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Formulation and *in vitro* Evaluation of a Cosmetic Emulsion Containing Apple Juice Extract

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The aim of this study is to prepare a stable water and oil emulsion (w/o) from the extract (3 %) of *Malus domestica* containing high percentage of antioxidant activity. Antioxidants has been widely used in the formulation of skin care product for human health. Due to its effects on collagen biosynthesis, it is considered as moisturizing and anti-aging active ingredient. In this study different solvents were used for the extraction of flavonoids from fruit. Formulation, preparation techniques and *in vitro* characterization methods of emulsions were observed. Different solvents were used and it was found that methanol: formic acid:water (MFW, 70:2:28) is the best for recovery of flavonoids (antioxidant) from fruit. Stability studies of this emulsion were carried out at different storage conditions *i.e.*, 8 ºC (in refrigerator), 25 ºC (in oven), 40 ºC (in oven) and 40 ºC + 75 % relative humidity (RH) (in stability cabin) for 28 days. Different parameters like pH, electrical conductivity and effect of centrifugation (simulating gravity) were determined during stability studies. Date obtained was evaluated stastistically using ANOVA two way analysis and LSD test. No phase separation was observed in sample during stable studies. It was found that both, the base and the formulation, were stable at all the accelerated conditions. Significant changes in the pH of base while insignificant changes in the pH of formulation were observed with the passage of time.

Key Words: Water and Oil emulsion, Stability, pH, Electrical conductivity.

INTRODUCTION

There is a great demand on the addition of natural antioxidants in pharmaceutical forms for topical application and flavonoids are known to exert this activity. Flavonoids are a group of polyphenolic compounds broadly distributed as secondary metabolism in plants and pharmaceutical, cosmetic and food industries present interest on their utilization, specially, for their reported biological activity[1].

Solvents, such as methanol, ethanol, propanol, acetone, ethyl acetate, dimethyl formamide and their combinations have also been used for the extraction of phenolic, often with different proportions of water[2,3].

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Methanol is more frequently used than ethanol due to its higher extraction efficiency. Aqueous methanol between 50 and 80 % has been used for extracting hydroxy cinnamic acids and many subgroups of flavonoids. Higher water composition in the solvent can aid in the extraction of glycosides of these compounds, although due to the complexity of heterosidic combinations, certain groups of flavonoids, such as flavones and flavanols, are not generally characterized as intact compounds but in the form of their aglycones. For that reason, a hydrolysis procedure before or during extraction is required[4].

An emulsion is a thermodynamically unstable system consisting of at least two immiscible liquid phases, one of which is dispersed as globule (the dispersed phase) in the other liquid phase (continues phase), stabilized by the presence of third agent, emulsifying agent[5]. Emulsions can offer promising applications in pharmaceutical, food and cosmetic industries because complete protection of the entrapped drug in an emulsion. The therapeutic properties and spreading ability of the constituents are increased. The absorption and penetration of medicament are controlled more easily if they are incorporated into an emulsion, emulsion action is prolonged and the emollient effect is greater than that observed with comparable preparations, water is an inexpensive diluents and a good solvent for many drugs and flavors that are incorporated into an emulsion[6].

EXPERIMENTAL

Abil® EM 90 was purchased from Franken Chemicals (Germany), double distilled water was prepared by using distillation plant (IM 100.00-0.43, IRMECO-GMBH, Germany). Digital pH-meter (WTW, Germany), digital conductivity-meter (WTW, Germany), stability chambers (Sanyo, Japan), water bath (HH-S 21-4, China), electrical balance (Precisa, Switzerland), digital humidity-meter (TES Electronic Corp., UK), centrifuge (Hettich, Germany), mechanical mixer (IKA, Germany), refrigerator (Dalwance, Pakistan), concentrator, microscopic software (MiniSee, Japan) and SPSS 10.0 were used during the study. Apples were purchased locally and double distilled water is used throughout.

Production of apple juice: The basic principle for apple juice production is very simple. It consists of the following steps[7] apple fruit selection (on basis of cultivar, maturity and quality), washing and inspection, crushing, milling or slicing to apple pulp. Raw juice is obtained by application of a force on the pulp and the juice is released (Fig. 1).

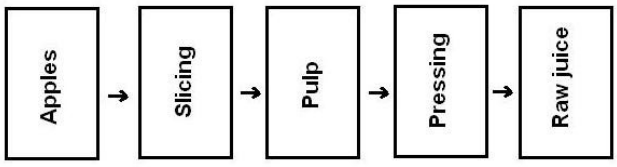

Fig. 1. Production of raw apple juice

Selection of solvent for extraction: Following solvents were selected for the extraction of antioxidants from apple. (i) 80 % (v/v) acetone in double distilled water, (ii) 70 % (v/v) ethanol in double distilled water 0.01 % (v/v) HCl, (iii) 70 % methanol in double distilled water 0.01 % (v/v) HCl, (iv) methanol:formic acid:water (MFW; 70:2:28). It was found that methanolic: formic acid:water (MFW; 70:2:28) contained high percentage of antioxidant.

Preparation of crude phenolic extract: Phenolic compounds of the fruits were extracted with MFW. Twenty grams of frozen fruit were added to 60 mL of MFW and homogenized in a blender for 2 min. The mixture was transferred to a 250 mL beaker, covered with parafilm and held for 24 h at 4 °C. The sample was vacuum filtered through a Whatman filter paper No. 41, 12.5 cm diameter filter paper and washed with 20 mL of MFW. The sediment was removed from the filter paper and re-suspended in 60 mL of fresh MFW. After mixing moderately for 10 min on a stirrer the mixture was vacuum filtered and the sediment was washed with 60 mL of MFW. The filtrates from each washing were pooled in a 200 mL volumetric flask and brought to volume with MFW. The above extract were collected and combined in rotary flask and then evaporated for dryness at 45 ºC under vacuum with a rotary evaporator.

Determination of antioxidant activity: Reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical radicals was used to investigate the radical scavenging potential of the test extracts. 1.5 mL methanolic solution of DPPH (100 μM) volume of test extracts dissolved in methanol. Appropriate controls were maintained. Ascorbic acid (50 mg/mL) was used as reference standard. The absorbance was measured at 517 nm after 20 min at room temperature against the respective blank, which contained the respective concentration of extract in ethanol. Control samples contained only in DPPH in ethanol. The free radical scavenging activity was determined by calculating the percent decrease in the absorbance of DPPH. The degree of DPPH scavenging activity of both extracts was evaluated by the formula:

$$\% \text{ Inhibition} = (A_0 - A_1) \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance.

Preparation of formulation: In this study, water/oil emulsion was prepared by the addition of aqueous phase to the oily phase with continuous agitation[8].

Oily phase that consisted of paraffin oil, beeswax and surfactant (Abil-EM 90), was heated up to 75 ± 1 ºC. At the same time, aqueous phase consisting of water was heated to the same temperature and then the apple juice extract was added in it. After that, aqueous phase was added to the oil phase drop by drop. Stirring was continued at 2000 rpm by the mechanical mixer for about 10 min until complete aqueous phase was added, 2-3 drops of lemon oil were added during this stirring time. After the complete addition of the aqueous phase, the speed of the mixer was reduced to 1000 rpm for homogenization, for a period of 5 min and then the speed of the mixer was further reduced to 500 rpm for 5 min for complete homogenization until the emulsion cooled to room temperature.

Formula of base: Oily phase: paraffin oil = 16 %, Abil-EM90 = 2 %, Beeswax = 3 %; aqueous phase: distilled water (q.s.) = 100 %; formula of formulation: oily phase: paraffin oil = 16 %, Abil-EM90 = 2 %, Beeswax = 3 %, aqueous phase = apple juices extract = 3 %, distilled water (q.s.) = 100 %.

Properties of emuslion: Emulsion was analyzed to assure the formulation of desired emulsion (Table-1).

Physical analysis: Emulsion was analyzed organoleptically (colour, thickness, look, feel) and physically (creaming and phase separation) (Table-1).

TABLE-1
ORGANOLEPTIC PARAMETERS AND CENTRIFUGATION TESTS FOR EMULSION

Time	Liquefaction				Colour				Phase separation				Centrifugation			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0 h	–	–	–	–	W	W	W	W	–	–	–	–	–	–	–	–
1 h	–	–	–	–	W	W	W	W	–	–	–	–	–	–	–	–
24 h	–	–	–	–	W	W	W	W	–	–	–	–	–	–	–	–
72 h	–	–	–	–	W	W	W	W	–	–	–	–	–	–	–	–
7 days	–	–	–	–	W	W	W	W	–	–	–	–	–	–	–	–
14 days	–	–	–	–	W	W	W	W	–	–	–	–	–	–	–	–
21 days	–	–	–	–	W	W	W	W	–	–	–	–	–	–	+	+
28 days	–	–	–	–	W	W	W	W	–	–	–	–	–	–	+	+

– = No change, + = slight change; A = 8 ºC, B = 25 ºC, C = 40 ºC, D = 40 ºC + 75 % relative humidity, W = white.

Types of emulsions: Type of emulsion was analyzed by diluting the emulsion with oil and water separately.

Microscopic tests: Emulsions were analyzed under microscope to confirm the stability. A drop of emulsion was placed on the glass slide and diluted with paraffin oil and covered by glass cover. A drop of immersion oil was placed on the cover slide and observed under the microscope. If the mean globule size increased with time (coupled with a decrease in globule number) it can be assumed that coalescence was the cause.

pH Determination: pH values of freshly prepared emulsions and emulsions kept at different conditions were determined by a digital pH meter.

Centrifugation tests: Centrifugal tests were performed for emulsions immediately after preparation. The centrifugal tests were repeated for emulsions after 12, 24, 36, 48 and 72 h, 7th, 14th, 21st and 28th day of preparation. The centrifugal tests were performed at 25 ºC and at 5000 rpm for 5 min by placing the 10 g of sample in centrifugal tubes (Hettich, Germany).

Stability tests: Stability tests were performed at different conditions for emulsions to note the effect of these conditions on the storage of emulsions. These tests were performed on samples kept at 8 ± 0.1 ºC (in refrigerator, Pakistan), 25 ± 0.1 ºC (in oven, Sanyo Germany), 40 ± 0.1 ºC (in oven, Sanyo Germany) and 40 ± 0.1 ºC (in oven, Sanyo, Germany) with 75 % relative humidity (RH). Physical characteristics of emulsions, *i.e.*, colour, creaming and liquefaction, were noted at various intervals for 28 days.

RESULTS AND DISCUSSION

Antioxidant activity of fruit: The antioxidant activity of fruit is shown in Fig. 2. It can be seen that fruit exhibited varying degree of antioxidant activity with different solvent extraction.

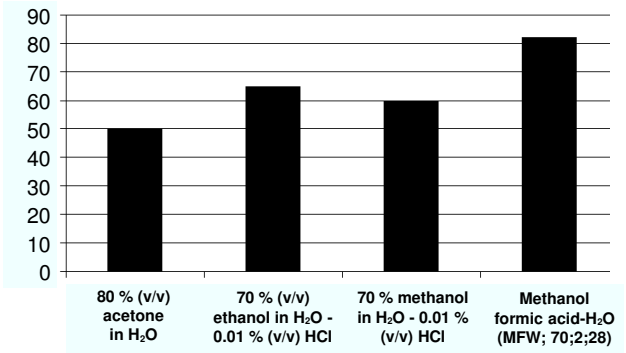

Fig. 2. Percentage antioxidant activity with different solvents

Stability of emulsions: Stability of base and formulation kept at different storage conditions was studied and physical characteristics regarding the stability of base and formulation have been discussed as.

Centrifugation tests: Centrifugation tests for base and formulation kept at different storage conditions were performed and phase separation in samples kept at different storage conditions was observed for 28 days at different time intervals. No phase separation was found in any sample of base and formulation.

Electrical conductivity tests: Electrical conductivity values of base and formulation kept at different storage conditions for 28 days have been determined. No change in electrical conductivity was found in any sample of base and formulation.

pH Tests: pH Values of base and formulation kept at different storage conditions up to 28 days have been determined and reported in Table-2.

TABLE-2
pH VALUES OF FORMULATION KEPT AT 8 ºC, 25 ºC, 40 ºC AND 40 ºC + 75 % RH

Time	8 ºC		25 ºC		40 ºC		40 ºC + 75 % RH	
	B	F	B	F	B	F	B	F
0 h	5.78	5.95	5.78	5.95	5.78	5.95	5.78	5.95
12 h	5.94	5.81	5.92	5.82	5.74	5.58	5.68	5.83
24 h	5.76	5.95	5.64	5.81	5.58	5.96	5.53	5.98
36 h	5.57	5.86	5.41	5.63	5.01	5.67	5.05	5.82
48 h	4.96	5.84	4.87	5.49	4.74	5.04	4.61	5.06
72 h	4.86	6.62	4.78	5.93	4.60	5.47	4.65	5.84
7 Days	4.69	6.62	4.58	5.84	4.51	5.75	4.47	5.93
14 Days	4.62	6.01	4.56	5.50	4.43	5.33	4.39	5.69
21 Days	4.56	6.07	4.47	5.85	4.41	5.26	4.36	5.84
28 Days	4.45	6.09	4.39	5.86	4.35	5.35	4.27	5.85

RH = Relative humidity, B = Base, F = Formulation.

Antioxidant activity: The antioxidant activity of fruit with different solvent extraction was observed. The antioxidant activity of fruit with 80 % (v/v) acetone in water, 70 % (v/v) ethanol in water 0.01 % (v/v) HCl, 70 % methanol in water 0.01 % (v/v) HCl and methanol:formic acid:water (MFW; 70:2:28) were 50, 65, 60 and 82 %, respectively (Fig. 2).

Stability: In the cosmetics industry, product stability is one of the most important quality criteria. The final acceptance of an emulsion depends on its stability and appearance. The readily apparent requirement in a well formulated emulsion is that the emulsion possesses adequate physical stability[9].

In this study, base and formulation each were divided in to four samples separately and these samples were kept at different storage conditions *i.e.*, at 8 ºC in refrigerator, at 25 ºC, 40 ºC and at 40 ºC + 75 % RH (relative humidity) in stability chambers. These samples at different storage conditions were observed for a period of 28 days at definite time intervals. The samples were observed with respect to change in colour, liquefaction and phase separation.

Liquefaction: The viscosity of external oil phase is the key factor contributing to the formation of stable emulsions. According to stokes law increased viscosity of the external phase is associated with improved shelf life of emulsions[10]. Although cosmetic creams appear as stable concentrated emulsions[11] but as soon as an emulsion has been prepared, time- and temperature-dependent processes occur to effect its separation leading to the decreased viscosity which results in increased liquefaction[9].

No liquefaction was observed in any sample of base and formulation kept at 8 and 25 ºC during whole observation period of 28 days but slight liquefaction was observed in the samples of base kept at 40 ºC and 40 ºC + 75 % RH from 21st day of observation; but there was no further increase in liquefaction till the end of study period. On the other hand, a slight liquefaction was observed in formulation samples kept at 40 ºC and 40 ºC + 75 % RH at 28th day of observation period.

Stokes law shows that the rate of creaming is inversely proportional to the viscosity. As creaming increased, the viscosities of the base and the formulation gradually decreased at increasing temperature resulting in liquefaction[10].

Phase separation: The instability of emulsions is basically clarified by a phase separation[11] *i.e.*, any emulsion reverts back to separate the bulk phases. The separated phase can either cream or sediment[9]. Destabilization is mostly compounded by coalescence and gives a first indication through extension of droplets[10]. The two instability processes, *i.e.*, coalescence and Ostwald ripening (the growth of large particles at the expense of small ones) result in droplet size growth with time[12]. According to Stock's law, larger droplets cream much more rapidly than smaller particles[10]. The concentration of the disperse phase and the droplet size are key parameters in determining the type and timescale of the instability process[12].

In the case of the base and the formulation no phase separation was observed in any of the samples kept at 8 ºC, 25 ºC, 40 ºC and 40 ºC + 75 % RH up to observation

period of 28 days. This indicated that the base and the formulation were stable at all the storage conditions for 28 days.

Centrifugation test: Centrifugation, if used judiciously, is an extremely useful tool for evaluating and predicting the shelf life of emulsions[9]. The cream volume or the separation of phases at a given time is taken as a measure of the physical stability of emulsion. However it is an example of a situation that exists in any accelerated test, namely, tendency to "overkill" the emulsions because the test used introduces a new mechanism of instability or causes an unreasonably high stress[9]. In this study centrifugation test was performed for the base and formulation kept at different storage conditions up to a period of 28 days at definite time intervals. No phase separation on centrifugation was seen in any of the samples kept at different storage conditions up to 28th day of observation. This indicated that the emulsions were stable at all the storage conditions for 28 days.

pH: The pH is a significant parameter in so far as the effectiveness of the cream is concerned and it can be used as an indicator of emulsion stability[11]. For the formation of stable emulsions pH value of aqueous phase is the key factor[9]. pH of skin ranges between 5 and 6 and 5.5 is considered to be average pH of the skin. Therefore, the formulations intended for application to skin should have pH closer to this range.

In this study, the pH of freshly prepared base and formulation was 5.78 and 5.95, respectively, which is very close to the skin pH. The pH values of the samples of base kept at different storage conditions were found to be decreasing continuously from the first day to the last day and on 28th day pH was 4.45, 4.39, 4.35 and 4.27, respectively. Whereas pH of the samples of formulation kept at different storage conditions *i.e.*, 8 ºC, 25 ºC, 40 ºC and 40 ºC + 75 % RH showed slight variations with time and slightly increased at the end of study period which might be due to the production of any metabolite. The pH values of samples of formulation kept at 8 ºC, 25 ºC, 40 ºC and 40 ºC + 75 % RH were 6.09, 5.86, 5.35 and 5.85 at 28th day, respectively.

By using two-way analysis of variance (ANOVA) technique at 5 % level of significance, it was found that the change in pH of different samples of base was significant at different levels of time and temperature but there was insignificant difference in change of pH of different samples of formulation at different levels of time and temperature. When LSD test was applied to check the individual average effects of the pH of the samples of base at different temperatures with the passage of time by taking average pH values of 0 h at different temperatures as standard, it gave significant change throughout the study period of 28 days. Again when LSD test was applied to check the individual average effect of the pH of the samples of formulation at different temperatures with the passage of time by taking average pH values of 0 h at different temperatures as standard, it gave insignificant change throughout the study period and only significant change was observed just at 48 h of study period. It was concluded that the change in pH of the samples of base at

different times is highly significant throughout the study period. The decrease in the pH value of samples of base may be due to any by-product which may be produced by the degradation of any ingredient of base. From LSD test it was concluded that there was significant change in pH of the samples of base at different storage conditions but insignificant change was observed in pH of the samples of formulation at different storage conditions with the passage of time.

Electrical conductivity: In this study, conductivity test was performed for all the samples of base and the formulation kept at different storage conditions up to a period of 28th days at definite time intervals. No electrical conductivity was seen in any of the samples of base and formulation kept at different storage conditions *i.e.*, 8 ºC, 25 ºC, 40 ºC and 40 ºC + 75 % RH up to 28th day of observation. No conductivity may be attributed to the non-conductive nature of oil phase[13].

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