

# Evaluating efficacy of *Punica granatum* L. peel extract gel in treating Cyclophosphamide induced alopecia in mice and *in vitro* anti-pediculicidal activity

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## ABSTRACT

The present study aimed to estimate the efficacy of *Punica granatum* L. ethanolic peel extract gel in treating hair fall in mice using the cyclophosphamide-induced alopecia method. *In vitro* anti-pediculicidal activity of peel extract was evaluated using the Filter paper diffusion method. Results indicated that high dose 20%w/v ethanolic peel extract gel of *P. granatum* was observed to have efficacy in treating alopecia and the results compared with that of standard minoxidil and it is also indicated that there is significant anti-pediculicidal activity to 20%w/v ethanolic peel extract was observed and results are compared with that of standard Benzyl Benzoate 25%w/v. These results provided an important basis for developing the peel extract as novel anti-pediculicidal activity and for treating alopecia and promoting hair growth, animals treated with high dose 20% gel showed better results compared to 10% low dose gel.

**Keywords:** *Punica granatum* L., alopecia, anti-pediculicidal, cyclophosphamide, minoxidil, benzyl benzoate.

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## INTRODUCTION

*Punica granatum* L. is the scientific name for Pomegranate (Anar) belongs to the family Punicaceae. Punicaceae is a genus of large deciduous shrubs that looks round and small trees with a single trunk, often seen with many low branching trunks; it is native to southern Europe and the Himalayas in Northern India, common names include Danimma in Telugu, Anar in Hindi, Pomegranate in English, Maadhulai in Tamil, Dareem/Dalim in Bengali, Dalimba / Dhalimbae in Kannada.

Alopecia is a sudden loss of hair that begins with one or more circular bald patches and progresses to a reversible disorder in which hair fall returns after a stressful situation, eventually leading to permanent baldness and looking patchy (Lutz and Sharp, 2015). Alopecia is most commonly a hereditary disorder (Matias et al., 1989). The enzyme 5 $\alpha$  reductase converts Testosterone to Dihydrotestosterone (Kaufman, 2002); in this case, this is an autoimmune disease (the immune

system attacks hair follicles and other parts of the body that grow hair, causing hair to fall out in round patches (Branisteanu et al., 2014).

Pediculosis is the term for the lice infestation in a healthy child, an infestation normally consists of less than ten live lice (Meinking, 1999). Asymptomatic infestations are possible. If the person becomes sensitised to antigenic components of louse saliva injected as the louse feeds, itching can occur. Sensitization can take four to six weeks (Frankowski and Weiner, 2002) during the first infestation. Some people are symptomatic and never scratch, secondary infection of the excoriated scalp may occur in neglected cases with severe infestations.

Different parts of *Punica granatum* have been screened for various pharmacological activities like astringent properties and also proved for its efficacy in treating wounds, diarrhoea, vaginal discharge, inflammations, and also used in traditional medicine, etc. Pomegranate peel

(Pop) is often considered as agricultural waste, comprising nearly 30 to 40% portion of fruit. Peel is a rich source of bioactive compounds and other biochemicals, fruit peel extract has antioxidant activity, antifungal, antibacterial, antiviral, antimicrobial activities, etc, but there is no report on the peel of *P. granatum* regarding treating Alopecia and anti-lice so far.

Therefore, the present study aims to evaluate the efficacy in treating Alopecia and the anti-pediculicidal activity of ethanolic peel extract of *P. granatum*.

## MATERIALS AND METHODS

### Plant collection

The fresh fruits of *P. granatum* L. were Purchased near local shops in Kakinada, Andhra Pradesh, India country, in December 2020. The plant was authenticated by Dr. T. Raghuram, Taxonomist at Maharani College Peddapuram.

### Preparation of extract

Purchased fruits were peeled off, washed, shade dried and made into a fine powder. The powder was macerated in ethanol for a period of 48 to 72 hours and then subjected to hot percolation for 8 hours, and then filtered. The filtrate was produced using a distillation process collected in a china dish and stored in a vacuum desiccator to dry.

### Preliminary phytochemical screening:

Some of the bioactive compounds derived from the plant are flavonoids, tannins, alkaloids, ellagic acid, phenols, amino acids, polysaccharides, etc.

### Quantitative phytochemical testing

#### Estimation of phenolic contents

This was calculated using the folin-ciocalteu method (Stratil et al., 2006) 3 ml folin-ciocalteu + 0.5 ml extract 1:10 v/v reagents, vigorously combine, and set aside for 5 min before adding 4ml 20 percent w/v sodium carbonate solution After that, the tubes were held at 30°C for 15 min to allow for colour development Read at 765 nm with a spectrophotometer. The maximum amount of phenols were examined and reported.

#### Estimation of total flavonoids

The total flavonoids content of an ethanolic extract *Punica granatum* l was calculated using the aluminium chloride process. Add 0.1 millilitres of 10% aluminium chloride, 1.8 millilitres of methanol, 0.1 millilitres of 1M sodium acetate, and 3 millilitres of distilled water to 0.6 litres of extract and hold at 30°C. Allow it to sit for 30 minutes before measuring the absorbance at 415 nm with a spectrophotometer. Complete flavonoids were calculated in methanol using a standard quercetin calibration curve, and the results were expressed as quercetin equivalent mg/100mg dry

weight of extract (Chang et al., 2002). The maximum amount of flavonoids were examined and Reported.

### 3 Estimation of total triterpenoids:-

Treat the ether and ethanolic peel extracts of *Punica granatum* L with a few drops of Sulphuric acid and shake well (Hossain et al., 2013). A no colour appears in these two, indicating the absence of sterols and triterpenoids; further, the same treatment of methanol and water extract was done, and the appearance of yellow colour in the lower layer indicates the presence of triterpenoids; the absorbance measured with a spectrophotometer indicated the presence of triterpenoids; the max amount of triterpenoids were examined and reported.

### Estimation of total tannins

A standard procedure was used to determine the tannin content of *P. granatum* L. peel extracts. 1 ml extract, 0.5 ml Folin-reagent (Jayaprakash and Sangeetha, 2015) A 1 ml saturated sodium carbonate solution from Ciocalteau, and 8 ml distilled water were combined. For 30 min, the reaction mixture was allowed to sit at room temperature. The supernatant was obtained by centrifugation, and the absorbance was measured using a UV-Visible Spectrophotometer at 725 nm. The maximum amount of tannins were examined and reported.

### In vitro anti-pediculicidal activity

#### Collection of head louse

Collect lice from school children's hair by combing through sections of the scalp using a clean teeth comb into Petri dishes.

#### Anti-lice activity (filter paper diffusion method)

The pediculicidal behaviour of ethanolic peel extracts of *P. granatum* was investigated using the filter paper diffusion method (Picollo et al., 2000). To obtain three different concentrations, all of the extracts were dissolved in distilled water (5, 10 and 20%). The louse was marked and separated. All of the test species were divided into 7 groups (5 lice each) and placed on a filter paper at the bottom of the petri dish, which was held open. A thin layer of 4 cm<sup>2</sup> was formed by pouring 0.5 ml of each test sample onto the test species. Group 1 was given 0.5 ml of distilled water to serve as a control, 0.5 ml of different concentrations of ethanolic extracts of 5, 10 and 20% were given to groups 2 to 4. Groups 5 to 7 were given 0.5 ml of benzyl benzoate 25% (w/v) in concentrations of 5, 10 and 20% (RidPed). All of the Petri dishes were placed in a dark chamber at 26 ± 0.5°C and 70% humidity for 1 h (Carpinella et al., 2007). After 1 hour, the dishes were removed and soaked in 0.5 ml of distilled water before being put back in the chamber under the same conditions as before. Furthermore, the lice were not exposed in an enclosed space because the petri dish was left open, reducing the risk of dangerous agents being ingested through the spiracles. The residue that remains in the head after rinsing with water for synthetic pediculicidal agents offers enhanced control against lice, but it is also correlated with the production of lice resistance. The dishes were examined under a dissecting microscope for any potential lice movement after 8 hours, and those that showed no movement were considered dead (Meinking et al., 1986). All of the treatments were done three times.

### ***In vivo* evaluating the efficacy of ethanolic peel extract gel of *Punica granatum* L. in treating cyclophosphamide-induced alopecia in mice**

Experimental animals: 6-8 weeks of C57BL -mice of 20-25 g, syngeneic if female

Chemicals used: Cyclophosphamide, minoxidil, ethanol (95%)

Selection of doses: 10-150 mg/kg body weight of cyclophosphamide is administered

#### **Induction of alopecia (*Cyclophosphamide-induced alopecia method*)**

A single intraperitoneal injection of Cyclophosphamide was given (10 to 150mg/kg body weight freshly dissolved in sterile water. Control mice received vehicles only ( distilled water ) a total of 6 mice were studied in 4 separate groups, group 1 for control, group 2 for standard, group 3 for test low dose, group 4 for high dose. For all these groups cyp was administered in the same dose based on their body weight 10 to 150 mg/kg body weight through ip route (Wheeler et al.,1962). The dose is injected only once after proper feeding and observation. The thinning of hair coat along the upper dorsum was seen after 1-2 weeks Of treatment after 2 weeks diffuse approximately the overall body if not treated (Paus et al., 1989).

#### **Preparation of herbal gel for topical application**

Two different formulations were prepared. containing *Punica granatum* peel extract of 10% w/w and 20% w/w. The gel was prepared using Carbopol, Carbopol 2 g was dispensed in 50 ml of distilled water with constant stirring in a 100 ml beaker. The beaker was kept aside overnight for a carbopol to swell. required quantity of polyherbal mixture 2 g for 2% and 5 g for 5% gel was properly mixed and triethanolamine was added dropwise to attain the required skin pH. sufficient quantity water was added to the final formulation to obtain the gel of required consistency (Bhingé et al., 2017).

#### **Evaluation tests for an herbal gel formulation**

##### ***Physical evaluation***

The prepared herbal gels were inspected visually for their colour, homogeneity, consistency as well as washability (Jain et al., 2016).

##### ***Measurement of pH***

An automated pH meter was used to calculate the pH of different gel formulations, 2.5 g of gel is measured and distributed in 25 ml of distilled water before being processed for hours.

##### ***Determination of spreadability***

At room temperature, the gel spreadability was assessed. After 1 min the spreading diameter of gel was determined between two glass plates, the gel was discovered to be semi-fluid.

##### ***Skin irritation test/sensitivity/allergy test***

Application of prepared formulation and observations (72 hours)

were made for erythema and edema

#### **Qualitative hair growth study**

##### ***Hair growth initiation time***

it is the minimum time taken to initiate hair growth initiation of hair growth was accessed as perceptible hair growth and observed against bright light preferably natural light. The appearance of hair over the skin is tested day after day of hair growth initiation.

##### ***Hair growth completion time***

The minimum time is taken to entirely cover the denuded region with new hair. Completion of hair is confirmed when the whole area is covered with newly grown and not with no patches (Adhirajan et al., 2003).

#### **Application of gel after initiation of hair fall patches**

Mice are divided into 4 groups:

- Group 1: Control (Blank gel)
- Group 2: Standard (minoxidil 5%)
- Group 3: Test (low dose 10%)
- Group 4: Test (high dose 20%)

The formulated gel is applied on the areas where hair loss patches are found in alternative days to notice the changes and hair growth initiation change is observed monitored during 30 days treatment and recording is started after 1 week on the day of 18, 24, 30 and number of days taken to initiated and complete hair growth was recorded and presented (Roy et al., 2007). The growth rate between treated and control was analysed after 1 week during days of 18, 24, 30 by means of physical evaluation and then both hair growth initiation and completion time in all four groups were evaluated and Reported (Jain et al., 2016).

#### **Statistical analysis**

The statistical values between the treated and control groups were determined using the student's t-test, with a p < 0.05 threshold considered significant.

## **RESULTS**

### **Screening of preliminary phytochemical screening**

Flavonoids, alkaloids, tannins, phenols, terpenoids, carbohydrates, coumarins, anthraquinones, saponins and amino acids were found. (Tables 1 to 4; Figures 1 to 6).

## **DISCUSSION**

### ***In vitro* anti-pediculicidal activity**

Review of literature, till date regarding *Punica granatum*

**Table 1.** Quantitative phytochemical testing of *Punica granatum* L.

S. no	Phytochemical	Quantitative analysis (mg/g)
1	Phenols	394.1 ± 0.13
2	Flavonoids	147.4 ± 0.10
3	Triterpinoids	112.1 ± 0.12
4	Tannins	210.5 ± 0.20

All the values are expressed as Mean ± SEM, n = 3.

**Table 2.** Quality control tests for ethanolic peel extract gel of *Punica granatum*.

	Parameters	Test low dose 10%	Test high dose 20%
Physical evaluation of gel	Appearance	Light Brown	Dark Brown
	Consistency	Soft	Soft
	Washability	Washable	Washable
	Homogeneity	Non Homogeneity	Non Homogeneity
Qualitative valuation of gel	pH	5	6
	Spreadability	Require Rubbing	Require Rubbing
	Skin Irritation	No irritation	No irritation

**Table 3.** *In vitro* screening of anti-lice activity of *Punica granatum* peel.

S. no	Test sample	Concentration (%W/W)	Average motility ± SEM (%)
1	Distilled water (0.5 ml)	10	40.1 ± 0.86
		5	40.1 ± 0.88
2	Ethanolic peel extract of <i>Punica granatum</i> L. (0.5 ml)	10	80.1 ± 1.22*
		20	100.1 ± 1.28*
		5	60.1 ± 0.98
3	Benzyl Benzoate 25% w/v (0.5ml)	10	80.1 ± 1.26
		20	100.1 ± 1.28

All the values are expressed as Mean ± SEM, n = 3; \*P < 0.001 when compared with standard values.

**Table 4.** Evaluating efficacy of *Punica granatum* L. ethanolic peel extract gel in treating cyclophosphamide-induced alopecia in mice and its hair growth initiation and completion time.

S. no	Group no.	Group Name	Treatment	No. of days taken for initiation of hair growth ± SEM	No. of days taken for completion of hair growth ± SEM
1	Group 1	Control (1 mice)	CYP + Blankgel	16.1 ± 1.06	27.1 ± 1.12
2	Group 2	Standard (1 mice)	CYP + MINOXIDIL5%	8.1 ± 0.71	23.1 ± 1.06
3	Group 3	Test low dose 10% (1 mice)	CYP+10% Ethanolic Peel extract of <i>Punica granatum</i> L.	12.1 ± 1.22	25.1 ± 1.33
4	Group 4	Test high dose 20% (1 mice)	CYP+ 20% Ethanolic peel extract of <i>Punica granatum</i> L.	9.1 ± 0.88*	23.1 ± 1.06*

All the values are expressed as Mean ± SEM, n = 3; \*P < 0.001 when compared with standard values.

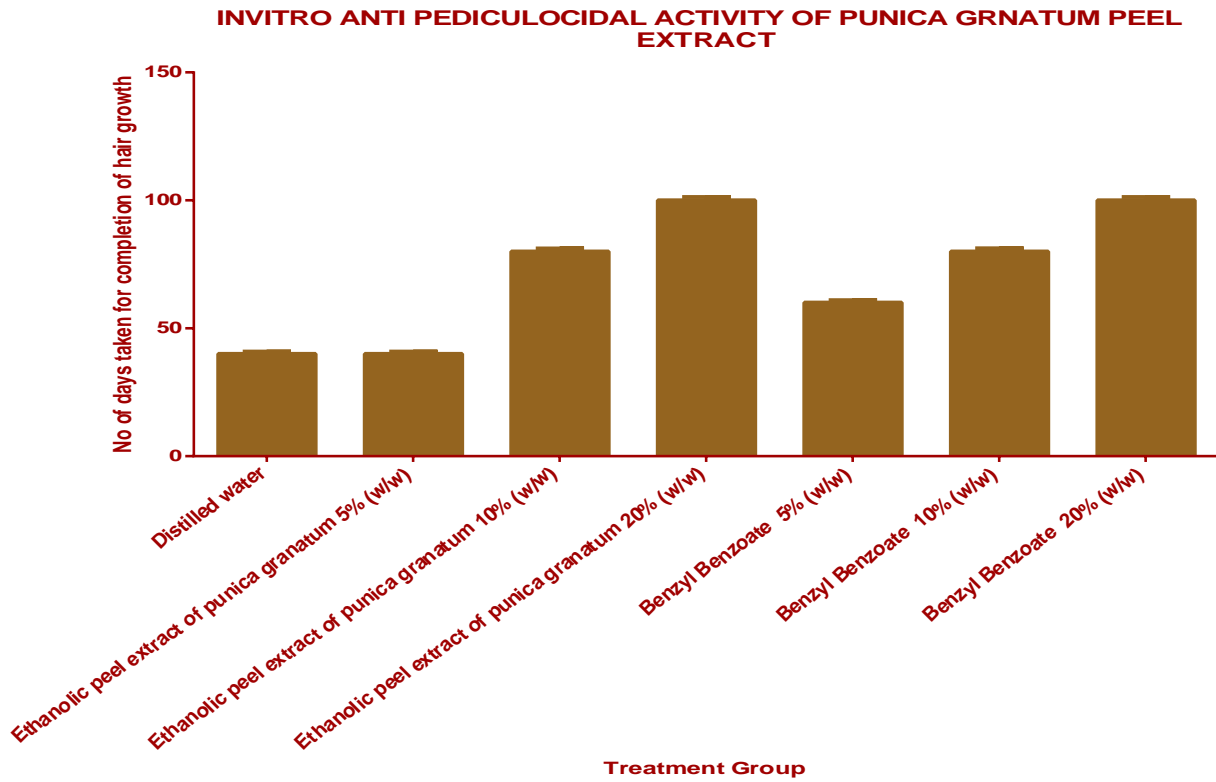


Figure 1. *In vitro* anti-pediculicidal activity of *Punica granatum* L peel extract.

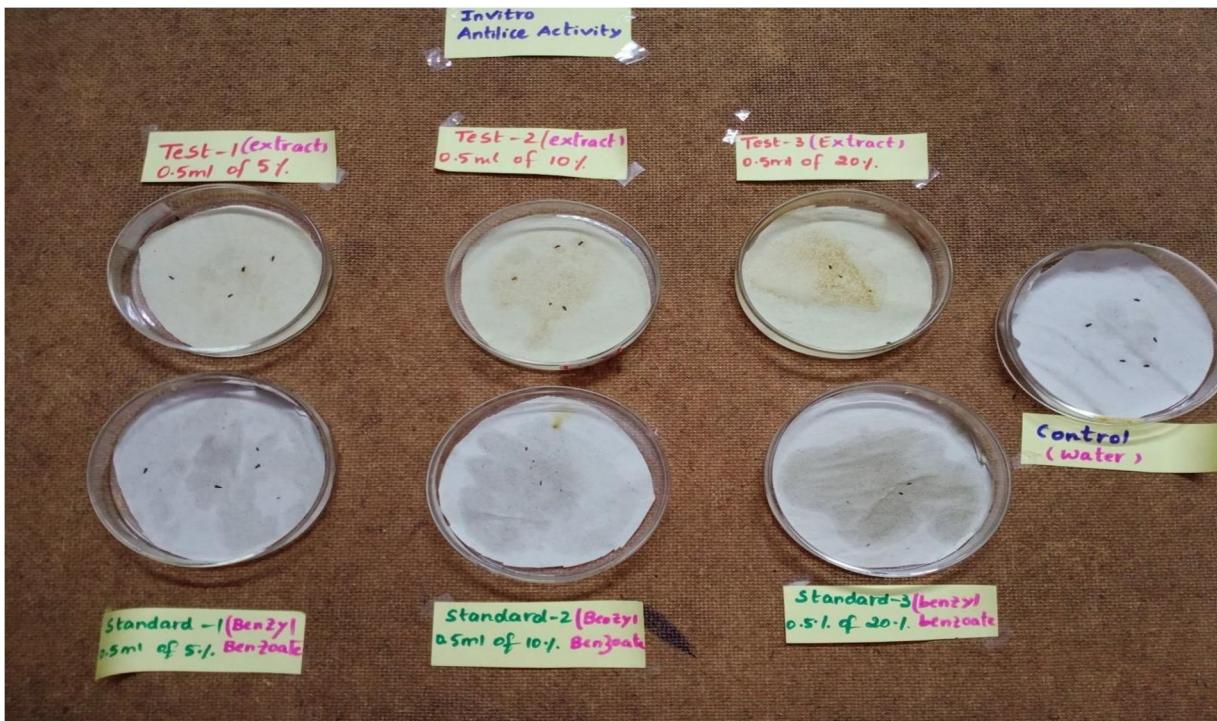


Figure 2. Visual experimental representation of *in vitro* pediculicidal activity of *Punica granatum* peel extracts.

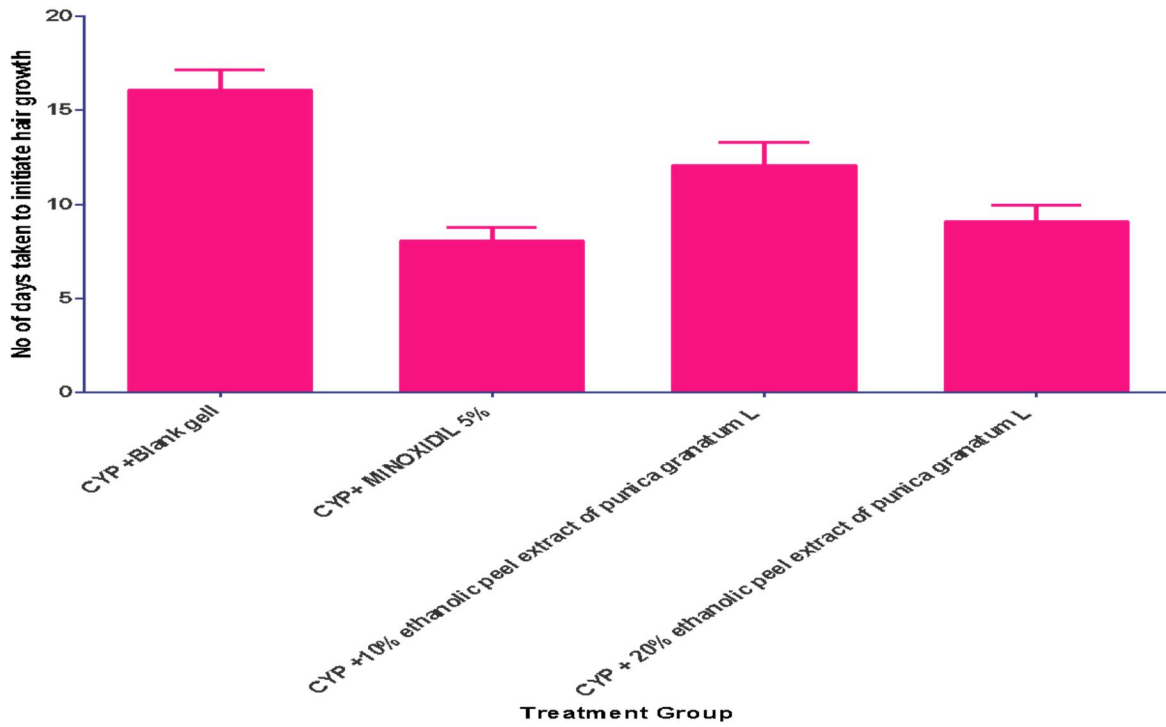


Figure 3. *In vivo* hair growth initiation time of *Punica granatum* L. peel extract.

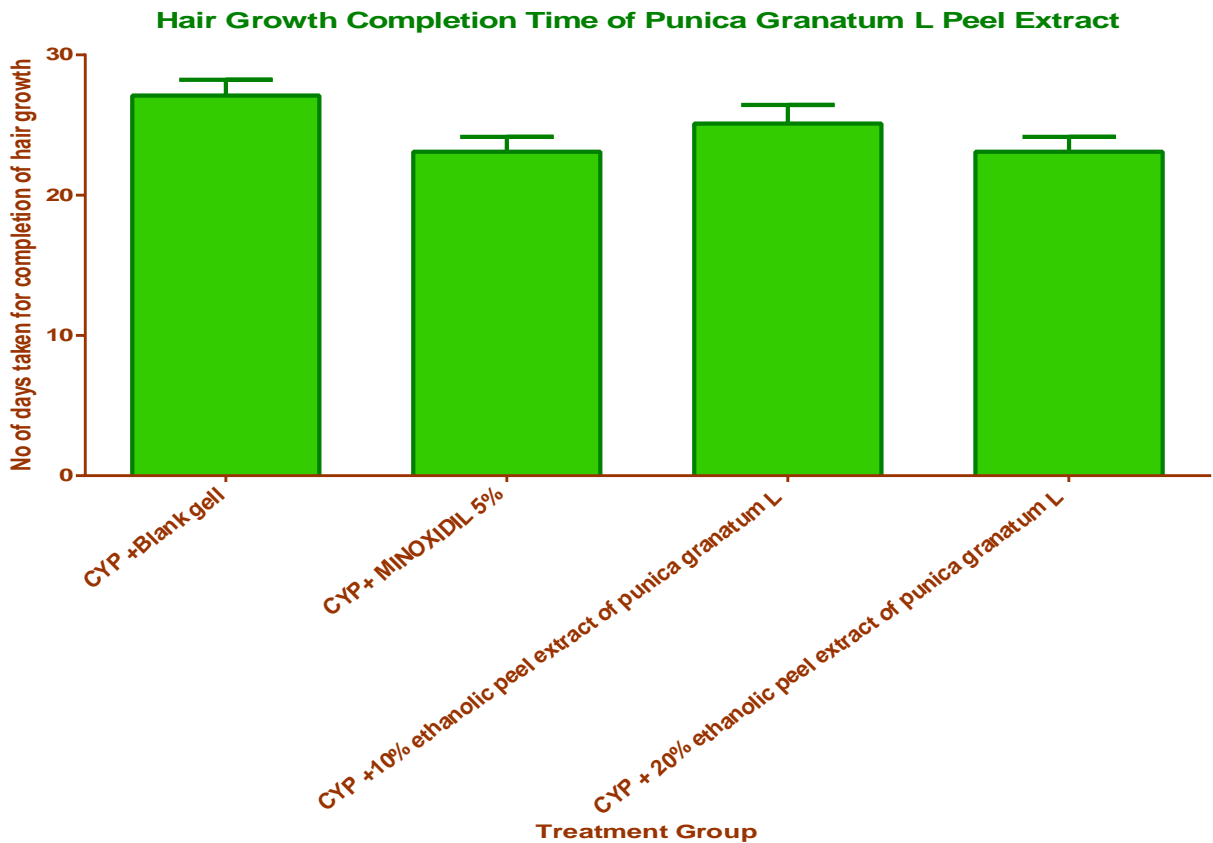
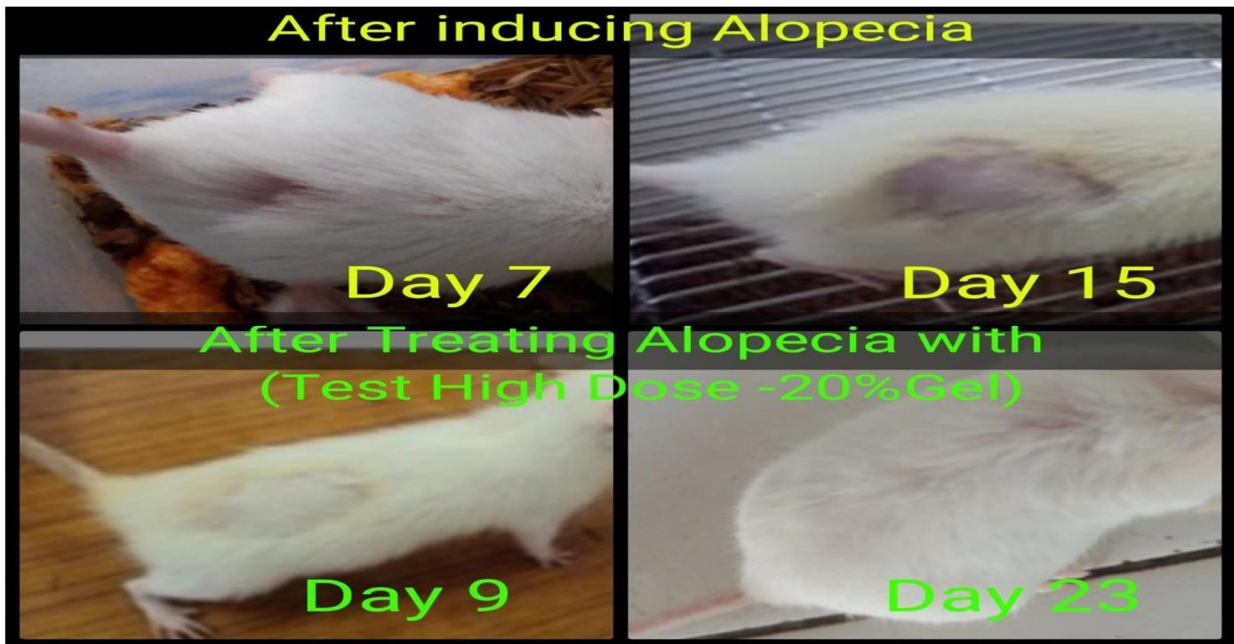
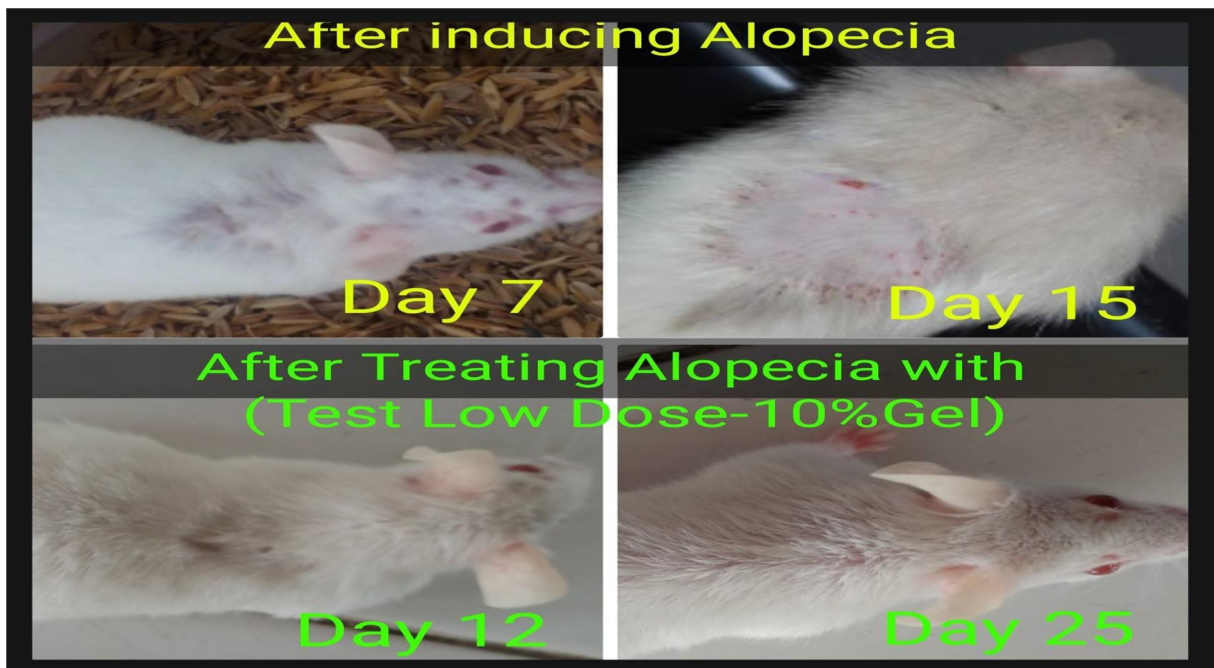


Figure 4. *In vivo* hair growth completion time of *Punica granatum* peel extract.





**Figure 5.** Visual representation evaluating hair growth initiation and completion time of high dose 20% of *Punica granatum* L. peel extract gel in mice.



**Figure 6.** Visual representation evaluating hair growth initiation and completion time of low dose 10 % of *Punica granatum* L peel extract gel in mice.

L, fruits, flowers, leaves, shoots, stems, barks, roots have been used in past. All parts have an astringent effect due to the presence of tannins. Several infusions of the plant have been used in herbal medicine to treating diarrhea,

vaginal discharge, pancreas inflammation, gall bladder disorders, wounds, etc. The different parts are screened for their pharmacological activities where fruit peel is proved for its antioxidant, antifungal, antibacterial,

antiviral, antimicrobial, anti-inflammatory activities, etc, but there is no report on peel regarding its efficacy in treating Alopecia and as anti-lice so far. Upon literature survey, *P. granatum* L is said to be very effective in treating or killing louse. The excellent results obtained after screening ethanolic extracts for possible anti-lice activity have validated the use of *P. granatum* peel extracts for managing lice infestations. According to Yang et al. (2005), bioactive compounds in the peel of *Punica granatum* L. that have anti-lice properties are tocopherols, phenols and phenolic ethers, and fatty acids. The use of *P. granatum* L. extract for controlling lice infestations has been authenticated from the excellent results obtained after the screening of ethanolic extract for its anti-lice activity. In filter paper bioassays, oils from natural sources such as tocopherols, phenols, fatty acids, etc showed important pediculicidal activity. The results of this study showed that ethanolic extract of *P. granatum* peel has excellent anti-lice activity, which may be attributed to the presence of these sterol compounds, which are responsible for the improved penetration and bioavailability of constituents into the body of the louse. Since all of the extracts were applied to lice mounted on filter paper, there was no significant spread of active constituents into the cuticle when the compound was applied directly to the insect's skin (Burkhart and Burkhart, 2001). The safe and successful use of *P. granatum* extract as a potent anti-lice agent has been noted, and the presence of resistance patterns was minimal due to its different mode of action (Breuer et al., 2003), which strongly supports the safe use of *P. granatum* extract as a potent anti-lice agent.

*P. granatum* extract was found to delay the emergence of lice, and its nature can aid in the detachment of lice from the hair before hatching. The ethanolic extract had the strongest pediculicidal effects of all the extracts, completely inhibiting nymph emergence at two different concentrations (10 and 20%). As a result, the findings of this study point to the possibility of using *P. granatum* peel extract as a viable treatment option for human head lice (Isman, 1997).

### **In vivo efficacy of *Punica granatum* Linn treating Alopecia**

In our work, the target was to promote hair growth activity by *Punica granatum* herbal plant peel. Using this extract, a gel is formulated to evaluate efficacy potency and therapeutic effects in the treatment of alopecia by visual observations, which have significant effects on hair growth. From the observed histopathological data, it is clear that the herbal treated groups showed the best results in hair growth and are compared to individual herbal gel formulations of treated groups, standard as well as control. The study provides an interesting

observation for the development formulation. *Punica granatum* L peel extract also showed the approximate result as standard minoxidil, when it is given in a high dose of 20% w/v. The hair growth-promoting activity of the aqueous extract was confirmed by visual observations and quantitative data in mice (Datta et al., 2009). Bioactive compounds in the peel of *Punica granatum*, extract gel for initiating hair growth are ellagic acid, polysaccharides, sterols include polysterols, saponins, vanillic acid, etc.

### **Conclusion**

By concluding that ethanolic extract of *Punica granatum* L peel is having efficacy in treating Alopecia and anti-pediculicidal activity because of the phytochemical compounds like ellagic acids, phenols, saponins, tocopherols, polysaccharides, vanillic acids, fatty acids, phenols, saponins, fatty acids, tannins, alkaloids, flavonoids which are soluble in ethanol.

Gel formulations of *P. granatum* promote hair growth by inducing follicles, Alopecia-induced mice were treated with herbal gel formulation for a prominent effect on hair. These results suggest that gel has the substantial potential of acting as a hair growth promoter apart from this it is completely inhibiting lice at different concentrations (10 and 20%). 20% is approximately equal to benzyl benzoate standard, as a result, the findings of this study point to the possibility of using *P. granatum* L. peel extract as a viable treatment option for humans head lice too. Further studies are required for the identification and isolation of active constituents from the extracts may provide new directions for the treatment of different problems of hair.

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