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EVALUATION OF ANTI-INFLAMMATORY AND OTHER BIOLOGICAL ACTIVITIES OF FLAVONOID BASED CREAM FORMULATION FOR TOPICAL APPLICATION USING *IN VITRO* MODEL

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ABSTRACT: Biologically active compounds identified as flavonoids, glycosides, alkaloids, saponins, terpenoids and polyphenolics from plant extracts possess health promoting properties. The use of natural antioxidants in the treatment of various skin diseases has gained utmost importance in recent years. Therefore, present study was designed and conducted with the aim to evaluate the anti-inflammatory activity of cream formulation containing fruit extracts of *Musa acuminata, Psidium guajava* and *Pyrus communis* using *in vitro* model. Fruit of *M. acuminata, P. guajava* and *P. communis* were extracted by cold percolation, comprising of ethanol, methanol and distilled water. The cream formulation and the reference drug (Diclofenac sodium) of varying concentrations were incubated with fresh egg albumin under specified experimental conditions and subjected to determination of absorbance and viscosity to assess the *in vitro* anti-inflammatory activity. Linear regression analysis was used to calculate IC_{50} value. The results revealed that cream formulation and diclofenac sodium inhibited protein denaturation in a dose-dependent manner. The IC_{50} value of cream formulation 1254.13±16.91µg/ml and diclofenac sodium was recorded as 845.82±16.94µg/ml at correlation coefficient value (r) of 0.900 and 0.983 respectively. The study gives an idea that the fruits extract can be used as a lead compound for designing a potent topical anti-inflammatory drug.

INTRODUCTION: Fruits represent a large natural source of valuable compounds that may serve as lead for the identification and development of new bioactive compounds. In modern era, there has been an increasing interest in diets comprising of fruits and vegetables due to their important role in the prevention of various degenerative disorders of skin. This preventive effect is primarily reported owing to the presence of bioactive compounds such as polyphenols, resveratrol, flavonoids, carotenoids, proanthocyanins, tannins and Vitamins [1]. These polyphenolic compounds are widely disseminated in nature as active constituents of herbs, shrubs, fruits, cereals, vegetables and plant derived products such as tea, coffee and instant energy juices. Due to established biological effects these are attractive substances for many areas of human life. Plants, herbs and their preparations and secondary metabolites have been used since the prehistoric time to modern medicine. Accordingly ethanomedicine have constituted one of the strong bases of treatment for healthcare all over the world.

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Fruits are the wellspring of various phytonutrients such as Vitamins, polyphenols, tannins, flavonoids and carotenoids. It is well established that fruits have many health related beneficial effect thus, consumption of such fruits will have a positive effect on health and will contribute in decreasing the mortality rate of cardiovascular and other degenerative diseases. Therefore, fruits extracts are mostly added to the cosmetic preparations and skin care products. It gives a natural divine fragrance and color and may help to treat dermatitis, acne, eczema and other skin problems [2, 3].

Flavonoids are the largest group of plant pigments, which are consumed with food as well as in the form of dietary supplements or herbal products. Epidemiological studies showed that flavonoids possess a number of pharmacological activities, such as anti-microbial, antioxidant, anti-allergic, anti-inflammatory, anti-cancer, anti-diabetic, cardio-protective, hepato-protective and detoxification activities [4 - 6]. As fruit extracts are usually rich in flavonoids, therefore, have great applications in cosmetology, phytopharmaceuticals and food supplements. These applications are well developed and documented mostly due to their anti-oxidant property. In addition, also reported to play an important role in topically applied products such as in anti-aging, anti-acne and in sunscreen formulations [2]. Thus, manufacturers are striving to develop a formulation containing phyto-nutrients and bioactive natural compounds and their use is continually fueling the cosmaceuticals and personal care industries [7].

Based on this scientific information, present work is aimed and designed to screen the extracts from fruits to develop a stable topical cream formulation. In present study various biological activities such as antioxidant, antimicrobial and anti-inflammatory activity of cream formulation was determined through *in-vitro* tests.

MATERIALS AND METHODS:

Drugs and Chemicals: All chemicals and solvents used were of analytical grade and purchased locally.

Instruments: Double beam Shimadzu UV-Vis spectrophotometer equipped with 1cm quartz cell, pH meter (Systronics), centrifuge machine (HERMLE Labortechnik GmbH, Germany), Vacuum rotary evaporator (BUCHI, Switzerland), weighing balance (Sartorius) and pH meter (Systronics), incubator were the instruments used in the study.

Plant Materials: The ripe fruit samples *Musa acuminata* Colla (Banana), *Psidium guajava* L. (Guava) and *Pyrus communis* L. (Pear) were purchased from a local market of Karachi, Pakistan, as per availability during the season.

Plant Extraction: The fruit pulp of banana, peeled fruit pulps of guava and pear were macerated separately in a mixture of solvent system comprising of ethanol and methanol (35%) and distilled water (30%) for 15 days at room temperature. The contents were filtered and concentrated under reduced pressure using rotary evaporator. Thick, viscous hydro-alcoholic extracts obtained from each fruit was kept into an air tight amber colored glass bottle.

Identification Test for Flavonoids: The hydro-alcoholic fruit extracts were subjected to qualitative analysis using an alkaline reagent test. 5ml of hydro-alcoholic fruit extracts were treated separately with 2 ml of sodium hydroxide solution. An intense yellow color precipitates was formed which turned into colorless, on addition of a few drops of diluted hydrochloric acid indicated the presence of flavonoids [8].

Determination of Total Flavonoid Contents (TFC): TFC was determined using aluminium chloride ($AlCl_3$) colorimetric method. The total flavonoid contents were expressed in term of quercetin equivalent (QE) µg/g using the standard calibration curve ($y= 0.0009x + 0.001$, $r^2= 0.9189$).

Cream Formulation and its Preparation: Cream formulation was prepared using ingredients shown in **Table 1**. The oil phase of cream was prepared by heating the ingredients (cetostearyl alcohol, stearic acid, cetomacrogol-1000, lanolin and glycerin) at 75 °C ± 2 with constant stirring using hot plate. While, for the preparation of aqueous phase purified water was heated separately in 2000 ml capacity beaker at 80 °C ± 2. To this methyl and propyl parabens were dissolved with occasional stirring and temperature was brought to 75 °C ± 2. The two phases (oil and aqueous) were mixed

together with vigorous stirring for about 1-2 minutes. Finally, the fruit extracts were added with constant stirring till a thick cream was formed. The temperature was further reduced to around 45 °C using cold-water bath and stirring was discontinued. The cream was stored in wide mouth air tight amber colored glass container and kept at room temperature.

TABLE 1: COMPOSITION OF CREAM FORMULATION

Ingredients	Uses	Components (% w/w)
Cetostearyl alcohol	Emulsifier	35
Stearic acid	Emollient, Co-emulsifier	40
Cetomacrogol-1000	Emulsifier	9
Lanolin	Emollient	50
Glycerin	Humectant	156.6
Methyl paraben	Preservative	4
Propyl paraben	Preservative	0.4
hydro-alcoholic fruit extract of *M. acuminata*, *P. guajava* and *P. communis*	Active ingredient	10
Distilled water	Vehicle	695
Total weight	-	1000

Antioxidant Activity: The *in vitro* anti-oxidant activity was determined by reducing power assay [9]. Ascorbic acid was used as a standard. The percentage of reduction was calculated from the following formula:

Percentage of reduction power = [1- (1- As/Ac)] x 100

Where, Ac is the absorbance of standard at maximum concentration tested and As is the absorbance of sample.

Anti-microbial Activity: The anti-microbial activity was conducted against standard ATCC cultures which include: *Staphylococcus aureus* (ATCC - 6538), *Bacillus subtilis* (ATCC - 6633), *Pseudomonas aeruginosa* (ATCC-27853), *Escherichia coli* (ATCC-14169) and *Candida albicans* (ATCC - 10231). The activity was executed using varying concentration ranges 100 - 400 µg per disc of test sample respectively. Cefotaxime (CFX), commercial antibiotic disc was employed as a positive control against *S. aureus*, *B. subtilis*, *E. coli* and Ceftriaxone (CTZ) was used against *P. aeruginosa* as a positive control for anti-bacterial screening, while, for anti-fungal screening Miconazole nitrate (MN) was used as a positive control against *C. albicans*.

Cream formulation (500 mg) was weighed accurately and transferred in a 25 ml volumetric flask. It was dissolved with ethanol using a vortex mixer. Accurately 5 µl, 10 µl, 15 µl and 20 µl solutions were applied to adequately label sterile discs to make a concentration of 100, 200, 300 and 400µg/disc respectively. To achieve a concentration of $1x10^6$ CFU/ml, approximately 1 ml of 1x 10^8 CFU/ml culture was poured into 100 ml media (SDA) and (TSA). 25 ml of media was then poured in each petri dish and allowed to solidify before use. Each prepared disc of cream infused with different concentrations, along with a standard selected antibiotic was placed gently on the agar surface. These plates were kept at 4 °C for 24 hours to allow maximum diffusion of test and reference materials. After this, the seeded plates were incubated at 37 °C for 24-72 hours to observe the zone of inhibition of anti-bacterial and anti-fungal activity [10].

Anti-inflammatory Activity: *In vitro* anti-inflammatory activity was determined by inhibition of protein (egg albumin) denaturation method [11, 12].

Control Solution (50 ml): Phosphate buffer saline (28ml) of pH 6.4 was transferred to freshly prepared egg albumin (2ml) and distilled water (20ml) was added to this, to prepared control solution.

Standard Solution (50 ml): Phosphate buffer saline (28ml) of pH 6.4 was transferred to freshly prepared egg albumin (2ml) and (20ml) solution of diclofenac sodium of different concentration ranges from 10 - 2000 µg/ml was added to this, to prepared standard solution.

Test Solution (50 ml): Phosphate buffer saline (28 ml) of pH 6.4 was transferred to freshly prepared egg albumin (2 ml) and (20ml) solution of cream formulation of different concentration ranges from 10 - 2000 µg/ml was added to this, to prepared test solution.

All the solutions were incubated at 37 ± 2 °C for 15 minutes and it was then heated at 70 °C on a water bath for 5 minutes. The solutions were allowed to cool at room temperature. The absorbance was then

measured using UV-Visible spectrophotometer at 660 nm using vehicle as blank. The percentage inhibition of protein denaturation was calculated from the control using below under formula:

Percentage of inhibition = 100 x (Abs $_{control}$ -Abs$_{test}$ /Abs $_{control}$)

Whereas, Abs is the absorbance.

The viscosity of solutions was determined by using Ostwald viscometer. The IC_{50} value represents the concentration (µg/ml) of test sample, required for 50% inhibition.

Statistical Analysis: Statistical Program for Social Sciences (SPSS Corporation, Chicago, IL), version 20.0 software was used for the analysis. The experimental data was presented as mean ± SD and one way (ANOVA) followed by Tukey's post- hoc test was used for multiple comparison between treatment groups ($p < 0.05$). Linear regression analysis was used to calculate the IC_{50} value. Regression analysis was used to determine correlation between the ferric reducing power and protein denaturation.

RESULTS AND DISCUSSION:

Total Flavonoid Content and Anti-oxidant Activity: The TFC of hydro-alcoholic fruit extract of *M. acuminata* was found to be 8.04 ± 3.4, *P. guajava* 11.37 ± 3.57, *P. communis* 11.37 ± 3.4 QE µg/g (**Table 2**). Flavonoids don't seem to be readily detectable therefore, aluminium chloride ($AlCl_{3)}$ was used as complexing reagent. The method is based upon the formation of stable complex between $AlCl_3$ and keto and hydroxyl groups of flavones and flavonoids. It is one of the most frequently used methods applied to the flavonoid content determination in various plants[13]. Plant secondary metabolites like phenolic acids, flavonoids, carotenoids, proanthocyanins and certain Vitamin such as Vitamin C and E contributing for the anti-oxidant activity, depicting a therapeutic potential. However, concentration of these phytochemicals are varying due to genetic makeup of the species, geographical region, climatic and soil conditions, cultivation time, pre and post-harvesting technique, ripening stage, processing and storage conditions [14, 15].

It is well established that the reducing capacity of bioactive compounds is related to the anti-oxidant activity. The reducing power of cream formulation was found to be 73.48 ± 1.30 ascorbic acid equivalents (AES) µg/g. The anti-oxidants present in the cream formulation caused their reduction of Fe/ ferricyanide complex to the ferrous form and thus, proved the reducing power. The anti-oxidant activity could be correlated with the polyphenolic components present in the cream.

TABLE 2: TOTAL FLAVONOIDS CONTENT OF FRUITS EXTRACT

Concentration (µg/ml)	*Musa acuminata*	*Psidium guajava*	*Pyrus communis*
25	4.33	8.78	7.67
50	8.78	9.89	12.11
100	11	15.44	14.33
Mean	8.04 ± 3.4	11.37 ± 3.57	11.37 ± 3.4

Anti-microbial Activity: Results shown in **Table 3** revealed that all the test organisms have some degree of dose - related sensitivity and thus the zone of inhibition increases with the increase in concentration of cream. Better sensitivity results have been observed against Gram- positive organisms compared Gram - negative organisms. The growth of *B. subtilis* and *S. aureus*, both were noted to be inhibited against all the four concentrations (100, 200, 300 and 400µg) of cream used, with the maximum zone of inhibition of 9.83 and 9.50mm respectively against 400µg. The standard drug Cefotaxime produced a zone of 15.0 mm with a concentration of 30µg.

In case of Gram- negative organisms (*E. coli and P. aeruginosa*) smaller zone of inhibitions were recorded at 200, 300 and 400µg, while no zone of inhibition was noted at 100 µg concentration. The maximum zone of inhibition of 7.86 mm and 7.03mm were recorded against *E. coli and Pseudomonas aeruginosa* with 400µg. The standard drug Cefotaxime produced a zone of 18.0 mm with a concentration of 30µg against *E. coli*, while Ceftriaxone produced a zone of 20mm against *P. aeruginosa* with 30µg. The antifungal effect on *C. albicans* was noted batter than antibacterial effect. A zone of inhibition of 13.50 mm was recorded against 400µg concentration,

while Miconazole nitrate produced 22mm zone of inhibition with 40µg concentration.

The active contents of the cream, *Musa acuminata, Psidium guajava* and *Pyrus communis* have been studied individually and reported to possess anti-microbial properties[16-20]. The overall anti-microbial activity of these fruits can be ascribed with the presence of flavonoids and phenolic substances present as bioactive constituent, while the mechanism of their anti-microbial activity have been proposed due to inhibition of nucleic acid synthesis, cytoplasmic membrane function, or energy metabolism [21-24].

The anti-microbial effect of cream formulation was effective however; it can be assumed that even a low degree of anti-microbial effect of the cream formulation will be helpful in the eradication of common microorganisms with its regular application, especially the Gram - positive bacteria and some fungi associated with skin problems.

TABLE 3: *IN VITRO* ANTI-MICROBIAL ACTIVITY OF CREAM FORMULATION

	Name of microorganisms / ATCC Number		Zone Inhibition (mm)				
			Disc concentration (µg) of Cream				Standard Antibiotic Disc
			400	300	200	100	(CFX* / CTX* / MN**)
Antibacterial screening	*B. subtilis* ATCC6633	Plate 1	10.0	9.0	7.0	6.0	15.0 mm
		Plate 2	9.5	8.0	6.3	5.9	15.0 mm
		Plate 3	10.0	8.0	7.2	6.2	15.0 mm
		Average zone of inhibition.	9.83 mm	8.33 mm	6.83 mm	6.03 mm	15.0 mm
	S. aureus ATCC6538	Plate 1	9.5	9.5	7.5	6.8	12.0 mm
		Plate 2	10.0	9.0	8.0	6.5	12.0 mm
		Plate 3	9.0	9.0	8.3	7.0	12.0 mm
		Average zone of inhibition	9.5 mm	9.16 mm	7.83 mm	6.76 mm	12.0 mm
	E. coli ATCC14169	Plate 1	8.0	6.7	5.2	R	18.0 mm
		Plate 2	7.8	6.2	4.8	R	18.0 mm
		Plate 3	7.8	6.0	5.0	R	18.0 mm
		Average zone of inhibition	7.86 mm	6.30 mm	5.0 mm	R	18.0 mm
	P. aeruginosa ATCC 27853	Plate 1	7.3	7.0	5.0	R	20 mm
		Plate 2	7.0	6.5	4.8	R	20 mm
		Plate 3	6.8	6.5	4.5	R	20 mm
		Average zone of inhibition	7.03 mm	6.66 mm	4.76 mm	R	20 mm
Antifungal screening	*C. albicans* ATCC 10231	Plate 1	13.2	9.8	7.6	6.5	22.0 mm
		Plate 2	13.5	10.0	7.5	7.0	22.0 mm
		Plate 3	13.8	9.5	8.0	6.8	22.0 mm
		Average zone of inhibition	13.5	9.76	7.70	6.76	22.0 mm

***** Cefotaxime (CFX) = 30µg/disc, Ceftriaxone (CTX) = 30µg/disc, ****** Miconazole nitrate disc MN (40µg/disc).

Anti-inflammatory Activity: Ethical issues are the main problem related to animal's model in experimental research [25]. Therefore, the protein denaturation bioassay was selected for *in vitro* assessment of anti- inflammatory activity of cream formulation based on their flavonoid content. It is simple and convenient method to evaluate the anti-inflammatory activity. Present findings revealed that cream formulation and diclofenac sodium (reference drug) were exhibited a concentration-dependent inhibition of protein denaturation ranges from 10-2000 µg/ml.

The % age inhibition of protein denaturation of diclofenac sodium was observed within the range of 26.3 - 74.8 % and cream formulation was 11 - 58.5 % throughout the concentration range of 10 to 2000µg/ml. Inhibitory concentration (IC_{50}) value of cream was recorded as 1254.13 ± 16.91 µg/ml and diclofenac sodium 845.82 ± 16.94 µg/ml at correlation coefficient value (r) of 0.900 and 0.983 respectively (**Table 4**). From the IC_{50} values it becomes evident that cream formulation was active as diclofenac sodium. A Tukey's post - hoc test revealed that inhibition of protein denaturation was

statistically significant when cream formulation and diclofenac sodium was compared to control. Therefore, from the results of the present study it can be concluded that cream formulation possessed anti-inflammatory activity. The anti-denaturation effect was further supported by the change in viscosities. It has been mentioned in various reports that the viscosities of protein solutions increase on denaturation [12, 26]. The viscosity of protein solutions are expected to be dependent on the concentration, shape, size, molecular weight, flexibility, intermolecular interactions, the degree of hydration and charge of the proteins. The viscosity can also be influenced by external factors such as temperature, pH and ionic strength and % age of solvent used.

TABLE 4: ANTI-INFLAMMATORY ACTIVITY BY PROTEIN DENATURATION

Concentration	Diclofenac sodium		Cream formulation	
µg/ml	% Inhibition	Viscosity (cps)	% Inhibition	Viscosity (cps)
Control	-	1.33	-	1.33
10	26.3 ± 0.15	0.96	11 ± 0.30	0.62
50	26.8 ± 0.20	0.99	18.5±0.55	0.70
100	31.6 ± 0.21	1.01	24±0.10	0.77
200	36.7 ± 0.50	1.05	30.6±0.6	0.81
400	40.9 ± 0.15	1.08	35.3±0.06	0.88
800	52.3 ± 0.32	1.10	48.5±0.21	0.97
1000	56.9 ± 0.15	1.12	51.7±0.1	0.98
2000	74.8 ± 0.20	1.14	58.8±0.06	1.02
IC_{50}	845.82 ± 16.94 µg/ml		1254.13 ± 16.91µg/ml	
Coefficient value (r)	0.983		0.900	

Values were expressed as mean ± SD (n=3).

Linear regression analysis was used to calculate IC_{50} value. One way analysis of variance (ANOVA) followed by Tukey's post- hoc test was used as the test of significance. The mean difference is significant at the 0.05 level. Denatured protein solution had a higher viscosity due to its larger molecule size. However, the viscosities were found to decrease with a concomitant decrease in concentration of test sample and reference drug as well. While, the viscosities of the cream formulation of all concentrations were always found less than that of control. The effect of concentration of test agent on the viscosity behaviour of denatured protein dispersion requires further studies [27, 28]. It was also noted that the reducing power anti-oxidant activity correlated strongly with anti-inflammatory activity as shown in **Fig. 1** ($y = 0.0821x + 70.907$, $R^2 = 0.903$).

In the present study, the *in vitro* anti-inflammatory activity of cream formulation can be attributed to its polyphenol contents. The flavonoids, tannins and saponins have the ability to bind cations and able to protect the protein membrane from denaturation. Therefore, polyphenolic compounds could be the possible reason for anti-denaturation property. Several flavonoids have been found to possess anti-inflammatory activity by inhibiting cycloxygenase-2 (COX2), tyrosine kinase and neutrophil degranulation. It has been reported that anti-oxidant activities of flavonoids can also help in establishing the anti-inflammatory activity [25]. Several anti-necrotic, digestive, neuroprotective hepato-protective and anti-inflammatory, drugs have recently been shown to have anti-oxidant and/or radical scavenging mechanism as part of their activity [29, 30].

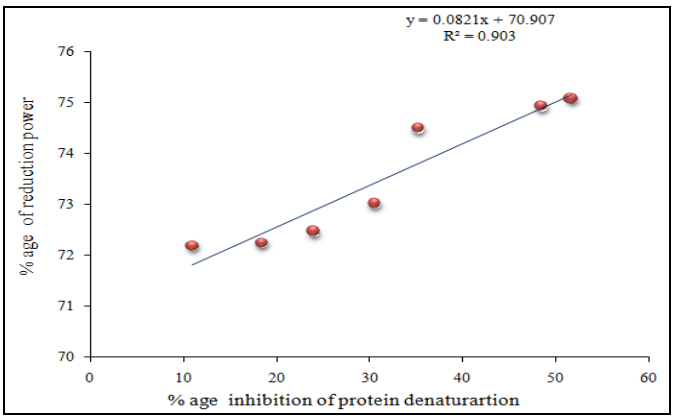

FIG. 1: CORRELATION BETWEEN FERRIC REDUCING POWER ANTIOXIDANT ACTIVITY AND PERCENTAGE INHIBITION OF PROTEIN DENATURATION

The *in vitro* results appear as interesting and promising and may be effective as potential sources of topical anti-inflammatory drugs. It is proposed that anti-inflammatory effect need to be further

assessed in other experimental models in pursuit of newer phyto-therapeutic against inflammatory diseases and to determine the mechanisms behind its anti-inflammatory actions.

CONCLUSION: The study provided reasonable data to conclude that seasonal fruits possess antioxidant property which is capable of protecting the skin from harmful effect of various physico-chemical factors. Despite great advancement within the field of synthetic drugs, plants still have their own unique place, having no side effects. Thus, a method should be made to find out the usefulness of plants against inflammation so as to use them as natural anti-inflammatory agents with low toxicity and higher therapeutic value. Present findings provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an anti-inflammatory agent from plant.

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