fruit browning and unwanted pigment associated esthetic problems, effective tyrosinase inhibition is desirable (Robins, 2009).

Anti-oxidants have shown important role in controlling melanogenesis (Takahiro *et al.,* 2011). ROS inhibitors can down-regulate UV-mediated melanogenesis (Yasui and Sakurai, 2003). Therefore, anti-oxidants being ROS inhibitors are frequently used in cosmetic industry to prevent hyperpigmentation (Funasaka *et al.,* 2000). Free radicals cause numerous degenerative disorders by interacting with biological molecules like lipid, protein, and DNA. Anti-oxidants are associated with several pharmacological activities including anti-aging, anti-mutagenicity, anti-carcinogenicity and skin whitening (Takahiro *et al.,* 2011). One approach to control oxidative stress and to achieve skin whiting is the use of natural or synthetic anti-oxidants and tyrosinase inhibitors.

Multiple natural and synthetic potent tyrosinase inhibitors are already available (Kim and Uyama, 2005; Robins, 2009; Ali *et al.,* 2019). However, safety concerns such as cytotoxicity offend the commercialization of most of the candidates. Therefore, search for safe and effective tyrosinase inhibitor is always desirable. To meet safety requirements, many researchers recommend the use of plant-based natural medical products (Tlili *et al.,* 2011). Many studies have been conducted using local plants to explore their important biological activities (Fatemeh *et al.,* 2007; Khan *et al.,* 2020; Rafiq *et al.,* 2020; Shah *et al.,* 2020; Naqvi *et al.,* 2020) Thus, in present study we selected *F. carica* and *C. bergamia* two edible plants for the isolation of extracts and evaluation of their anti-tyrosinase and anti-oxidant activities, important to control tyrosinase rooted pigmentation.

MATERIALS AND METHOD

Plant material and chemicals

F. carica and *C. bergamia* leaves and fruits were collected from Mirpur, AJK, Pakistan. Mushroom tyrosinase and L-DOPA were purchased from Sigma Aldrich.

Preparation of plant extracts

Selected parts of *F. carica* (leaves and fruit) and *C. bergamia* (leaves, fruit peel and pulp) were cleaned, dried and ground. The powdered samples were dipped in methanol 1:10 (g:mL) ratio for 15 days with gentle shaking twice per day. Later, sample was filtered and solvent was evaporated by using rotary evaporator at 36^{o}C (Fig. 1). The obtained MeOH-crude extracts were further air dried at room temperature and processed for fractional distillation in n-hexane (*n*-Hx), ethyl acetate (EA) and chloroform ($CHCl_3$) 1:1 (µg:mL) ratio separately to obtain respective extracts for evaluation of anti-tyrosinase activity.

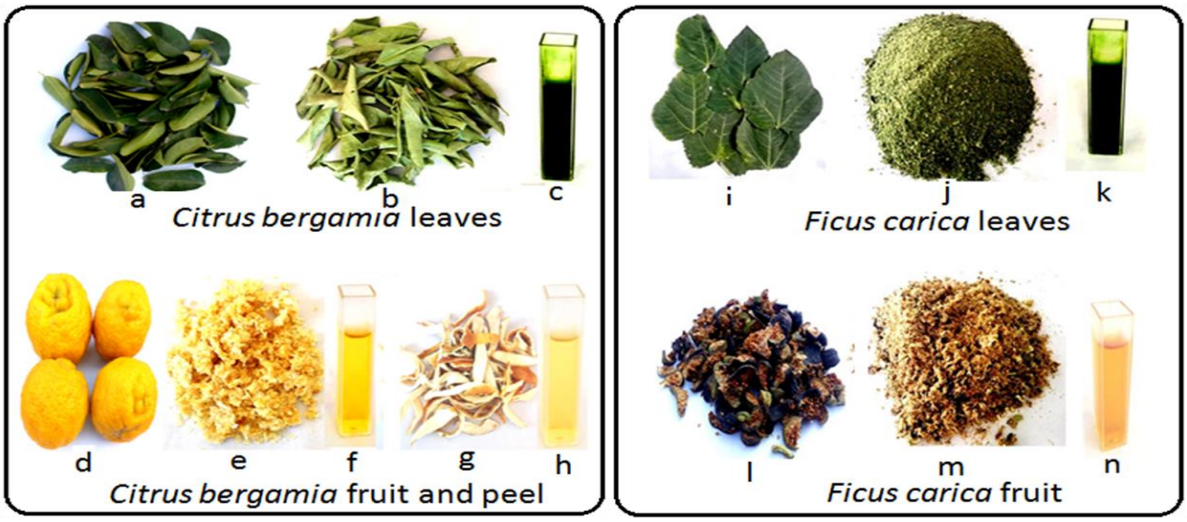

Fig. 1: Extraction steps for *C. bergamia* (a-h) and *F. carica* (i-n). *C. bergamia* leaves (a) fresh (b) dried and (c) MeOH filtrate for extraction are shown. *C. bergamia* fruit (d) fresh (e) dried pulp and (f) MeOH pulp filtrate for extraction while (g) dried fruit peel and (h) MeOH peel filtrate for extraction are shown. *F. carica* leaves (i) fresh (j) dried and (k) MeOH leaves filtrate for extraction are shown. *F. carica* fruit (l) dried (m) powder and, (n) MeOH fruit filtrate for extraction are shown.

Tyrosinase assay

Tyrosinase assay was performed as previously reported (Ali *et al.,* 2019). Briefly, phosphate buffer, mushroom tyrosinase and extract solutions were mixed and sample was incubated for 10 min. Later, L-DOPA was added, incubated again (20 min at 25 °C) and change in absorbance was checked at 450 nm.

Anti-oxidant activity

To test anti-oxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed by following Kanwal *et al.* (2015) with slight modifications. Briefly, DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 mL methanol and OD was adjusted 0.98 ± 0.02 at 490 nm to get working solution. Plant extract was mixed with DPPH working solution, incubated at 20 ^{o}C in dark and absorbance was noted at 490 nm to calculate scavenging percentage (%) as given below.

Scavenging (%) = (Control absorbance - Sample absorbance)/(Control absorbance) x 100

Sample = Test sample (serial dilution) along with DPPH working solution,

Control= DPPH working solution along with methanol

Finally, IC_{50} from scavenging (%) was calculated to compare scavenging potential of tested extracts.

RESULT

In present study, methanolic (MeOH) extracts from *C. bergamia* (leaves, fruit peel and pulp) and *F. carica* (leaves and fruit) were prepared aiming to evaluate their anti-oxidant and anti-tyrosinase activities. Moreover, extracts were further processed for fractional distillation using ethyl acetate (EA), n-hexane (*n*-Hx) and chloroform ($CHCl_3$) preparing total 20 extracts and anti-tyrosinase activity was analysed further.

Anti-tyrosinase activity

Our results confirmed that *C. bergamia* leaves MeOH-crude, MeOH-*n*-Hx, MeOH-EA and MeOH-$CHCl_3$ extracts showed IC_{50} values as 151.3, 24.6, 6.1 and 12.9 µg mL^{-1}, respectively (Table 1).

Table 1: Anti-tyrosinase activity of *C. bergamia* leaves extracts.

Extract of *C. bergamia* leaves	**Tyrosinase inhibition IC_{50} ± SEM (µg mL^{-1})**
MeOH-crude	151.3 ± 29
MeOH-*n*-Hx	24.6 ± 1.1
MeOH-EA	6.1 ± 1.2
MeOH-$CHCl_3$	12.9 ± 1.9

C. bergamia peel MeOH-n-Hx, MeOH-EA and MeOH-$CHCl_3$ extracts showed tyrosinase inhibition IC_{50} values as 142.7, 4.1 and 35.2 µg/mL, respectively (Table 2).

Table 2: Anti-tyrosinase activity of *C. bergamia* fruit peel extracts.

Extract of *C. bergamia* peel	**Tyrosinase inhibition IC_{50} ± SEM (µg mL^{-1})**
MeOH-crude	*nd
MeOH-*n*-Hx	142.7 ± 21.2
MeOH-EA	4.1 ± 0.3
MeOH-$CHCl_3$	35.2 ± 5.2

*not determined

Moreover, *C. bergamia* pulp MeOH-crude, MeOH-EA and MeOH-$CHCl_3$ extracts showed IC_{50} values for anti-tyrosinase activity as 366.8, 17.4 and 10.6 µg/mL, respectively (Table 3). However, IC_{50} for *C. bergamia* pulp MeOH-*n*-Hx was not determined.

Table 3: Anti-tyrosinase activity of *C. bergamia* fruit pulp extracts.

Extract of *C. bergamia* pulp	**Tyrosinase inhibition IC_{50} ± SEM (µg mL^{-1})**
MeOH-crude	366.8 ± 36.5
MeOH-*n*-Hx	*nd
MeOH-EA	17.4 ± 2.6
MeOH-$CHCl_3$	10.6 ± 1.5

*not determined

Second plant, *F. carica* leaves and fruit were also tested for possible anti-tyrosinase activity. *F. carica* leaves MeOH-crude, MeOH-*n*-Hx, MeOH-EA and MeOH-$CHCl_3$ extracts showed IC_{50} values as 156.2, 123.3, 127.9 and 16.7 µg mL^{-1}, respectively (Table 4).

Table 4: Anti-tyrosinase activity of *F. carica* leaves extracts.

Extracts *F. carica* leaves	**Tyrosinase inhibition IC_{50} ± SEM (µg mL^{-1})**
MeOH-crude	156.2 ± 12
MeOH-*n*-Hx	123.3 ± 24.4
MeOH-EA	127.9 ± 6.3
MeOH-$CHCl_3$	16.7 ± 3.3

Moreover, *F. carica* fruit MeOH-crude, MeOH-*n*-Hx, MeOH-EA and MeOH-$CHCl_3$ extracts showed IC_{50} values as 132.0, 141.3 and 105.7 and 15.1 µg mL^{-1}, respectively (Table 5).

Table 5: Anti-tyrosinase activity of *F. carica* fruit extracts.

Extract of *F. carica* fruit	**Tyrosinase inhibition IC_{50} ± SEM (µg mL^{-1})**
MeOH-crude	132.0 ± 10.5
MeOH-**n**-Hx	141.3 ± 7.1
MeOH-EA	105.7 ± 15.7
MeOH-$CHCl_3$	15.1 ± 2.9

Interestingly, *F. carica* fruit MeOH-crude, *C. bergamia* leaves MeOH-*n*-Hx, *C. bergamia* peel MeOH-EA and *C. bergamia* pulp MeOH-$CHCl_3$ extracts showed highest anti-tyrosinase activity from all MeOH-crude, MeOH-*n*-Hx, MeOH-EA and MeOH-$CHCl_3$ extracts, respectively (Table 6).

Table.6 Anti-tyrosinase activity trend with respect to their solvents.

Extract type	Trend in tyrosinase inhibition
MeOH-crude	*F. carica* fruit MeOH-crude > *C. bergamia* leaves MeOH-crude > *F. carica* leaves MeOH-crude > *C. bergamia* pulp MeOH-crude
MeOH-*n*-Hx	*C. bergamia* leaves MeOH-*n*-Hx > *F. carica* leaves MeOH-n-Hx > *F. carica* fruit MeOH-n-Hx > *C. bergamia* peel MeOH-*n*-Hx
MeOH-EA	*C. bergamia* peel MeOH-EA > *C. bergamia* leaves MeOH-EA > *C. bergamia* pulp MeOH-EA > *F. carica* fruit MeOH-EA > *F. carica* leaves MeOH-EA
MeOH-$CHCl_3$	*C. bergamia* pulp MeOH-$CHCl_3$ > *C. bergamia* leaves MeOH-$CHCl_3$ > *F. carica* fruit MeOH-$CHCl_3$ > *F. carica* leaves MeOH-$CHCl_3$ > *C. bergamia* peel MeOH-$CHCl_3$

Anti-oxidant activity

All the extract showed anti-oxidant activity with potential as tabulated below (Table 7). The *C. bergamia* MeOH-crude leaves, pulp and peel showed anti-oxidant IC_{50} values as 192.7, 384.2 and 125.9 µg mL^{-1}, respectively. However, *F. carica* leaves and fruit MeOH-crude extracts showed anti-oxidant IC_{50} values as 300.3 and 77.3 µg mL^{-1}, respectively (Table 7).

Table 7: Anti-oxidant activity MeOH-crude extracts.

Extract	DPPH activity IC_{50} ± SEM (µg mL^{-1})
C. bergamia leaves MeOH-crude	192.7 ± 28
C. bergamia pulp MeOH-crude	384.2 ± 19
C. bergamia peel MeOH-crude	125.9 ± 4
F. carica leaves MeOH-crude	300.3 ± 29
F. carica fruit MeOH-crude	77.3 ± 10

Thus, tested samples showed anti-oxidant as well as anti-tyrosinase activity, important for pigmentation and cosmetic industry.

DISCUSSION

In present study, we prepared methanolic (MeOH) extracts from *C. bergamia* (leaves, fruit peel and pulp) and *F. carica* (leaves and fruit) which were further processed for fractional distillation using ethyl acetate (EA), n-hexane (n-Hx) and chloroform ($CHCl_3$) (total 20 extracts) aiming to inhibit tyrosinase activity important for tyrosinase rooted pigmentation.

Later, anti-oxidant activity of all MeOH-crude extracts, reported to be associated with tyrosinase inhibition was evaluated. Interestingly, all the test samples were found significant anti-oxidant. Our results confirmed that ~~and~~ all the MeOH-crude *C. bergamia* leaves, fruit peel, pulp and *F. carica* leaves and fruit crude extracts showed anti-oxidant activity with IC_{50} range of 384.2 ± 19 to 77.3 ± 10 µg mL^{-1} collectively. Our results confirmed the previous finding that reported anti-oxidant properties of *Citrus* peel oil and *F. carica* (Tsai *et al.,* 2017; Patil *et al.,* 2009). *F. carica* leaves are enrich with flavonoids, sesquiterpenes, tannins, alkaloids and saponins (Tchombe and Louajri, 2015) responsible for various pharmacological activities i.e. anti-

oxidant, anti-cancer, anti-viral, anti-bacterial and anti-inflammatory activities (Badgujar *et al.,* 2014). Moreover, citrus fruits peel is used in Chinese medicines. In addition, citrus peel essential oil is shown to have mixture of bioactive volatile compounds like monoterpene hydrocarbons. Essential oils are used for aromatherapy and for certain psychological and physical conditions (Susan, 1996). Interestingly, anti-oxidant properties are reported to have close association with tyrosinase rooted melanin inhibition (Takahiro *et al.,* 2011). Anti-oxidants such as N-acetyl cysteine are reported to abolish UVB-induced α-melanocyte stimulating hormone (α-MSH) and to study the association of ROS with melanin synthesis (Funasaka *et al.,* 2000). Elevations of endogenous anti-oxidants are shown to suppress melanin production (Kameyama *et al.,* 1996) and ROS are reported to contribute in melanin synthesis (Ali *et al.,* 2019; Chou *et al.,* 2013; Yanase *et al.,* 2001). The cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) or mitogen-activated protein kinase (MAPK), melanocortin 1 receptor (MC1R), and microphthalmia-associated transcription factor (MITF), important melanogenic signaling are reported to down regulated with the ROS suppression which subsequently reduce tyrosinase, critical for melanin synthesis (Panich, 2011; Chou *et al.,* 2010; Yanase *et al.,* 2001).

All 20 test *F. carica* and *C. bergamia* extracts showed various levels of anti-tyrosinase activity (Table 1 – Table 5). Anti-tyrosinase activity trend among all tested extracts was as *C. bergamia* peel MeOH-EA > *C. bergamia* leaves MeOH-EA > *C. bergamia* pulp MeOH-$CHCl_3$ > *C. bergamia* leaves MeOH-$CHCl_3$ > *F. carica* fruit MeOH-$CHCl_3$ > *F. carica* leaves MeOH-$CHCl_3$ > *C. bergamia* pulp MeOH-EA > *C. bergamia* leaves MeOH-n-Hx > *C. bergamia* peel MeOH-$CHCl_3$ > *F. carica* fruit MeOH-EA > *F. carica* leaves MeOH-n-Hx > *F. carica* leaves MeOH-EA > *F. carica* fruit MeOH-crude > *F. carica* fruit MeOH-n-Hx > *C. bergamia* peel MeOH-n-Hx > *C. bergamia* leaves MeOH-crude > *F. carica* leaves MeOH-crude > *C. bergamia* pulp MeOH-crude.

Anti-oxidants and tyrosinase inhibitors are critical for melanin inhibition. Inhibitors can be achieved from diverse synthetic sources. However, their safety issues prevent their commercialization. Therefore, isolation of extracts from natural sources is desirable. Thus, we selected leaves and fruits of edible *F. carica* and *C. bergamia* for the isolation of extracts. Extracts from both selected plants showed anti-oxidant as well as anti-tyrosinase activity. Interestingly, *C. bergamia* MeOH-EA peel extract showed highest tyrosinase inhibition (IC_{50}) 4.1 ± 0.3 µg/mL among all tested extracts proposing it most potential candidate to control tyrosinase rooted hyperpigmentation.

CONCLUSION

Present study confirmed that all *C. bergamia* and *F. carica* tested MeOH crude extracts had significant anti-oxidant and anti-tyrosinase activities. Moreover, crude extracts processed for fractional distillation i.e EA, n-Hx and $CHCl_3$ also showed potent tyrosinase inhibition, *in-vitro*. However, *C. bergamia* MeOH-EA peel extract showed highest tyrosinase inhibition among all tested extracts out-reaching as most potent candidate for tyrosinase rooted hyperpigmentation in future.

CONFLICT OF INTEREST

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

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