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# Anti-melanogenesis potential of a new series of Morita-Baylis-Hillman adducts in B16F10 melanoma cell line

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ABSTRACT

Melanin is a natural polymer pigment which provides skin photoprotection against ultraviolet radiation. An excessive synthesis of melanin leads to hyperpigmentation disorders. Tyrosinase catalyzes the rate limiting steps on melanogenesis. Therefore, tyrosinase inhibitors have potential applications in medicine and cosmetic fields. We carried out herein the screening of a family of cyclic Morita-Baylis-Hillman adducts (MBH) to find out their effects on tyrosinase activity and on melanogenesis in murine melanoma B16F10 cell line. Kinetic analysis of tyrosinase inhibition showed that compounds **1a** (2-hydroxymethyl) cyclohex-2-enone) and **3f** (diethyl (1-(6-oxocyclohex-1-en-1-yl) ethyl-phosphonate) were competitive inhibitors, whereas the compound **2b** (6-oxocyclohex-1-en-1-yl) ethyl acetate) was a non-competitive one. Additionally we have found that (**1a**, **2b** and **3f**) compounds had a strong melanogenesis inhibition effect in isobutylmethylxanthine (IBMX)-treated murine melanoma B16F10 cells when tested at low and non cytotoxic dose (10–50 μM), by attenuating the melanin production, intracellular tyrosinase activity and tyrosinase expression. Thus, we suggest that these compounds could be used as effective skin-whitening agents.

## 1. Introduction

Tyrosinase (EC 1.14.18.1), belonging to the metalloenzymes family, is a copper-containing multifunctional oxydase [1,2] that is widely found in nature. It catalyzes the hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones, both depending on the molecular oxygen [3,4]. Tyrosinase is a key enzyme in melanin biosynthesis involved in determining the color of mammalian skin, eyes and hair [5]. Its abnormal expression is responsible for the various dermatological disorders such as melasma, age spots, and sites of actinic damage. It also contributes to neuro-melanin formation in the human brain and the neuro-degeneration associated with Parkinson's disease [6]. Furthermore, tyrosinases catalyze the browning of vegetables and fruits [7] and the cuticle formation in insects [8]. The browning is an undesirable reaction that is responsible for lower product quality and, becomes a major problem in food industry and one of the main causes of quality-loss throughout post-harvest action.

Melanin is a biological pigment that play a preponderant role in determining the color of the skin, hair and eyes, and protecting the skin against ultraviolet radiation from the sun which accentuate the aging process and the risks of skin cancer [9]. Excessive production of melanin causes abnormal pigmentation such as freckles, age spots, melasma and melanoma. Therefore, the search for moderating agents of pigmentation is important for treating abnormal skin pigmentation.

Therefore, tyrosinase inhibitors have become increasingly important in medicinal, cosmetic and in the agro-food fields.

Many tyrosinase inhibitors have been used to treat skin hyperpigmentation such as arbutin [10], kojic acid [11], hydroquinone [12] and quercetin [13,14]. Synthesis of new and diverse structures of compounds may serve to find-out more effective and safe wightening agents.

The compounds used in our study were produced by the reaction of "Morita–Baylis–Hillman" which is a versatile C—C bond forming reaction that provides functionalized adducts [15,16]. These adducts and their derivatives have potential biological activities [17], and they have been explored for the synthesis of various biologically active natural products.

Cyclic Morita-Baylis-Hillman adducts have various and potent biological activities such as Antimicrobial [18,19], antifungal [20], anti-

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malaria [21,22], Leishmanicidal [23] and anti-proliferative activity on human tumor cell lines [24]. Thus these compounds may be useful as therapeutic agents in the treatment of various human diseases. However there is no report on effect of MBH adducts on tyrosinase and melanogenesis in B16F10 melanoma cell line.

In our study, we were interested by studying the effect of three series of cyclic Morita-Baylis-Hillman (MBH) adducts, on tyrosinase activity and on melanogenesis, i.e., α-(hydroxyl-alkyl) cyclohexenones whish is firstly synthetized in the presence of various aldehydes with moderate to good yields [25 − 29], the α-(acetoxy-methyl) cyclohexenones synthetisized from the α-(hydroxyl-alkyl) cyclohexenones [30,31] and finally the γ-keto allylic phosphonates [32] synthetisezed by the substitution of acetate with a phosphanate. In a recent study, the Morita-Baylis-Hillman (MBH) adducts have potent antioxidant activity [33] and the γ-keto allylic phosphonates were used in the synthesis of new tin tetrachloride adducts [34].

In the first part of our study, we performed the screening of inhibitory effect of these compounds on tyrosinase activity from the free ink of *Sepia officinalis* which exhibits a powerful tyrosinase activity [35] and represents a convenient model system for investigating melanogenesis [36]. It has been shown indeed that Sepia tyrosinase can be inhibited by arbutin and *p*-coumaric acid [37], Aryl butenes compounds [38,39] and some triazolylnucleosides [40].

The second part is dedicated for the search of the effects of synthetic compounds on murine tyrosinase activity and on melanogenesis using cultured B16F10 murine melanoma cell line which is the closest model to human, for the purpose of developing novel hypo-pigmenting agents in mammalian.

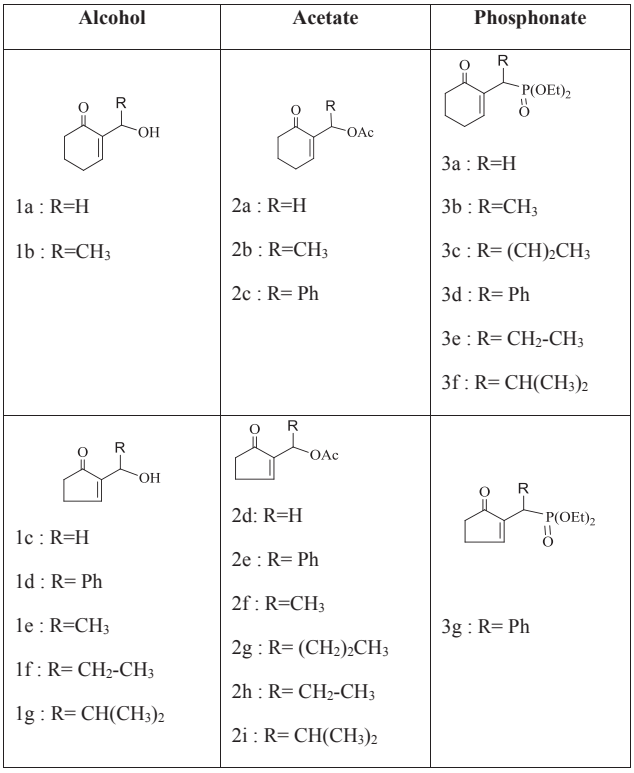


**Fig. 1.** Chemical structures of cyclic Morita-Baylis-Hillman adducts.

## 2. Experiments

### 2.1. Reagents

L-Tyrosine was purchased from Fluka Biochimika, Germany. Dimethyl sulfoxide (DMSO) was obtained from Lobal Chemie, Mumbai, India. L-DOPA, Gentamicin and MTT (Thiazolyl Blue Tetrazolium Bromide) were purchased from Bio Basic, Canada. 3-Isobutyl-1-methylxanthine (IBMX) and melanin were obtained from Sigma-Aldrich, Germany. RPMI-1640 Medium, Fetal Bovine Serum and trypsin-EDTA (0.05%) were purchased from Gibco Life Technologies, Paisley, UK. A series of cyclic (MBH) adducts were kindly given by Prof. Farhat Rezgui and synthesized as described in previous Refs. [26–32] (Fig. 1).

### 2.2. Extraction and purification of Sepia tyrosinase

The tyrosinase was isolated [37] from the ink sacs of the mollusk *Sepia officinalis* collected from the Gulf of Gabes (south of Tunisia). The ink was collected and stored at 4 °C. To separate enzymatic extract from the melanin, the ink was centrifuged at 20.000 rpm at 4 °C and the supernatant (melanin-free ink) was collected and stored at -20 °C until further use. The tyrosinase was partially purified by chromatography on Phenyl Sepharose column equilibrated with 0.5 mM ammonium sulfate and 50 mM sodium phosphate buffer pH 7. The enzyme was eluted afterward with an inverse linear gradient of 0.5–0 mM ammonium sulfate in 50 mM sodium phosphate buffer pH 7. The active fractions were collected. All steps were carried out at 4 °C.

The proteins concentration was determined using the Bradford method with bovine serum albumin as standard.

### 2.3. Effects of inhibitors on Sepia tyrosinase activity

Tyrosinase activity was measured using a previously described method with some modifications [37]. The standard reaction medium contained 50 mM phosphate buffer (pH 6.8), the compound to be tested and the enzyme solution. The compounds were dissolved in DMSO. The final concentration of DMSO in the test solution was 3.3% [41], the assay mixture without inhibitor was used as a negative control.

After a pre-incubation period of 5 min at room temperature, 0.4 mM L-Tyrosin was added and the generated dopachrome was measured by a Shimadzu spectrophotometer (UV-1650PC) at 475 nm every 10 s for at least 5 min and at 58 °C [37,42]. One unit (U) of enzymatic activity was defined as the amount of enzyme transforming 1 µmole of L-DOPA per minute. The extent of inhibition by added sample was expressed as the amount necessary for 50% inhibition ($IC_{50}$).

$$\text{Inhibition } (\%) = (1 - A1/A0) \times 100$$

(A0 = negative control absorbance, A1 = assay absorbance). Experiments were performed in duplicate.

### 2.4. Determination of the inhibition type and kinetic parameters

To determine the nature of inhibition and kinetic parameters, we used the program "Sigma Plot version 12" after establishing the Lineweaver-Burk representation of double reciprocal plots (1/v versus 1/[S]).

The inhibition constant $K_I$ was derived from a plot of the apparent Michaelis-Menten constant ($K_m$) or maximal velocity ($V_{max}$) versus the concentration of the inhibitor.

Apparent $K_m$ and $V_{max}$ parameters can be obtained from Eq. (1) for the various inhibition types by letting $K_{iu}$ and $K_{ic}$ approach infinity and then comparing (1) with the Michaelis-Menten Eq. (2).

$$V = V_{max}S/[K_m(1 + I/K_{ic}) + S(1 + I/K_{iu})] \quad (1)$$

$$V = V_{max}S/(K_m + S) \quad (2)$$

$K_{ic}$ and $K_{iu}$ are the EI and ESI dissociation constants (Cornish-Bowden notation [43]). The Enzyme Kinetics Module uses the notation:

$K_i = K_{ic}$

$\alpha K_i = K_{iu}$

For competitive inhibition, $K_{iu}$ is infinite to give $K_m^{app} = K_m(1 + I/K_{ic})$ and $V_{max}^{app} = V_{max}$.

For non competitive inhibition, $K_{ic} = K_{iu}$ which gives $K_m^{app} = K_m$ and $V_{max}^{app} = V_{max}/(1 + I/K_{ic})$.

### 2.5. Murine melanoma cell line culture

The murine B16F10 melanoma cell line was maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and Gentamicin (50 μg/ml) at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### 2.6. Extraction and effects of inhibitors on murine tyrosinase activity

The melanoma B16F10 cells treated with 100 μM of IBMX, were lysed in the buffer (10 mM sodium phosphate pH 6.8, 1% Triton X-100, and 1 mM PMSF). The lysates were centrifuged at 13.000 rpm for 20 min, and the supernatant was used as a crude cellular tyrosinase. The proteins content in the supernatant was determined by Bradford assay using BSA as a standard. Fifty micrograms of proteins were placed in each well of a 96-well plate and the enzymatic assay was initiated by adding L-DOPA, the inhibitory compounds (**1a**, **2b** and **3f**) and 50 mM sodium phosphate buffer pH 6.8. The dopachrome formation was monitored by measuring the absorbance at 475 nm every 10 min for at least 1 h using a microplate reader (Thermo Scientific Varioscan Lux). The tyrosinase activity was expressed as percent change relative to the control.

### 2.7. Murine cell viability

Cell viability was determined using the MTT assay. Briefly, the B16F10 cells were seeded at a density of $2 \times 10^4$ cells/well in 96-well tissue culture plate. After 24 h of incubation, the cells were treated with various concentrations of compounds for 24 h. After media removal, fresh media containing MTT solution (1 mg/ml) was added and incubation was pursued for 4 h at 37 °C. Subsequently, formasan crystals formed were dissolved in SDS and the absorbance was measured at 570 nm using a microplate reader (Thermo Scientific Varioscan Lux). Tested compounds was dissolved in DMSO solution. In consideration of the possible effect of DMSO on cell viability, a maximum amount (1%) of DMSO was added to culture media and used as positive control. DMSO at this amount was found not to affect the viability of B16F10 cells. Data were representative of three independent experiments.

### 2.8. Measurements of melanin content in murine melanoma cells

The melanin content was measured using a previously described method with slight modifications [44]. Briefly, cells were seeded at a density of $2 \times 10^5$ cells/well in a 6-well plate. After 1 day of incubation, the murine B16F10 cells were treated with various concentrations of compounds (**1a**, **2b**, and **3f**) and in the presence of IBMX (3-isobutyl-1-methylxanthin) (100 μM) that induced melanogenesis. After 48 h of incubation, the B16F10 cells were harvested and washed twice with PBS. The pelleted cells were dissolved in 1 N NaOH for 1 h at 60 °C. Then, the absorbance at 410 nm was measured, and the melanin content was calculated against a known standard of synthetic melanin. The proteins content in the supernatant was determined by Bradford assay. Specific melanin content was related to the amount of proteins in the same reaction.

### 2.9. Measurements of intracellular tyrosinase activity in treated cells with allyl compounds

The effect of the compounds on tyrosinase activity from IBMX-induced B16F10 cells was investigated. After 24 h incubation, in a 6-well plate, the cells were treated with various concentrations of the compounds and 100 μM IBMX for another 24 h incubation. The cells were lysed in cold lysis buffer (10 mM sodium phosphate pH 6.8, 1% Triton X-100, and 1 mM PMSF) and the crude cellular tyrosinase was prepared as described previously. Fifty micrograms of protein were placed in each well of a 96-well plate and the enzymatic assay was initiated by adding L-DOPA and the sodium phosphate buffer pH 6.8; the generated dopachrome was measured at 475 nm every 10 min for at least 1 h using a microplate reader (Thermo Scientific Varioscan Lux). The tyrosinase activity was expressed as percent change relative to the control induced by IBMX.

### 2.10. Western blots

After treatment, cell lysates were prepared using RIPA buffer. Thirty micrograms of protein (determined by the Bradford protein assay) were mixed with loading buffer and boiled at 100 °C for 5 min. The samples were separated by SDS-PAGE and transferred onto PVDF membrane (Amersham, GE Healthcare Life Science,UK). The membrane was blocked with 5% non fat dry milk in PBST buffer (0.1% Tween 20 in PBS) for over-night at 4 °C and incubated for 2 h with primary antibody anti-tyrosinase (Life technology, USA) in 1% blocking buffer at room temperature. After the membrane was washed with PBST, it was incubated with anti-mouse-IgG-HRP (Vector Laboratories, Burlingame, California) for 1 h at room temperature. After washing the membrane, the enhanced chemiluminescence (ECL) detection reagents (GE Healthcare,UK) were used to develop the signal of the membrane.

### 2.11. Statistical analysis

The differences between the results of the melanin content assay and intracellular tyrosinase activity assay were assessed for statistical significance using the Student's test using SPSS 13.0 statistical software (SPSS). Differences were considered statistically significant at $^{*}p < 0.05$ and $^{**}p < 0.01$.

## 3. Results

### 3.1. Effects of MBH derivatives on the monophenolase activity of Sepia tyrosinase

The effects of various MBH adducts (Fig. 1) on the tyrosinase activity from the free ink of *Sepia officinalis* were determined and summarized in Table 1. Among the twenty six compounds, only three cyclohexenone molecules were found to be potent tyrosinase inhibitors: (**1a**) (2-hydroxymethyl) cyclohex-2-enone), (**2b**) (6-oxocyclohex-1-en-1-yl) ethyl acetate) and (**3f**) (diethyl (1-(6-oxocyclohex-1-en-1-yl) ethyl) phosphonate). The $IC_{50}$ value of these three compounds were calculated from the plot of inhibition percentages in function of inhibitor concentrations and these were determined to be 22, 7.5 and 42 mM, respectively (Table 1 and (Fig. 2A–C), indicating that compound (**2b**) is the most potent inhibitor.

All cyclopentenones derivatives were not effective against monophenolase activity of Sepia tyrosinase at 50 mM.

### 3.2. A competitive mechanism for the inhibition of the tyrosinase activity by the compounds (1a) and (3f)

The form of inhibition exerted by compounds (**1a**) and (**3f**) of tyrosinase activity was deduced from Lineweavere-Burk double reciprocal plots (Fig. 3). In the presence of compounds (**1a**) and (**3f**), plots produced a family of straight lines with the same y-axis intercept, indicating that these compounds are competitive inhibitors of tyrosinase. This means that each of these compounds can bind to the free enzyme preventing, in that way, the substrate from binding to the active site. The inhibition constants $K_i$ of the inhibitor binding to the free enzyme (E) were determined and summarized in Table 1.

**Table 1**
Effects of Morita-Baylis-Hillman adducts on tyrosinase activity.

| Group | Number | Residual activity % | | IC50 (mM) | Inhibition type | Ki (mM) |
|---|---|---|---|---|---|---|
| | | 20 mM | 50 mM | | | |
| Alcohol | **1a** | 58.5 ± 1.5 | 3.8 ± 0.8 | 22 | Competitive | 4.7 |
| | **1b** | 95 ± 3.8 | 88 ± 2.5 | | | |
| | **1c** | 100 ± 1.6 | 97 ± 2.1 | | | |
| | **1d** | 94 ± 1.5 | 90 ± 5.1 | | | |
| | **1e** | 90 ± 2.2 | 75 ± 3.1 | | | |
| | **1f** | 80 ± 2.6 | 63 ± 1.9 | | | |
| | **1g** | 91 ± 2.0 | 74 ± 3.1 | | | |
| Acetate | **2a** | 60 ± 1.2 | 58 ± 1.5 | | | |
| | **2b** | 0 ± 0.0 | 0 ± 0.0 | 7.5 | Non competitive | 7.5 |
| | **2c** | 97 ± 2.3 | 95 ± 2.5 | | | |
| | **2d** | 100 ± 2.1 | 98 ± 2.1 | | | |
| | **2e** | 100 ± 2.3 | 91 ± 2.6 | | | |
| | **2f** | 100 ± 1.8 | 99 ± 1.8 | | | |
| | **2g** | 100 ± 0.2 | 95 ± 1.1 | | | |
| | **2h** | 98 ± 1.6 | 91 ± 3.6 | | | |
| | **2i** | 95 ± 1.9 | 75 ± 0.8 | | | |
| Phosphonate | **3a** | 95 ± 2.2 | 86 ± 2.0 | | | |
| | **3b** | 89 ± 1.1 | 70 ± 1.6 | | | |
| | **3c** | 100 ± 1.2 | 95 ± 2.3 | | | |
| | **3d** | 100 ± 1.0 | 100 ± 2.2 | | | |
| | **3e** | 94 ± 1.5 | 83 ± 2.9 | | | |
| | **3f** | 70 ± 2.0 | 42.1 ± 1.9 | 42 | Competitive | 21.3 |
| | **3g** | 100 ± 2.2 | 98 ± 2.3 | | | |

### *3.3. A non-competitive mechanism for the inhibition of the tyrosinase activity by compound (**2b**)*

The tyrosinase kinetics were determined in the presence of compound (**2b**) using double reciprocal Lineweaver–Burk plots. The plots of 1/v versus 1/[S] gave a family of straight lines with different slopes which intersected one another at the X-axis (Fig. 3); the $K_m$ remained the same while $V_{max}$ decreased. The compound (**2b**) is therefore considered as a non-competitive inhibitor of the enzyme. This means that compound (**2b**) can bind not only to the free enzyme, but also to the enzyme-substrate complex.

### *3.4. Effects of compounds (**1a, 3b** and **5f**) on murine tyrosinase activity*

The previously discovered tyrosinase inhibitors have been assayed using Sepia tyrosinase because of its availability and powerful activity [35]. However, the obtained tyrosinase inhibitors could have some limitations in their application as depigmenting agent for human skin, because of the differences between Sepia and human tyrosinases. Therefore, we used a crude murine tyrosinase, that is analogous to the human one, to carry out the inhibitory effects of MBH adducts on enzymatic activity assays *in vitro*. Various concentrations of compounds (**1a**, **3b** and **5f**) were added to a crude enzyme containing tyrosinase activity extracted from IBMX-induced B16F10 murine cells. We have noticed that in the presence of 1 mM compound (**2b**), the murine tyrosinase activity decreased to 80% while the compounds (**1a**) and (**3f**) were much less actives against tyrosinase activity (Fig. 4).

### *3.5. Evaluation of depigmentation activity of compounds (**1a, 2b** and **3f**) in murine B16F10 melanoma cells*

Compounds (**1a**, **2b** and **3f**) were tested *in vitro* on melanogenesis of a mammalian system: the murine melanoma cell line B16F10.

Foremost, cytotoxicity was evaluated through cell viability determined by the MTT assay after 24 h incubation with the compounds (**1a**, **2b** and **3f**) in a concentration ranging from 10 to 100 μM. Fig. 5(A) indicates that these compounds did not alter cell viability even at 100 μM.

Then, the effectiveness of melanization process was estimated by measuring the melanin content of cultured melanoma cells after treatment with these compounds. Fig. 5(B) shows the melanin pellets and the specific melanin content of B16F10 cells, either untreated or treated

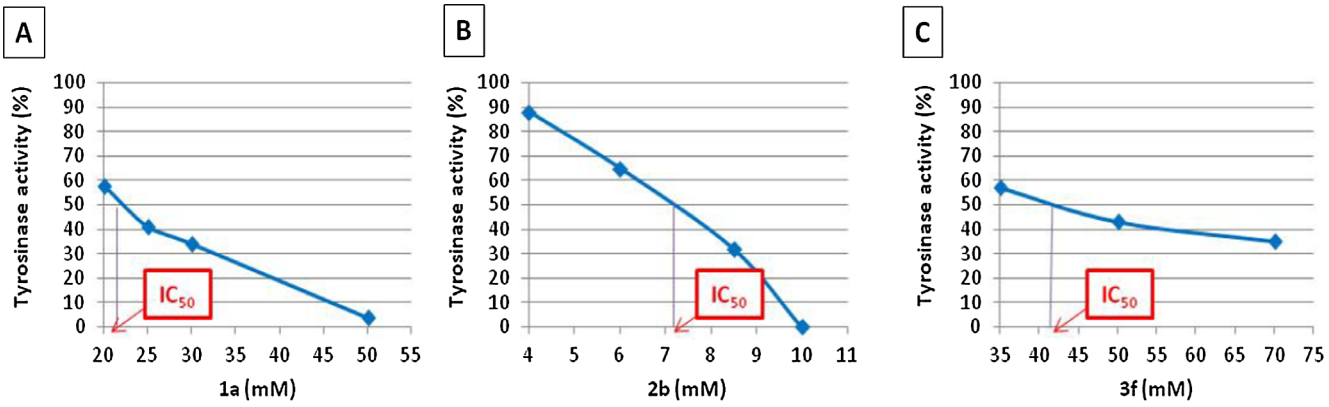


**Fig 2.** The tyrosinase activity in function of the inhibitor concentration allowing the determination of $IC_{50}$ values of compounds 1a, 2b and 3f.

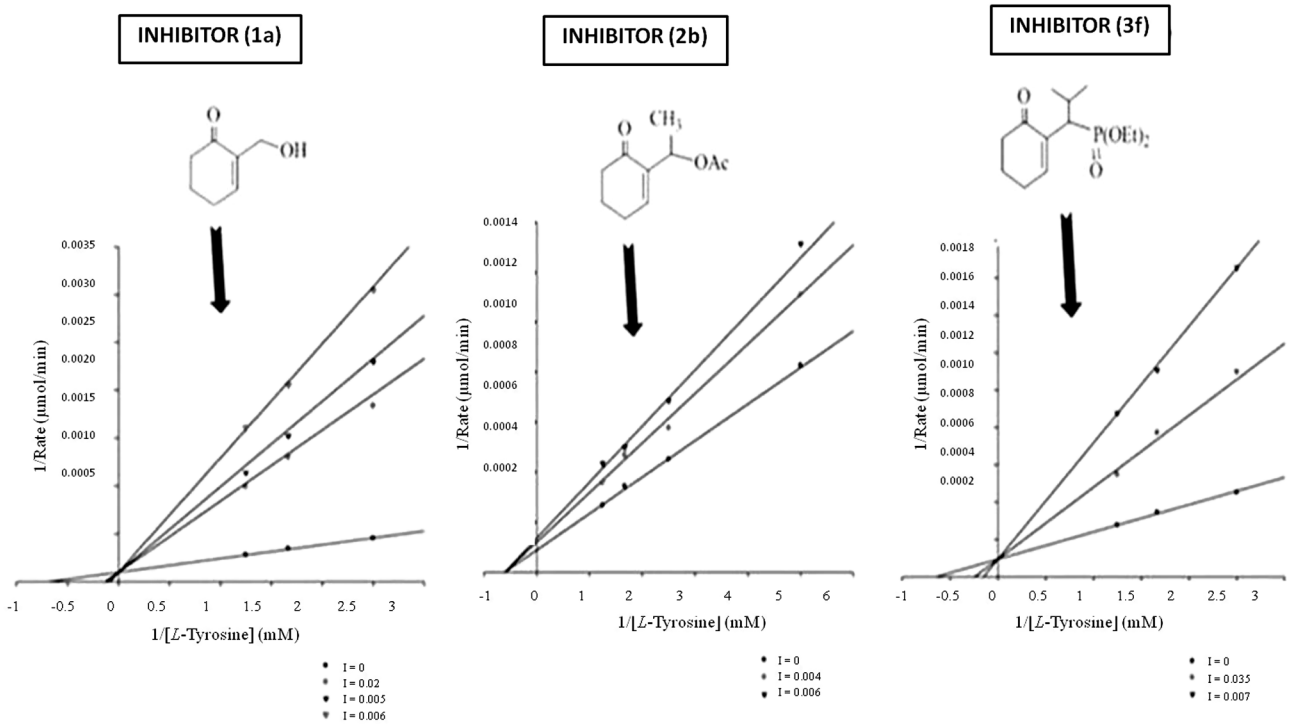


**Fig. 3.** Lineweaver-Burk inhibition plots of three compounds (1a, 2b and 3f) on tyrosinase activity. The structure of each compound is shown on the top-left of each corresponding plot.

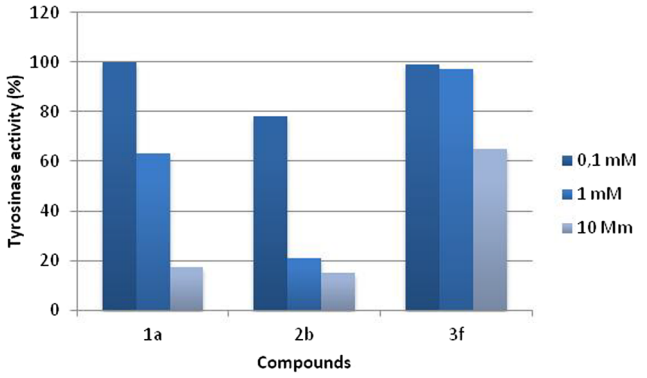


**Fig. 4.** Effects of compounds (1a, 2b and 3f) on murine tyrosinase activity extracted from B16F10 cells at 0.1, 1 and 10 mM.

with 100 µM of IBMX and various concentrations of compounds (**1a**, **2b** and **3f**). The melanin content and color of pellets of cells treated with IBMX are higher than that of untreated cells, which confirms that melanogenesis was successfully induced by IBMX. Treated cells with compounds (**1a**, **2b** and **3f**) presented a dose-dependent decrease in their melanin content at low and non-cytotoxic concentrations (10 and 50 µM). Compound (**2b**) had the strongest effect on melanin production, it decreased almost half-amount of melanin at a dose of 50 µM after 48 h of incubation. Melanin content in treated cells with (**1a**) and (**3f**) decreased to 21 and 29% at 50 µM respectively (Fig. 5B).

Afterward, the tyrosinase activity of treated murine cells with various concentrations of the 3 compounds (**1a**, **2b** and **3f**), was then determined. These compounds exhibited tyrosinase inhibitory effect which correlated with that found for the decrease in the amount of melanin (Fig. 5B and D). Tyrosinase activity in B16F10 was strongly decreased by 26, 44.5 and 23% at 50 µM of compound **1a**, **2b** and **3f** respectively (Fig. 5D).

Finally, the protein expression of tyrosinase in treated cells was measured by western blot analysis. We found that the tyrosinase expression was repressed with increasing concentration of tested compounds from 10 to 50 µM (Fig. 5C).

## 4. Discussion

Cyclic Morita-Baylis-Hillman adducts (MBHA) are a new class of bioactive compounds, most of them can be prepared by fast and in a single synthetic step in high yields [17]. The chemical synthesis of various derivatives led to the discovery of new cheaper and efficient drugs.

Herein, we looked, for the first time, the effects of the MBHA derivatives on Sepia tyrosinase activity and on melanogenesis in murine B16F10 melanoma cell line.

In the first part, we have noticed that among the twenty three compounds tested, only three (**1a**) (2-hydroxymethyl) cyclohex-2-enone, (**3f**) (diethyl (1-(6-oxocyclohex-1-en-1-yl) ethyl) phosphonate) and (**2b**) (6-oxocyclohex-1-en-1-yl) ethyl acetate) presented significant inhibition on tyrosinase activity. In particular, the compound (**1a**), belonging to the hydroxymethyl-cyclohexenone family, showed an important inhibitory effect (42%) on tyrosinase activity at a dose of 20 mM (Table 1). This might be explained by the presence of hydroxyl group in this molecule. Indeed, previous studies have shown that a hydroxyl group can be responsible for a better inhibitory effect on the monophenolase activity of mushroom tyrosinase [13].

Among the α-(acetoxymethyl) cyclohexenones, we found that compound (2b) presented the strongest effect with 100% inhibition at a dose of 20 mM and an IC50 value of 7.5 mM. By comparing its structure with that of compound (2a), devoid of inhibiting activity, we believe that the presence of a methyl group at the allyl position could be responsible for the inhibitory effect.

Among the δ-keto allyl phosphonates, the compound (3f) exhibited 58% inhibition of tyrosinase activity at a dose of 50 mM; this could be the result of the presence of the isopropyl group at the allyl position.

We noticed that all cyclopentenones derivatives did not have an inhibitory effect (Table 1 and (Fig. 1), see D, E and F). This can be explained by the fact that the 6-membered ring is more reactive than the 5-membered one.

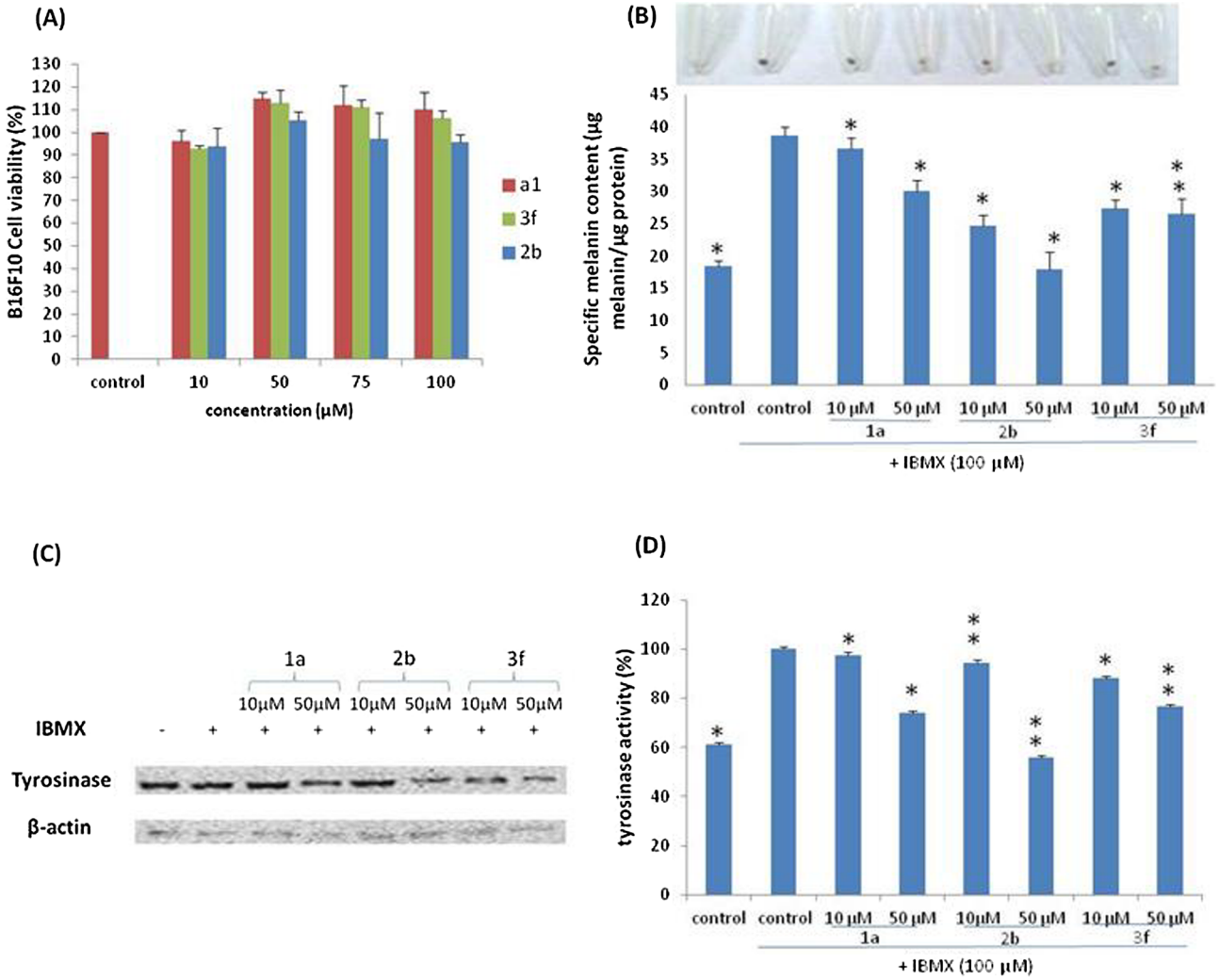


**Fig. 5.** Effects of compounds (1a, 2b and 3f) on cell viability, tyrosinase and melanin synthesis in murine B16F10 melanoma cells. (A) Viability of B16F10 cells treated with three compounds at various concentrations (from 10 µM to 100 µM); (B) Photograph of melanin pellets and the corresponding specific melanin content determined in B16F10 treated cells with 10 and 50 µM concentrations of compounds; (C) Western blot analysis of tyrosinase secretion in B16F10 cell line after treatment with compounds (1a, 2b and 3f) at 10 and 50 µM and (D) Intracellular tyrosinase activity in B16F10 cells treated with 10 and 50 µM concentrations of compounds. Data shown represent the mean ± S.D of three separate experiments. Values are significantly differents by comparison with IBMX-treated control. $^{*}p < 0.05$ and $^{**}p < 0.01$.

The kinetic study showed that the type of inhibition for (1a) and (3f) compounds is competitive and that of compound (2b) is non-competitive (Fig. 3). The examination of the compounds' structures shows that the competitive compound (1a) had a hydroxyl group and could be considered somehow as an analog of the substrate L-Tyrosine, therefore competing with the substrate at the active site. Other studies have shown that the presence of an aromatic portion on MBHA derivative is essential for their bioactivity [24].

In the second part, the inhibitory effect on murine tyrosinase and melanogenesis activity of compounds (**1a**, **2b** and **3f**) was investigated in IBMX-stimulated murine B16F10 cells. IBMX has been reported as an intracellular cAMP elevator by inhibiting phosphodiesterase, a cAMP degrading enzyme [45].

The screening and kinetics study of Sepia tyrosinase correlated with the effect of MBHA compounds on crude murine tyrosinase activity in B16F10 cells (Fig. 4). The compound **2b** is the potent Sepia tyrosinase and melanogenesis inhibitor, but its inhibiting concentration on Sepia and murine tyrosinase activity (at mM) is greater than that exerted on cellular murine tyrosinase activity (Fig. 5D) and melanin production in B16F10 cell (at µM) (Fig. 5B). Morever, the western blot analysis showed that the treatment of B16F10 cells by compounds (**1a**, **2b** and **3f**) decreased tyrosinase expression with a concentration-dependant manner (Fig. 5C). Therefore, we suggest that the decrease of melanin content in the IBMX-stimulated B16F10 cells would not be due to a direct tyrosinase inhibition only; it might rather affect the expression of tyrosinase gene and other melanogenesis proteins (at the level of transcription or translation). Indeed, other studies showed that the treatment of hyperpigmentation could be done through the inhibition of the expression of other proteins involved in the melanogenesis process such us the tyrosinase related protein 1 (TRP1), tyrosinase related protein 2 (TRP2) and microphtalamia-associated transcription factor (MITF) [46]. The latter factor regulates the expression of tyrosinase, TRP1 and TRP2 genes at transcriptional level via binding to the M-box motif in their promoter regions [47]. Moreover, the depigmentation can be done by the inhibition of maturation of tyrosinase [48] or by the acceleration of tyrosinase degradation [49].

Melanoma B16F10 cell viability is not affected by the compounds (**1a**, **2b** and **3f**) at the concentrations ranging from 10 to 100 µM which was employed in our experiments of melanogenesis inhibition (Fig. 5A). Other MBHA derivatives had a cytotoxic activity against NCIADR cell [24] and cervical cancer (Hela) cell line [50] at low concentrations.

Most importantly, we have discovered three Sepia tyrosinase

inhibitors from MBHA derivatives, and we investigated their anti-melanogenesis effect in IBMX-treated murine melanoma B16F10 cell line. They inhibited melanin production through the down-regulation of tyrosinase activity and tyrosinase expression. We suggest that these melanogenesis inhibitors could be used in the cosmetic field as skin-depigmenting agents.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.11.028.

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