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Original Article

Bioactive compounds from *Malva rotundifolia* L.

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ABSTRACT

Eight compounds were isolated for the first time from the methanolic extract of *Malva rotundifolia* L. Based on spectroscopic data and mass techniques, the structures of these compounds were identified as palmitic acid (**1**), β-sitosterol (**2**), 5α,8α-epidioxyergosta-6,22-dien-3β-ol (**3**), di-(2-ethylhexyl)phthalate (**4**), β-sitosterol 3-O-β-D-glucoside (**5**), methyl linoleate (**6**), 3-oxo-α-ionol β-D-glucopyranoside (**7**) and megastigman-7-ene-3,5,6,9-tetrol (**8**). All compounds except **2** and **5** were examined for their anti-allergic and melanin synthesis activities on RBL-2H3 and B16 melanoma cells, respectively. The most active compound was **3** which significantly suppressed the release of β-hexosaminidase and therefore it can be used as anti-allergic substance. The same compound also showed melanin synthesis inhibition but other compounds (**1**, **4**, **6**, **7** and **8**) showed melanin synthesis stimulatory effects at the same concentration.

Keywords: *Malva rotundifolia* L., Palmitic acid, β-Hexosaminidase, Melanin

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INTRODUCTION

Family Malvaceae includes over 75 genera and 1000 species of tropical and temperate distribution, especially in America [1, 2]. This family is represented by several genera, such as, *Althaea* (hollyhock, marshmallow); *Sida*; *Abutilon* (Indian mallow); *Gossypium* (cotton); *Hibiscus* (rose mallow) [2]. Other well-known plants are the mallows or cheeseweeds (*Malva*). The name cheeseweeds is due to the separation of the fruit compartment into sections resembling pieces of circular cheeses of old fashioned grocery stores [3]. Genus *Malva* is reported to have flavonoids [4, 5], anthocyanins [6], steroids [7, 8], phenolics [9], aliphatic compounds [10, 11], coumarins [9] and others. *Malva* genus has demonstrated different biological effects such as antioxidant [12, 13], anti-inflammatory [14, 15], antimicrobial [16, 17], hypoglycemic [18], hepatoprotective [19], and other biological activities include counter-irritant activity [20]. Furthermore, it is also reported to reduce the occurrence of the cardiovascular diseases and prevent thrombus formation [21], and treatment of hand eczema [22].

There is no report concerning the chemical composition of *M. rotundifolia* L. However, little biological studies were published [23]. Therefore, the purpose of this study was to investigate the chemical constituents of *M. rotundifolia* L. and evaluate their effects on melanin synthesis and their anti-allergic activities.

On the other hand, we measured the anti-allergic activities of the isolated compounds by using rat basophilic leukemia mast cells (RBL-2H3) which used as a pattern of human mast cells. Mast cells have vital roles in allergic diseases. Activation of mast cells by an allergen causes exocytosis. Upon exocytosis, the allergic mediator β-hexosaminidase released linearly with histamine from mast cells [24]. Any substance that suppresses the release of these inflammatory mediators is considered to be a potential anti-allergic substance [25].

Besides, we evaluated the effect of the isolated compounds on melanin synthesis. Production of melanin and skin pigmentation are the function of melanocytes. These melanocytes synthesize melanin upon irradiation by ultraviolet and then melanin transports to keratinocytes to absorb energy and protect against sunburn. Nevertheless, abnormal melanogenesis results in pigmentary disorders, such as hypopigmentation or hyperpigmentation, which are of clinical and cosmetic concerns. Many lightening compounds such as arbutin, are used to modulate melanogenesis [26]. For elimination of the influence of the cytotoxicity of the compounds, the MTT assay was done in parallel for both experiments.

MATERIAL AND METHODS

General: 1D and 2D spectra of compounds were recorded on Bruker AVANCE III 400 MHz equipped with a 5-mm PABBO probe head. Samples were dissolved in $CDCl_3$ or CD_3OD and the chemical shifts are expressed in ppm relative to the TMS acting as an internal standard. TLC was performed on silica gel F_{254} aluminum plate (20 × 20 cm, Fluka) (Merck, Germany). The detection was done at UV_{254} nm using ultra violet lamp, (Desaga, Germany) working at 254 and 366 nm. Using 0.5% vanillin (Sigma) in conc. H_2SO4-EtOH (1:19) as spraying reagent followed by heating, spots were visualized. Isolation and purification procedures were performed using solvents of reagent grade, purified by distillation (Fischer chemicals). Column chromatography (CC) was performed on silica gel G 60-230 mesh (Merck, Germany).

Plant material: The plant material used in this study consists of all aerial parts (with flowers and seeds) and roots of *M. rotundifolia* L. This plant was collected from Saint Catherine's Monastery, Egypt in the South Sinai Governorate on March, 2013. The plant identity was kindly confirmed by Dr. Ibrahim Mashaly, Professor of Ecology, Faculty of Science, Mansoura University, Egypt. A voucher specimen (No. 2013/MR) was kept at the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University.

Extraction and isolation procedures: The powdered dried plant (400 g) was extracted by percolation with MeOH (5 x 1 L). The combined extracts of methanol were concentrated under reduced pressure at 40 °C until a syrupy consistency is obtained. Drying was done in a desiccator over anhydrous $CaCl_2$ till a constant weight (66 g). The dried extract was suspended in water-methanol and subjected to successive extraction with petroleum ether (5 x 200 mL), methylene chloride (4 x 200 mL), ethyl acetate (4 x 200mL) and *n*-butanol (5 x 200 mL). Under reduced pressure, the collected extracts were evaporated to dryness to afford 7 g (10.6%) petroleum ether extract (Fraction A), 1.4 g (2.1%) methylene chloride extract (Fraction B), 1 g (1.5%) ethyl acetate extract (Fraction C) and finally 5 g (7.5%) *n*- butanol extract (Fraction D).

1. Isolation of compounds: Fraction A was loaded on silica gel column (350 g, 50 x 3.5 cm) and subjected to gradient elution with petroleum ether –

EtOAc starting with 100% petroleum ether. Similar fractions were pooled together on the basis of similarity in R_f values. Collected fractions were subjected to purification through chromatography and repeated crystallization to give compounds **1 (**10 mg), **2** (30 mg), **3** (7.1 mg), **4 (**10.2 mg) and **5** (10 mg).

Fraction B was loaded on silica gel column (100 g, 40 x 2.5 cm) and subjected to gradient elution with petroleum ether – EtOAc starting with 5% EtOAc. The latter elution afforded fractions which were collected on the basis of similarity in R_f values. Collected fractions were subjected to purification through chromatography and repeated crystallization to give compound **6 (**8 mg).

Fraction D was loaded on silica gel column (150 g, 45 x 2.5 cm) and subjected to gradient elution using ethyl acetate : methanol : water (100 : 0 : 0, 100 :5 : 4, 100 : 8 : 6, 100 : 10 : 7 and 100 : 13 : 11). Similar fractions were pooled together on the basis of similarity in R_f values. Collected fractions were subjected to purification through chromatography and repeated crystallization to afford compounds **7 (**2.5 mg) and **8 (**3.8 mg).

2. Identification of the isolated compounds: Eight compounds were isolated for the first time from *Malva rotundifolia* L. methanolic extract. Compounds **1-8** (Figure 1) were identified by comparing their data with published data in literature [27-35]. These compounds are palmitic acid **1**, β-sitosterol **2**, 5α,8α-epidioxyergosta-6,22-dien-3β-ol **3**, di-(2-ethylhexyl)phthalate **4**, β-sitosterol 3-O-β-D-glucoside **5**, methyl linoleate **6**, 3-oxo-α-ionol β-D-glucopyranoside **7** and megastigman-7-ene-3,5,6,9-tetrol **8**.

Cell line and culture conditions: Rat basophilic leukemia (RBL-2H3) and B16 melanoma cells were obtained from Riken Bioresource Center, Japan and preserved in 10% FBS brought from Thermo Fisher Science, Tokyo, Japan in Eagle's minimal essential medium (EMEM) purchased from Nissui Seiyaku, Tokyo, Japan. Optimum pH and temperature were maintained by providing 5% CO_2 and keeping cells at a constant temperature 37 °C. p-nitrophenyl-N-acetyl-β-glucosaminide and calcium ionophore A23187 were obtained from Sigma-Aldrich, St. Louis, MO, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Tokyo Chemical Industry, Tokyo, Japan.

Anti-allergic assay: The anti-allergic effects of the compounds were investigated by the β-hexosaminidase inhibition assay. A modified RBL-2H3 was used as a cell-based model of allergic response [36]. Using a 96-well plate, the cells were placed at a density of 1×10^6 cells/well. After incubation in EMEM for 48 h, the medium was replaced with 100 µL of Tyroid buffer (5 mM KCl, 130 mM NaCl, 1 mM $MgCl_2\cdot6H_2O$, 1.4 mM $CaCl_2$, 1 mg BSA, 10 mM HEPES, 5.6 mM glucose, pH 7.2) and 1 µL of each compound (n = 3) was added. Simultaneously, 1 µL 100% DMSO (negative control) and quercetin 50 mg/mL in DMSO (positive control) were tested and the plate was incubated again for 30 min in CO_2 incubator at 37 °C. Thereafter, the sample was removed and to each well, 100 µL of the Tyroid buffer and 2 µL calcium ionophore A23187 were added and incubated for 30 min. After incubation, 50 µL of the supernatant were gathered from each well and in a new 96-well plate mixed to 50 µL of 1 mM substrate solution (p-nitrophenyl-N-acetyl-β-glucosaminide). On a shaker, the mixture was incubated for 1 h at room temperature. 100 µL of stop solution (100 mM sodium carbonate, pH=10) were added. Finally, the absorbance was measured at 405 nm. In another well plate MTT experiment was performed in parallel for measurement of the cytotoxicity of compounds. To each well of the other well plate, 50 µL of MTT reagent in phosphate buffered saline (PBS, 250 µg/mL) were added to cells (1×10^6 cells/well) after the cells were treated with each compound for 1 h. In a humidified atmosphere of 5% of CO_2 at 37 °C, the plates were incubated for 4 h. After removal of the medium, 1.0 mL isopropanol (containing 0.04N HCl) was added, and after overnight keeping in dark, **t**he absorbance was measured at 570 nm using a microplate reader (Shimadzu, Japan).

B16 melanoma cell line assay: This assay was carried out as previously described [37, 38] with little modifications [39]. Using two 24-well plates, a mouse B16 melanoma cells were placed at a density of 1×10^5 cells/well in media free from samples for 24 h. After 24 h incubation, 2 µL of the test sample (n = 3) and 998 µL of fresh media replaced the old media. Simultaneously, 2 µL 100% DMSO (negative control) and arbutin 50 mg/mL in DMSO (positive control) were tested. After 48 h incubation, the medium was replaced with fresh medium containing each sample. The remaining adherent cells were assayed after 24 h then, the medium was removed and the cells were washed with PBS from one plate. The melanin content was determined by dissolving the cell pellet in 1.0 mL of 1N NaOH. The crude cell extracts were kept overnight in dark and then were assayed for melanin content by using a microplate reader (Shimadzu, Japan) at 405 nm. To each well of the other well plate for determination of cell viability, we added 50 µL of MTT reagent in PBS (250 µg/mL). In a humidified atmosphere of 5% of CO_2

at 37 °C, the plates were incubated for 4 h. After removal of the medium, 1.0 mL isopropanol (containing 0.04N HCl) was added, and after overnight keeping in dark, the absorbance was measured at 570 nm.

RESULTS AND DISCUSSION

Anti-allergic assay: In the present study, the inhibitory activities of the isolated compounds on β-hexosaminidase release were shown at different concentrations (Table 1). The most active compound was **3** which significantly suppressed the release of β-hexosaminidase without cytotoxicity at final concentrations of 50, 100 and 200 μM. Compounds **1, 4, 7** and **8** showed moderate activities in inhibition of β-hexosaminidase release at a concentration of 50 μM. The least active compound was **6** that caused a minor suppressive effect on the release of β-hexosaminidase (~4% inhibition) at 100 μM.

B16 melanoma cell line assay: In this study, the effects of the isolated compounds on melanin synthesis in B16 melanoma cells were shown at different concentrations (Table 2). The most active compound was **3** that showed melanin synthesis inhibition (~49%) but caused little melanocytotoxicity (~13%) at a final concentration of 10 μM. Other compounds (**1**, **4**, **6**, **7** and **8**) showed melanin synthesis stimulatory effects at the same concentration.

CONCLUSION

This study resulted in isolation of eight compounds for the first time from the methanolic extract of *Malva rotundifolia* L. 5α,8α-epidioxyergosta-6,22-dien-3β-ol is a promising compound that can be used as a safe anti-allergic substance. Also, the same compound is a promising compound as a new skin whitening agent for treating hyperpigmentation through inhibition of melanin synthesis. Moreover, the study resulted in isolation of some compounds with melanin synthesis stimulatory effect.

Table (1): Cell viability and effects of the isolated compounds on β-hexosaminidase release from RBL-2H3 basophilic leukemia cells.

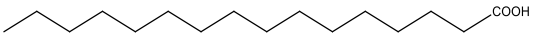
	50μM		100μM		200μM	
	β-hexosaminidase release	CV	β-hexosaminidase release	CV	β-hexosaminidase release	CV
1	72.340 ±3.250	97.560 ±2.812	76.595 ±9.594	98.943 ±4.263	78.723 ±10.054	99.837 ±3.088
3	63.551 ±5.665	113.771 ±8.632	53.271 ±6.321	112.280 ±7.265	60.280 ±9.121	93.421 ±5.359
4	71.495 ±7.193	110.877 ±2.528	92.990 ±13.025	116.184 ±6.315	103.271 ±2.428	108.771 ±5.896
6	111.214 ±7.971	103.728 ±1.331	96.261 ±8.411	109.210 ±4.091	126.168 ±4.282	105.789 ±7.033
7	81.775 ±12.720	106.096 ±1.449	74.299 ±27.231	108.859 ±3.352	86.915 ±23.886	103.903 ±2.187
8	71.028 ±7.009	112.412 ±2.729	91.588 ±5.307	117.500 ±3.034	123.364 ±16.958	118.464 ±2.210

Data presented as means ± SD (n=3), CV, cell viability (%). Quercetin was used as a positive control at 5μM, CV=101.27±4.634, β-hexosaminidase release =50.00±12.141.

Table (2): Cell viability and effects of the isolated compounds on melanin synthesis using B16 melanoma cells.

	10μM		20μM		40μM	
	MC	CV	MC	CV	MC	CV
1	130.270 ±6.895	105.3 ±2.540	128.468 ±3.689	91.571 ±6.734	115.135 ±1.178	94.147 ±11.652
3	51.699 ±2.568	87.414 ±3.638	50.323 ±2.021	81.555 ±1.382	39.077 ±1.699	74.972 ±3.202
4	153.398 ±13.507	106.437 ±3.910	100.809 ±2.684	104.303 ±1.783	84.061 ±1.560	88.571 ±2.216
6	143.408 ±2.283	102.496 ±2.409	179.378 ±4.348	102.389 ±3.561	80.131 ±5.249	90.477 ±1.884
7	178.154 ±11.406	94.008 ±6.424	161.770 ±8.717	101.248 ±3.0115	175.800 ±4.846	92.617 ±3.590
8	127.053 ±16.090	100.610 ±1.956	136.714 ±0.911	97.254 ±1.685	88.888 ±8.375	94.317 ±2.861

Data presented as means ± SD (n=3), MC, melanin content (%); CV, cell viability (%). Arbutin was used as a positive control at 50 mg/mL, CV=99.227±1.507, MC=47.477±2.707.



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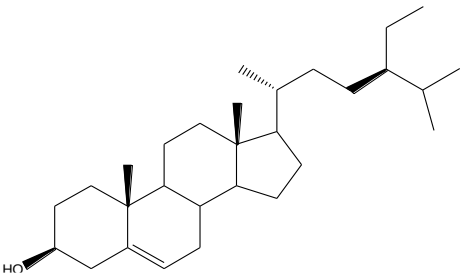

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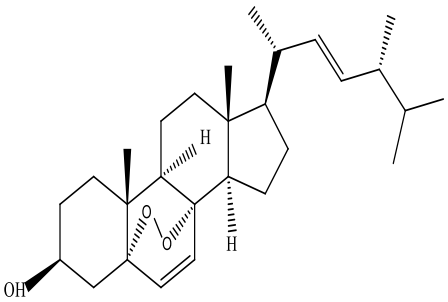

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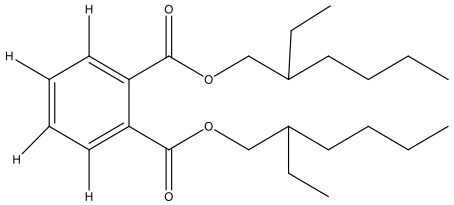

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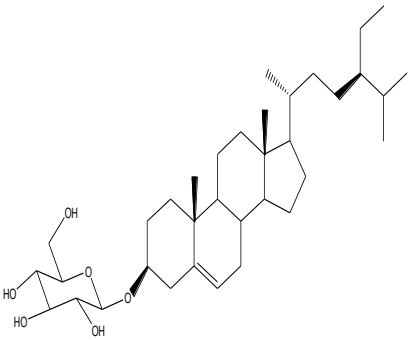

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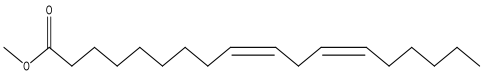

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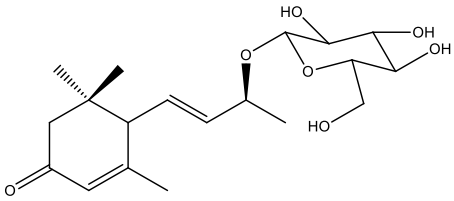

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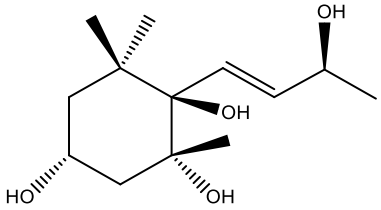

8

Figure (1) Structures of the isolated compounds (1-8)

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