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Determination of Arbutin Content and In-vitro Assessment of Anti-hyperpigmentation Activity of Microemulsion Prepared from Methanolic Extract of the Aerial Part of *Arbutus Pavarrii Pampan* Growing in East of Libya

Fatma Elshibani¹, Seham El Hawary², Abd El Rahaman El Shabrawy², Shahira Ezzat²,

Mohamed Alsharkasi³, Naema Elremali⁴, Fateh Eltaboni⁵

Lecturer, Department of Pharmacognosy, Faculty of Pharmacy, University of Benghazi, Benghazi, Libya¹

Professor, Department of Pharmacognosy, Faculty of Pharmacy, University of Cairo, Cairo, Egypt²

Lecturer, Department of Chemistry, Faculty of Pharmacy, University of Benghazi, Benghazi, Libya³

Assistant Lecturer, Department of Chemistry, Faculty of Pharmacy, University of Benghazi, Benghazi, Libya⁴

Assistant Professor, Department of Chemistry, Faculty of Science, University of Benghazi, Benghazi, Libya⁵

ABSTRACT: Arbutin is found as a major component in the aerial parts of *Arbutus pavarrii Pampan*. It is a natural harmless skin-whitening and anti-aging agent intended for external use. The compound is non-toxic, it neither has a stimulating effect nor disgusting odour. Hydrophilic arbutin can be combined in lipophilic media. In this study, the arbutin content of the methanolic extract of *Arbutus pavarrii Pampan* was determined using HPLC/UV assay. Two microemulsions were prepared with different surfactants and equal ratios to estimate their physical properties. The tyrosinase inhibitory activity of the extract and microemulsions was determined using mushroom tyrosinase as a suitable model system and measured in vitro by observing the appearance of dopachrome, which is an intermediate in the melanogenesis process. The results were matched keeping the same total concentration of inhibitor. The efficiency of the different preparations was compared with arbutin (1%) as reference substances. It was concluded that the microemulsions and methanolic extract of the plant could act as an effective carrier for treating uneven skin pigmentation.

KEYWORDS: *Arbutus pavarrii Pampan*, arbutin, microemulsion, tyrosinase.

I. INTRODUCTION

The skin Hyper-pigmentation is a chief problem in middle-aged and old aged people. This problem is initiated due to the accumulation of melanin pigment, which is responsible for the colour of hair and skin in humans. Chronic exposure to the sun is the main reason which leads to the overproduction of melanin. Tyrosinase is the crucial enzyme in the production of melanin [1-3]. The enzyme tyrosinase (monophenol; oxidoreductase; polyphenol oxidase, PPO; dihydroxy-L-phenylalanin) is a copper-containing enzyme belongs to the oxidase superfamily. It catalyses the biosynthesis of the melanin and other pigments and considered to be a key enzyme for two types of reactions in melanin synthesis, which are the hydroxylation of L-tyrosine to form 3,4-dihydroxyphenylalanine (L-DOPA) and the

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subsequent oxidation of L-DOPA to generate o-dopaquinone. O-quinone is an extremely sensitive complex that can be naturally polymerized to form the pigment melanin causing severe problems in human beings [4-6]. Hydroquinone, arbutin, kojic acid, or azelaic acid are commonly used in cosmetic preparations as active substances for their whitening properties [7]. Pharmaceutical cosmetic preparations that contain plant extracts that have a good inhibitory effect on melanin formation are a suitable option for whitening facial skin and protecting against darkening with relatively fewer side effects [8]. Hydroquinone glycoside which known as Arbutin was isolated from the *Ericaceae* (bearberry, strawberry tree, huckleberry, and heather), *Saxifragaceae*, *Asteraceae*, *Rosaceae*, *Lamiaceae*, and *Apiaceae* families. Numerous studies showed that arbutin is just as effective as hydroquinone, but less toxic [9]. Arbutin is found as a major constituent in the aerial parts of *Arbutus pavariiPamp*

II. OBJECTIVES

- To determine the arbutin content by using HPLC/UV assay.
- To investigate physical properties of the prepared microemulsion.
- To examine tyrosinase inhibitory effect of *Arbutus pavariiPamp*.

III. MATERIALS

Tyrosinase enzyme from mushroom, 25000units was stored at -20°C (SigmaAldrich), L-tyrosine substrate was stored at room temperature (Sigma), Potassium dihydrogen orthophosphate buffer was stored at room temperature (Himedia), Potassium hydroxide (SigmaAldrich), Arbutin (SigmaAldrich) as a positive control was stored at 2-8°C, Methanol (SigmaAldrich), Hydrochloric acid (SigmaAldrich), Tween 80 and Propylene glycol (PG) as surfactant/cosurfactant mixture (SigmaAldrich), Oleic acid (SigmaAldrich), Sucrose laurate (SigmaAldrich).

IV. METHODS

Preparation of plant extracts

The aerial parts of *Arbutus pavariiPamp* (500g) was dried in an open environment and was separately comminuted into small pieces, homogenized in a mixer and macerated in boiling methanol 90% for 30 minutes (for inactivation of enzymes). Extraction was carried out by maceration in methanol 70% till exhaustion. The collected methanolic extract for the plant was evaporated under reduced pressure at temperature not exceeding 50°C to yield 50g.

Determination of the arbutin content of *Arbutus pavariiPamp* methanolic extract

The content of arbutin in plant extracts was determined by reversed-phase high performance liquid chromatography technique RP-HPLC. Recommended RP-HPLC procedure. A standard solution and/or test sample containing arbutin were separated using a reverse-phase ODS Hypersil® C8 column (125mm x 4mm, 5.0pm) and measured at 222nm. An aliquot of 100pl of a series of arbutin standard solutions and 100pl of sample extract was injected into the LC system and eluted with the mobile phase, water:methanol:0.1M HCl (89:10:1, v/v/v) (the flow rate = 1.0ml/ min •). Arbutin peak area was measured. The concentration of Arbutin in the plant extract was obtained by referencing with the calibration curve obtained under the exact experimental conditions. The ideal setting of HPLC for the determination of arbutin were performed under isocratic conditions. Different mobile phase systems with various composition were examined. Firstly, the optimal wavelength for the detection of arbutin was considered, and the UV spectrum of the standard compound showed the absorption maxima at 222nm. A wavelength of 222nm showed the highest sensitivity for arbutin. Secondly, amongst the mobile phases investigated, a mixture comprising of water:methanol:0.1M hydrochloric acid (89:10:1, v/v/v) was used, and it was found that this mobile phase was the most suitable as it resulted in a high resolution, considerable peak profiles and accepted retention times. Lastly, the ideal flow rate was 1.0ml/min, as it gave a good sensitivity, accepted resolution, as well as a short analysis time. During the RP-HPLC analysis, arbutin (internal reference) displayed a symmetrical single peak.

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Preparation of standard solutions

A 1,000 μ g/ml stock solution of arbutin standard was prepared in methanol.

Preparation of sample solutions

Crude extracts (5g) was extracted with 100ml 75% methanol and filtered. The filtrate was evaporated to dryness and dissolved in 10ml of methanol.

Establishment of arbutin standard calibration curve

Five different concentrations (1, 5, 10, 20, 50 μ g/ml) were prepared from standard stock solution of arbutin (1mg/ml). The calibration curve was obtained by plotting the areas of the peaks against the concentrations. The data points were fitted into a line of best fit by linear regression method.

Data used to establish calibration curve of arbutin standard are displayed in Table 1 and standard calibration curve of arbutin is shown in Figure 1.

Validation of the HPLC method

The analytical HPLC method was validated in terms of linearity, which was evaluated with the standard solutions. Linear regression analysis for the standard calibration curve was performed.

The regression equation: $Y = AX+B$

X: The concentration mg/ml Y: Peak area A: Intercept B: Slope

Table 1 Peak areas recorded for the different concentrations of arbutin standard

Concentration (μ g/ml)	Peak area
1	398
5	3507
10	7335
20	15194
50	39212

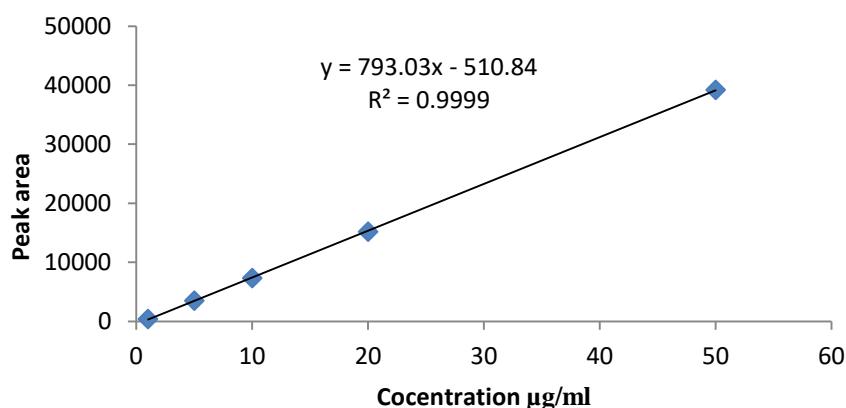


Fig. 1. Standard calibration curve of arbutin

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Preparation of the microemulsion

Microemulsions (MEs) are optically isotropic thermodynamically stable clear systems. They are formed spontaneously upon mixing a suitable oil, water, and an amphiphile blend (surfactants either alone or in combination with a cosurfactant[10]. In comparison with the traditional lotions and creams, MEs offer more advantages as topical drug delivery system. They were used to solubilize drugs and to improve topical drug availability[11]. They can increase the rate and depth of moisturizing agents into the skin. It has been suggested that MEs may dissolve the ordered structure of the stratum corneum lipids, leading to the loss of barrier properties of the skin[12].

Preparation of the Microemulsion Systems Containing Different Concentrations of *Arbutus pavarii*Pampan methanolic extract

Two MEs systems were prepared with different surfactants and the same ratio, the first ME (S1) composed of 10% oleic acid, 25% water, and 32.5% Tween 80: propylene glycol (PG) as surfactant/cosurfactant mixture (S/CoS) in a ratio of 1:1, was prepared, the second ME (S2) the surfactant was replaced by natural one which is known as sucrose laurate with the same ratio. The composition of the ME base was chosen according to preliminary trials explained in previous work[13]. S/CoS mixture was mixed in the chosen concentrations, and then water was added dropwise with continuous stirring (water titration method). The system was kept in a well closed glass container for three days to achieve equilibrium before the assessment. Different concentrations of plant extract (PE) were incorporated in the prepared ME. Three systems were prepared: S_{2%}, S_{5%}, and S_{10%} which contain 2%, 5%, and 10% of plant extract, respectively. S/CoS was mixed by a magnetic stirrer (Wheaton, Rc-2, Japan), then the PE which mixed with water was added, and the mixture was stirred till the system completely dissolved, stirring was sustained for approximately 10 minutes.

Evaluation of the Systems

The ME systems were subjected to the following tests:

Visual Inspection

Visual examination of the systems was carried for optical clarity, homogeneity, and fluidity.

Transmittance test

Percent transmittance of the optimized microemulsion along with its diluted formulations was obtained by measuring the transmittance at 650nm using UV spectrophotometer (UV-1601-220X, Shimadzu).

Thermodynamic Stability Studies

The thermodynamic stability of the developed emulsions was evaluated, on three phases, according to the procedure designed by Shafiq et al. [14]. Primarily, the formulations were exposed to six heat (45°C)-cool (4°C) cycles with storing at each individual temperature for about 48h. Then, they were centrifuged at 3,500rpm for 30minutes. Lastly, they were subjected to three freezes (-21°C)-thaw (25°C) cycles with keeping at each degree of temperature for 48h.

Globule size and Zeta potential determination

The average globule size and zeta potential of the microemulsion were determined by dynamic light scattering, using a Zetasizer HSA 3000. The protocol of analysis was explained with detail in the literature [15].

V. RESULTS AND DISCUSSIONS

HPLC/UV assay was used to determine the concentration of arbutin in the alcoholic extract of *Arbutus pavarii*Pampan aerial part (300g extract obtained from 3000g of dried leaves). Based on three independent sample injections, arbutin content of the extract amounted to an average peak area which corresponds to arbutin concentration of 50.48±1.98mg per gram of alcoholic leaves extract: 2.14% of dry plant powder.

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	Size (d.n...	% Intensity	Width (d.n...
Z-Average (d.nm): 531.8	Peak 1: 10.29	76.1	1.640
Pdl: 0.597	Peak 2: 196.4	23.9	19.41
Intercept: 0.944	Peak 3: 0.000	0.0	0.000

Result quality Refer to quality report

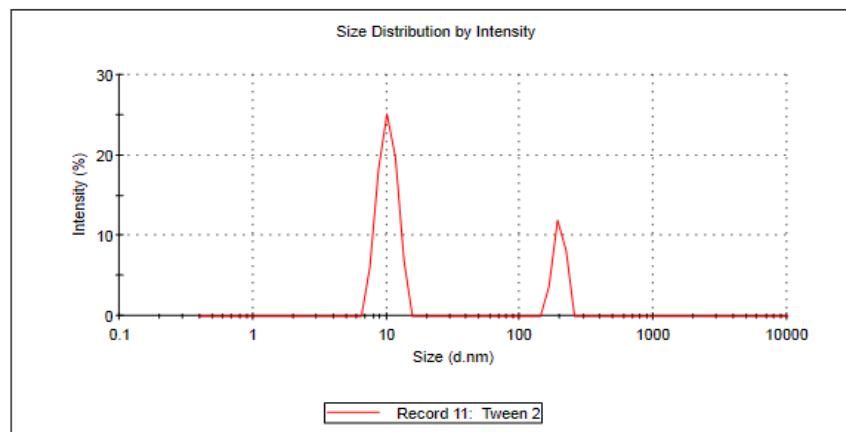


Fig. 2. Globule size of S1 microemulsion

The thermodynamic stability of investigated microemulsions (S1 and S2) was evaluated using heat-cool cycles, centrifugation, and freeze-thaw cycle stress tests. All emulsions completed the tests with no signs of creaming, phase separation or cracking. This could prove that the developed systems have a considerable thermodynamic and physical stability. Globule size measurement showed that the S2 microemulsion was smaller with a particle size of 8.954nm compared with S1(10.29 nm). Additionally, the difference in concentration of the ME exhibited very little change in particle size, the optimized microemulsion formulation remained stable and did not convert into macroemulsion. Results for globule size have been shown in Figures 2 and 3.

	Size (d.n...	% Intensity	Width (d.n...
Z-Average (d.nm): 336.6	Peak 1: 9.184	71.0	1.916
Pdl: 0.396	Peak 2: 306.4	29.0	43.65
Intercept: 0.930	Peak 3: 0.000	0.0	0.000

Result quality Refer to quality report

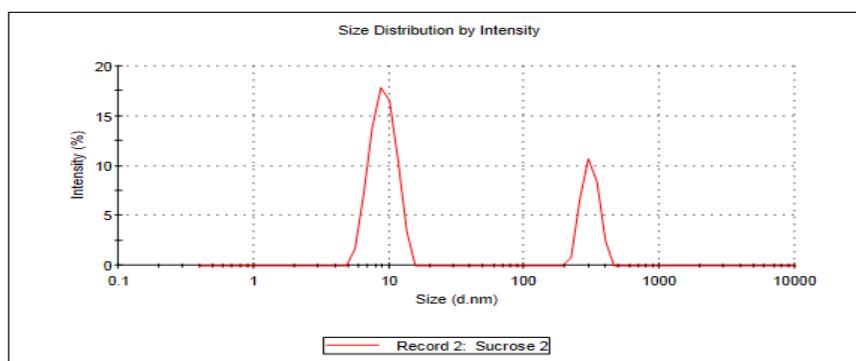


Fig. 3. Globule size of S2 microemulsion

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Zeta potential data represented in Figures 4 and 5 displayed that zeta potential values peaked at zero milli volts of the two systems S1 and S2, this indicated that globules of microemulsion had no charge, thus, the system was stable. As there was no charge on globules, no flocculation of globules occurred and hence, microemulsion was found to be stabilized.

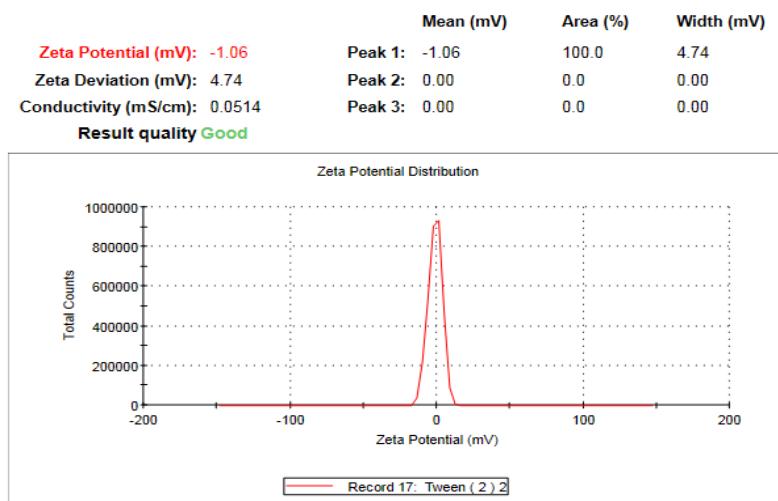


Fig. 4. Zeta potential of S1 microemulsion

Figure 5 represents the relation between the zeta potential in mill volts (mV) versus the total counts. It was clearly observed that the net charge for S2 microemulsion equal to zero due to the fact that the peak was maximized at zero mV.

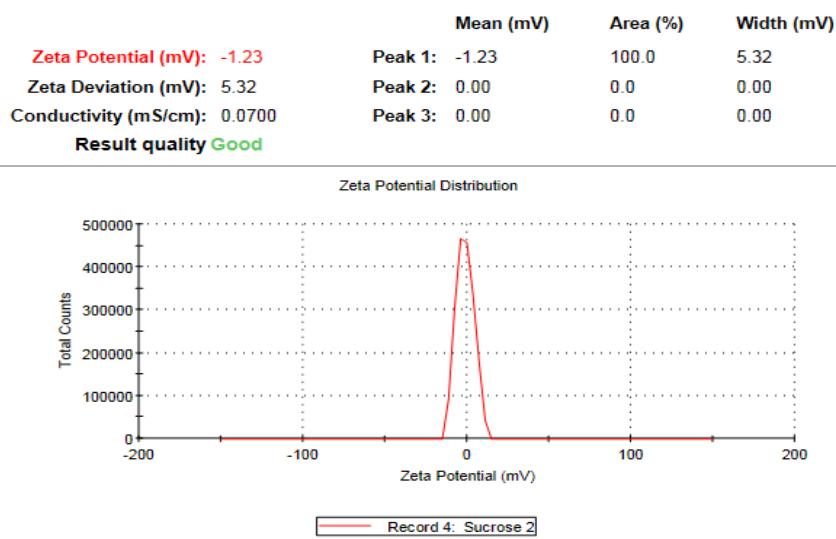


Fig. 5. Zeta potential of S2 microemulsion

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Tyrosinase inhibitory properties are frequently investigated with the aid of a spectrophotometer. The procedure described by Vanni, Gastaldi *et al.* [16] was applied in this study. Tyrosinase inhibitory activity of methanolic extract and the microemulsion of the two different formula was determined and compared with the tyrosinase inhibitory activity of arbutin as a reference substance. For the assessment of tyrosinase inhibitory activity, a mixture composed of 2mL L-tyrosine solution (0.244mM) and 0.9mL 50% methanol solution of inhibitor in aqueous phosphate buffer (pH ¼ 6.8; I ¼ 0.01M) was prepared; for the control sample, an equivalent volume of 50% methanol solution was used instead of the inhibitor solution. The oxidation of L-tyrosine was carried by adding 0.1mL of the aqueous mushroom tyrosinase solution (0.1mg=mL). ME, microemulsion and control mixture were incubated for 10min at 37°C. Dopachrome appearance was monitored spectrophotometrically at 475nm.

The effect of the test compound on tyrosinase inhibition was determined by IC₅₀, the concentration at which the compound inhibits half the original tyrosinase activity. The percent inhibition of tyrosinase activity was determined using the following equation:

$$\% \text{ inhibition} = 100 - \{(A_{475} \text{ of test sample} / A_{475} \text{ of control}) * 100\}$$

A sample 475 and A control 475 were the absorbance values in the absence and presence of inhibitor. Tyrosinase inhibitory activities of ME and microemulsion were evaluated with the same method.

Table 2 depicts tyrosinase inhibitory effect of two microemulsions and methanolic extract of *Arbutus pavarii*Pamp, and arbutin was also tested as a reference. Different amounts from each formulation were added to mushroom tyrosinase which was utilized as an appropriate model system. Hence the percent of inhibition was determined for the extract, S1, and S2 samples. As an overall trend, the efficiency of inhibition increased by adding more sample, that was observed for all formulations. But the percent of inhibition reached at maximum (83%) when 2.5 mg of S2 formulation was added, whereas respectable inhibition was recorded for the extract of *Arbutus pavarii*Pamp (67 %) compared to S1 formulation (62 %). Additionally, evaluation of the formulation effects on tyrosinase inhibition demonstrated that the S2 formulation contained the highest IC₅₀ concentration which gave 1.86mg of IC₅₀ concentration, while extract showed the lowest effect. According to the results, it was suggested that the inhibitory effect of the extract, S1, and S2 formulations were dose dependent. The inhibitory effect of S1 and S2 formulations and extract on tyrosinase activity were described for the first time for this biological property. The considerable extract amount and inhibitory effect proposed that the plant could be suitable as new sources of skin-whitening agents.

Table 2 Tyrosinase inhibitory effect of MEs and methanolic extract

	Conc (mg).	% inhibition	IC ₅₀
Extract	0.5	39	0.96 mg
	0.75	47	
	1	51	
	1.5	62	
	2	67	
Microemulsion S1	1	53	1.86 mg
	1.5	56	
	2	59	
	2.5	62	
Microemulsion S2	1	35	6.06mg
	1.5	48	
	2	63	
	2.5	83	
arbutin	2	83	

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VI. CONCLUSION

The tyrosinase inhibitory activity of the extract and microemulsions was determined by using mushroom tyrosinase as a suitable model system and measured *in vitro* by observing the appearance of dopachrome, an intermediate in the melanogenesis process. It was concluded that the inhibitory effect of the extract and two microemulsions was dose dependent. The substantial extract amount and its inhibitory efficiency on suggested that plant might be appropriate as new sources of skin-whitening agents.

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