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Full Length Research Paper

Lipoxygenase assay and cutaneous erythema test have discovered a potent anti-inflammatory activity shown by some genus *Prunus* plants

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Medicinal plants have gained considerable interest in treatment of various medical conditions including inflammation. *Rosaceae* (the rose family) family of flowering plants having largest genus *Prunus* (plums, peaches, cherries and almonds) is one of the most economically important families. In current study, anti-inflammatory activities of three fruit species *Prunus persica, Prunus avium* and *Prunus domestica* of family *Rosaceae* and genus *Prunus* were evaluated by both *in-vitro* and *in-vivo* assays. Non-invasive bioengineering probe Mexameter MPA-5 was used to measure cutaneous erythema level *in-vivo*, while for *in-vitro* evaluation lipoxygenase assay was used. These medicinal plant extracts were entrapped in oil-in-water emulsions and applied on 33 healthy human female volunteers (22 years) to assess cutaneous erythema level. Methanol extracts of *P. persica, P. avium* and *P. domestica* depicted significant (P ≤ 0.05) lipoxygenase activity *in-vitro*, that is, 58.73, 51.29 and 47.87%, respectively. Results of *in-vivo* assay revealed that active formulations loaded with botanical extracts produced statistically significant decrease in skin erythema over respective controls after 48 h *P. persica, P. avium and P. domestica* extracts can be used for isolation of new lead compounds with better anti-inflammatory activity. Results indicate that studied plants present potential for many natural products studies with high safety profile.

Key words: Anti-inflammatory, *Prunus persica*, *Prunus avium*, *Prunus domestica*, Lipoxygenase, *Rosaceae*.

INTRODUCTION

Inflammation is a protective response of the body towards various injuries and infections (Vijayalakshmi et al., 2011). Most anti-inflammatory drugs used to alleviate the cardinal signs of inflammation particularly pain, redness and swelling are synthetic agents having various side effects (Dharmasiri et al., 2003; Bepary et al., 2008). In many applications, it is possible to use crude plant materials or extracts to screen lipoxygenase enzyme

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(Taner and Aslıhan, 2007). The screening of medicinal plants for lead anti-inflammatory compounds may guide to the discovery of more safer and effective compounds (Fazli et al., 2014).

Lipoxygenase (LOX) is an enzyme found in many plants which catalyses the oxygenation of polyunsaturated fatty acids (PUFA) to form fatty acid hydroperoxides that plays important roles in wound response and defense mechanism. Lipoxygenases have antioxidant status in plant-based foods. Lipoxygenase in vegetative tissues provide hydroperoxide substrates that can be metabolized to compounds that play important roles during wound response in plants. Linoleic acid is the most common substrate in plant-based foods (Taner and Aslıhan, 2007).

In peaches, LOX activity increases in conjunction with the ripening processes (Mingyu et al., 2011). Peaches (*Prunus persica*) are enriched in water, potassium and Vitamin C. Potassium helps to maintain the water balance, counteracting the negative effect that sodium could have in the retention of liquids; therefore it is appropriate to eliminate liquids of the organism. Peach protects us from dryness in the skin, juvenile acne or a bigger easiness for the infections (Daymi et al., 2010). Cherries boost the immune system. Cherry (*Prunus avium*) is rich in water, Vitamins C, phenolics and anthocyanins (Bourquin et al., 2003). Several studies have demonstrated that cherry intake inhibits inflammatory pathways (Kelley et al., 2006). It is skin cleanser and reduces inflammation (Usenik et al., 2008). Plums (*Prunus domestica L.*) are good sources of natural antioxidants. Masks of these juicy fruits are usually applied on skin to produce soothing and moisturizing effects (Dae et al., 2003). But limited data is available on the Lipoxygenase activity of these botanicals.

Lipoxygenase assay (*in vitro*) is related to the anti inflammatory activity (Fazli et al., 2014) of *P. persica, P. avium* and/or *P. domestica* fruits of family *Rosaceae*. For *in-vivo* analysis of skin irritation, redness or swelling subjective visual assessment methods are commonly applied but better results can only be interpreted by trained researchers (Rogiers et al*.,* 1999). Thereby, non-invasive bioengineering techniques could be used to make the data more objective and to measure subclinical symptoms like skin erythema, redness or inflammation. Primary skin irritation test or patch test is the most valid and widely used test for evaluation of irritancy or erythema potential of skin. 24 to 48 h occlusive patch test is usually applied to observe the type of any allergic/inflammatory reaction to a particular topical pharmacological or dermatological agent (Nigam, 2009).

This novel work was aimed to screen and correlate the *in-vitro* anti inflammatory activity of natural medicinal extracts prepared from Peach, Cherry and Plum fruits by Lipoxygenase assay, to evaluate *in-vivo* cutaneous erythema of topical soothing and cooling oil-in-water emulsions; loaded with those pleasant fruity medicinal extracts; by the use of non-invasive method; Mexameter MPA-5.

MATERIALS AND METHODS

Chemicals

Linoleic acid and Baicalein (Sigma, USA), Methanol (Merck KGaA Darmstadt, Germany), Hydrochloric Acid and Sodium Phosphate Buffers (Merck, Germany), Stearic-acid and Paraffin oil (Merck, Germany), Propyl and Methyl paraben (Acros, USA) and Potassium Hydroxide (Riedel, USA) were the chemicals used in this study.

Apparatus

Ultraviolet (UV) Spectrophotometer (Shimadzu Japan), Elisa Plate Reader (Biotek Synergy HT), Rotary evaporator (Eyela, Co. Ltd. Japan), Incubator (Shimadzu Japan), Electrical Balance (Precisa BJ-210, Switzerland), Refrigerator (Dawlance, Pakistan), micropipettes, separating funnel and water bath (HH.S214, China) were used.

Collection and identification of fruit materials

P. persica and *P. domestica* were collected from local market of Bahawalpur, Pakistan. The identification of the fruit sample; *P. persica,* voucher number 705-12-2119; was performed at the Cholistan Institute of Desert Studies (CIDS), the Islamia University of Bahawalpur, Pakistan. Identification of the fruit sample (*P. domestica,* voucher number 18247) was performed at Herbarium Department of Pharmacology, Sir Sadiq Muhammad Khan Post Graduate Medical College, The Islamia University of Bahawalpur, Pakistan. *P. avium* (voucher number IUB 7-821) was identified at Herbarium University College of Conventional Medicine, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan.

Extraction methods

Maceration and hot percolation methods were used in extraction of fruit samples. *P. persica, P. avium* and *P. domestica* fruits were extracted whole (without peeling); after removing the stones.

Preparation of *P. persica* extract

One hundred gram fresh fruits of *P. persica* (pulp with peel) were crushed and extracted three times by maceration with 80% aqueous methyl alcohol, by acidifying with a 0.25 ml of 1% hydrochloric acid, at room temperature for 24 h. The extract residue was removed by filtration through multiple layers of muslin cloth to get a coarse filtrate. The coarse filtrate was then filtered through a Whattman No.1 filter paper to get particle free extract. The methyl alcohol was removed under vacuum at 40°C. The concentrated extract was collected by rotary evaporator and stored under refrigeration.

Preparation of *P. avium* extract

P. avium fruit sample (100 g) was crushed and successively macerated (individually) in a mixture of 400 ml of 80% methanol and water in a ratio of 80:20 respectively; with 0.25 ml of 1% HCL; for 24 h at room temperature in dark. Then, the mixture was filtered through sixteen layers of muslin cloth and then through Whattman

Table 1. Ingredients of the active formulation (O/W emulsion) loaded with *Prunus persica* / *Prunus avium* / *Prunus domestica* extract.

	S\N	**Ingredients**	**Quantity (g)**
A	1	Stearic acid	22
	2	Paraffin oil	6
	3	Propyl paraben	0.02
B	4	Potassium hydroxide	1.5
	5	Methyl paraben	0.10
	6	Active ingredient (i.e. Peach/Cherry/Plum Extract)	5
	7	Distilled water	65.38
	8	Perfume	Nil

Where; A indicates the composition of oily phase and B indicates aqueous phase.

No.1 filter paper paper to remove all the coarse particles and to get a clear filtrate. The extract was then concentrated at rotary evaporator under reduced pressure at 40°C and stored in a refrigerator for further use.

Preparation of *P. domestica* extract

Fruit sample (*P. domestica*) was accurately weighed to 100 grams. After carefully removing stones; the whole fruit was crushed with spatula and quantitatively transferred to a measuring flask with about 200 mL of a methanol: water (80:20) solvent mixture and homogenized in a blender. The homogenized fruit mixture was transferred to a large beaker, by repeating the addition of 200 mL methanol: water (80:20) and 0.25 ml of hydrochloric acid (1%); for 24 h at room temperature in dark. The macerated fruit materials were filtered through 16 layers of muslin cloth for coarse filtration. The coarse filtrates were then filtered through a Whattman No.1 filter paper to get particle free extract. To obtain concentrated extract, the filtrates was evaporated under vacuum at 40°C in a rotary evaporator and stored in amber containers; under refrigeration (-15°C) until used for further analysis.

In-vitro evaluation of anti-inflammatory activity

Lipoxygenase (LOX) inhibition assay: Lipoxygenase (LOX) activity was assayed according to the method (Tappel, 1953; Baylac and Racine, 2003) with slight modifications. A total volume of 200 µL lipoxygenase assay mixture contained 150 µL sodium phosphate buffers (100 mM, pH 8.0), 10 µL test compound and 15 µL purified lipoxygenase enzyme (600 units well-1, Sigma Inc.). The contents were mixed and pre-read at 234 nm and pre-incubated for 10 min at 25°C. The reaction was initiated by addition of 25 µL substrate solution. The change in absorbance was observed after 6 min at 234 nm using 96-well plate reader Synergy HT, Biotek, USA. All reactions were performed in triplicates. The positive and negative controls were included in the assay. Baicalin (0.5 mM well-1) was used as a positive control (Khalid et al., 2013).

The decrease in absorbance indicates increased enzyme activity which was determined by the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of test solution})}{\text{Absorbance of control}} \times 100$$

Where; Absorbance of Control = Total enzyme activity without inhibitor, Absorbance of Test = Activity in the presence of test compound.

IC_{50} values were calculated using EZ–Fit Enzyme Kinetics software (Perrella Scientific Inc. Amherst, USA). LOX activity (% inhibition) was measured in triplicate.

In-vivo evaluation in healthy human volunteers

Alternative to animal testing in present work; 33 healthy human volunteers (all female of 22 years average age) were included after their written informed consent. Individuals were divided into three groups (11 each) for the application of three different formulations loaded with *P. persica* (peach), *P. avium* (cherry), and/or *P. domestica* (plum) extracts. Subjects were strictly prohibited to use any topical or oral antihistamine or steroidal drug during and one week preceding the study period to void any false perception of the results. Participants could unclose the patches due to any irritant or uncomfortable feeling during the study period but all volunteers completed the study safely and effectively.

Anti-inflammatory activity of all the three medicinal plant extracts was evaluated *in-vivo* by entrapping them in topical oil-in-water emulsion formulations. Three oil-in-water emulsion formulations were tested along with their respective vehicle controls (placebo). All the three formulations were having different composition and named as Peach Extract Cream, Cherry Extract Cream and Plum Extract Cream; with 5% extract of *P. persica, P. avium* and *P. domestica*, respectively. Vehicle control of each emulsion formulation was without any active ingredient (extract), for comparative analysis of actual inflammatory or erythema potential of active ingredients in the formulations. Oil-in-water emulsion formulations were prepared by the addition of oil-phase (A) to aqueous-phase (B) with regular stirring (Table 1). Both phases were individually heated to 70 ± 1°C. Then oil-phase was added drop by drop to aqueous-phase with stirring at 1300 rpm for about 10 min. No fragrance was added to the formulations to avoid any irritancy or allergic reaction on topical application. After complete mixing, the speed of mechanical stirrer was minimized to 800 rpm for 8 to 10 min then final homogenization was carried out at 500 rounds per minute for another 10 min. Vehicle control formula was also prepared by same procedure but without any respective active ingredient.

To decide the final stable formula of active formulation (Table 1), 25 approx. formulas with varying quantity of different ingredients of both oil phase and aqueous phase were prepared. Stability tests such as change in pH, electrical conductivity test and centrifugation test for phase separation were performed for active formulations and respective control formulations at different storage conditions, that is, 4, 25, 40 and 40°C with 75% relative humidity (RH) for a

Table 2. Lipoxygenase activities of *Prunus persica, Prunus avium* and *Prunus domestica* extracts.

	Lipoxygenase assay		
Crude extract	***Prunus persica***	***Prunus avium***	***Prunus domestica***
Inhibition (%)	58.73±0.78	51.29±0.23	47.87±0.54
IC50 (µg/ml)	15.31±0.76	18.01±1.21	21.07±1.13

Data are expressed as means ± standard deviations (n = 3); values are not significantly different ($P \geq 0.05$).

study period of 90 days.

Vehicle control was prepared by using same composition but without any active ingredient or extract. For single application closed patch test (48 h), area (1 × 1 cm) was marked on the inner right and left forearms of all human female volunteers; for the applications of active formulations on right forearms and their respective vehicle controls on left forearms. A small amount of each active formulation was applied on the right forearms and respective vehicle controls (without active ingredient) on left forearms of all volunteers in the three groups, that is, in Group-I (11 volunteers) Peach Extract Cream was applied on right forearms and its vehicle control (without peach extract) was applied on left forearms, in Group-II (11 volunteers) Cherry Extract Cream was applied on right forearms and its vehicle control (without cherry extract) was applied on left forearms. Similarly, in Group-III (11 volunteers) Plum Extract Cream was applied on right forearms and its control (without plum extract) was applied on left forearm for comparison. Area was covered with semi-occlusive cotton bandage, fixed with adhesive tape (closed patch test). Non-invasive biophysical technique used was Mexameter MPA-5 (Courage + Khazaka, Germany) for measurement of erythema index. Erythema level is expressed in arbitrary units which range from 0 to 1000 erythema index. The higher the erythema level observed the higher the capacitance was and vice versa. The skin erythema level on both forearms (right and left) of each volunteer was noted on zero hour, that is, before application of any product on the marked sites. Then after 48 h, the enclosed patches for primary skin irritation testing were removed and measurements were performed for erythema testing to observe any increase or decrease in erythema index.

Ethical considerations

This work was approved by the Board of Advance Studies and Research (BASR) and Ethical Review Committee (ERC), The Islamia University of Bahawalpur. Study was conducted according to ethical guidelines of GCP (Good Clinical Practice). Human volunteers were included in this study after taking their written informed consent. They were informed about any possible adverse reaction, protocols and objectives of this work.

Statistical analysis

All the measurements were done in triplicate. At 0.05 level of significance; data was compared to assess the significance of difference using Graph Pad Prism (GPP) version 5.01. The results obtained were expressed as Mean ± S.D.

RESULTS AND DISCUSSION

Lipoxygenase (LOX) assay

The LOX activities (% inhibition and & IC_{50} µg/ml) of various medicinal plant extracts were determined and shown in Table 2. More the percentage inhibition more the Lipoxygenase activity whereas, less the IC_{50} µg/ml (minimum inhibitory concentration required) more good is the activity. Various crude extracts of same family (*Rosaceaae*) and same genus (*Prunus*) were found to have good and comparable activities; maximum in *P. persica L.* extract and then in *P. avium L.* and *P. domestica L.* extracts when Baicalein was used as control.

Cutaneous erythema testing

Patch tests for erythema were performed for *P. persica*, *P. avium* and *P. domestica* formualtions with their respective vehicle controls and percentage of changes in erythema index for all the three formualtions with their vehicle controls has been shown in Figure 1. Where; A_1 indicates *P. persica* active formulation with its respective control (A_2), B_1 indicates *P. avium* active formulation with its respective control (B_2) and C_1 indicates *P. domestica* active formulation with its respective control (C_2).

All the active formulations (A_1, B_1 and C_1) significantly decreased cutaneous erythema level after performing patch test of 48 h; whereas their respective vehicle controls (A_2, B_2 and C_2) statistically produced insignificant change ($P \geq 0.05$) in skin erythema index, so the active formulations loaded with medicinal plant extracts of *P. persica, P. avium* and *P. domestica* can be safely used on human skin for *in-vivo* evaluation. All the three medicinal plant extracts produced significant anti-inflammatory effects ($P \leq 0.05$). No skin irritation was observed rather all the volunteers felt very soothing, cooling and moisturizing feeling during the application of closed patch of medical plant extract formulations. It may be attributed to the presence of various natural antioxidants and/or lipoxygenase in these fruit extracts which has the ability to reduce skin erythema or redness.

Conclusion

P. persica, P. avium and *P. domestica* extracts have positive significant correlation of Lipoxygenase activity (*in-vitro*) and produced no skin erythema after *in-vivo* testing. All those fruit extracts produced cooling and soothing effects on human (female) skin; thereby could

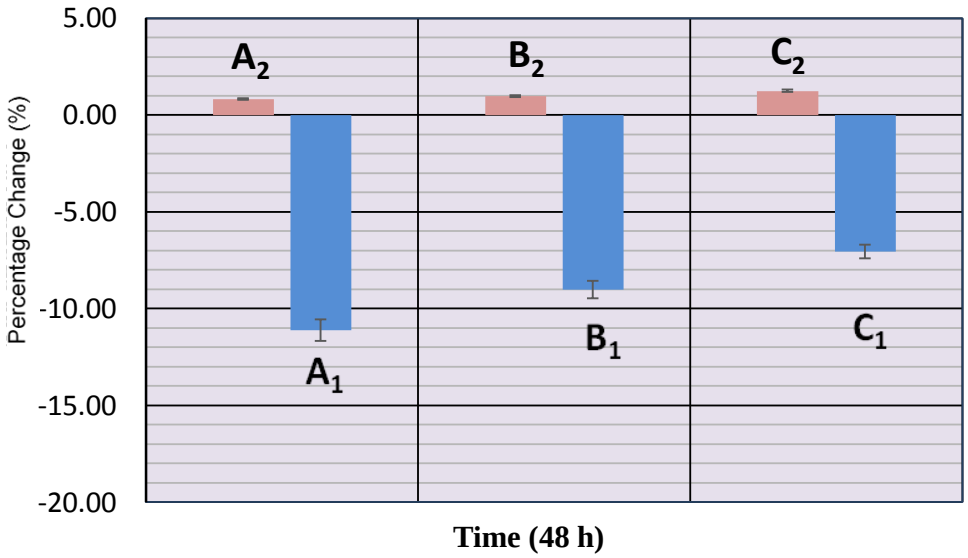

Figure 1. Percentage of change in erythema in case of active formulations (A_1, B_1, C_1) and respective controls (A_2, B_2, C_2) after 48 h.

be considered for use in skin care safely. The fruit sample extracts were proved to be pleasant, economical, non-irritant and anti-erythemic for topical use which makes them acceptable by the consumer. However, this study can be further elaborated to study the effects of these botanical extracts on other aspects of inflammation in future.

Conflict of Interest

The authors have not declared any conflict of interest.

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