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Formulation and evaluation of polyherbal anti-acne combination by using *in-vitro* model

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 # Sharing first authorship

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ABSTRACT

Acne is one of the most common problem faced by today's youth and is listed in eighth position in the prevalence. It is affecting almost 9.4% of the global population in the world. This skin ailment is mostly occurring in the teenagers. There is no medicine is available in the market which can cure acne. And also, there are some limitations to these available management therapies as well as side effects are associated with them. The overcome these drawbacks of the available therapies a herbal formulation can a better choice due to the easily availability, synergistic activity, better bioavailability and less side effects of phytoconstituents. For our study we have selected Raphanus sativum (Mulak), Piper nigrum (Marich) and Allium sativum (Rason) due to its uses and availability. We have estimated the chemical constituents and phytochemicals present in these plants and their activity against Propionibacterium acne. We have also prepared a formulation using all the three plants and tested them for their activity and drug release with the time. In the case of permeation study, after the 12 h study the percentage of drug release is calculated 76.65%. Finally, we are concluding that the prepared formulation and the herbs used both have the anti-acne activity and the skin penetration is good.

Keywords: *Marich; Mulak; Rason; Propionibacterium acne; Acne; Antibacterial.*

1. INTRODUCTION

Acne is the common well being complication in the current situation [1]. It has been reported that around the world 79-95% reported cases of acne in the age group of 16-18 years and in India, the age group of 12-17 years; 50.6% of boys and 38.13% of girls have the complication of acne out of all reported incident. These reported incident ratio and prevalence provide a search of potential target for treatment of acne [2-3].

It is a persistent provocative skin complication and four major sites of pathophysiology have been recognized in acne: hyper keratinization and obstruction of sebaceous follicles, irregular desquamation of follicular epithelium, an androgen-stimulated increase within the era of sebum, and multiplication of *Propionibacterium acnes*, which creates irritation [4]. Disturbance of the preclinical forerunner lesion known as the microcomedo produces irritation, which leads to the pustules and papules of clinical illness and may eventually result in scarring. *Propionibacterium acnes* are the target sites of anti-acne drugs [5-6].

Ayurvedic system of medicine mentioned the acne as Shalmali thorn like eruptions on the face of adolescents, due to disturbance of Kapha, Vata and Rakta and named as Yuvana Pidika or Tarunya Pitika or Mukhadushika [7]. Moreover three type of chikitsa (treatment) is advised for the acne in Ayurvedic system of medicine i.e Shodhana (purification of body) and Shamana (preservationist treatment) Chikitsa or combination of both [4-5].

From thousand years back human start's using natural treatment or therapy i.e. herbal medicine for acne. The most of modern antibacterial and antimicrobial drugs having basic drawbacks like drug resistance, side effects, tolerance, so to overcome this problem the recent study more focus on herbal medicine research [5]. The present work is hypothesized to evaluate the potential of herbal combination in the management of acne. For this work we have selected three commonly used plants such as Raphanus sativum (Mulak), Piper nigrum (Marich) and Allium sativum (Rason).

Raphanus sativum (Mulak) is an indigenous herb belonging to the family Cruciferousis. Its common name is reddish and cultivated throughout the 3000-meter altitudes [8]. It is reported that it contains glycoside, flavonoids, tannins and sulphur containing compounds etc. It is used as antimicrobial activity, antioxidative activity, antitumor activity, antiviral activity, cardiovascular disease, antibacterial activity and antiinflammatory activity [9-10].

Piper nigrum (Marich) is a climber belonging to Piperaceae family. It is cultivated in north and south region of India. It is reported that it contains alkaloids, flavonoids, tannins, glycosides and essential oils [11]. It is used as anticancer, antioxidant, anti-inflammatory, anti-diarrheal, analgesic, antimicrobial and anti-acne activity etc [11-13].

Allium sativum (Rason) is a perennial bulbous plant and belonging to Liliaceae family. It is widely cultivated as the most condiment crop in India. It contains volatile oils, alkaloids, flavonoids, tannins, glycosides, sterols, tri-terpenes and fixed oils. It is used as hypocholesterolaemic action, antimicrobial, cholesterol and lipid-lowering effects, antithrombotic effects, hypotensive effect, pesticide and anticancer activity [14-15] (Table 1).

In vitro disc diffusion method is widely used for the determination of antibacterial as well as antimicrobial activity of the drugs. The organisms are inoculated in the suitable media which helps to growth of that particulate organism. The discs are made with the known concentration and to be tested against the organism [26]. Media is prepared in the aseptic conditions and autoclave to avoid the growth another organism. Prepared media is poured in the Petri plated and broth culture media of the organism is added, after that the prepared discs are diffused in the media and intubate for the specific time period [27].

In vitro transdermal drug delivery system is important tools for the evaluation of drug administration via skin for both local as well as systemic delivery of drug. Franz diffusion cell widely used static designs for the studying *in vitro* conditions, it is made with the glass having the donor cell where the semipermeable membrane is attached followed by the application of cream, it contains one sampling arm and having the inlet and outlet arm for the water to control the temperature 37 °C, in between there is a receiver cell which is directly contact with the semipermeable membrane and a metal pin is kept inside the receiver cell and with the help of magnetic stirrer it will rotate and the rotation is adjusted at 250 rpm [28].

Table 1. Reported pharmacological activities of different parts of *Mulak*, Marich and Rason.

Mulak (*Raphanus sativum*) [8, 16-20]		Marich (*Piper nigrum*) [12-13, 21]		Rason (*Allium sativum*) [15, 22-25]	
Pharmacological activity	**Part used**	**Pharmacological activity**	**Part used**	**Pharmacological activity**	**Part used**
Anti-microbial Activity	Root	Anticancer	Fruit (Alcoholic extract)	Hypocholesterolaemic action	Bulb
Anti-oxidative Activity	Root	Antioxidant	Fruit (Alcoholic extract)	Antimicrobial	Bulb (Aqueous extract)
Anti-tumor Activity	Root (Aqueous extract)	Anti-inflammatory	Fruit (Alcoholic extract)	Cholesterol and lipid-lowering effects	Bulb (Aqueous extract)
Antiviral Activity	-	Anti-diarrheal	Fruit (Aqueous extract)	Antithrombotic Effects	Bulb (Powder)
Cardiovascular Disease	-	Analgesic	Fruit	Hypotensive effect	Bulb (Juice)
Antibacterial activity	Seed (Ethanolic extract)	Immuno-modulatory	Fruit	Pesticide	Bulb (Oil)
Anti-Inflammatory activity	Leaf (Hydro alcoholic extract)	Antimicrobial	Fruit (Alcoholic extract)	Anticancer	Bulb (Juice)
		Anti- acne	Fruit (Alcoholic extract)		

2. MATERIALS AND METHODS

Raw herbs such as Rason, Mulak and Marich, were purchased from the local market Jalandhar, Punjab. The drugs were authenticated by the Nation Institute of Pharmaceutical Education and Research (NIPER) Mohali.

Method for extraction:

(a) Marich: Soxhlet extraction is done for the extract of Marich. 100 gm of Marich fruit are taken and pound to make course powder and 150 ml of ethanol is added. Ethanolic extract of Marich has been collected after the drying in water bath.

(b) Mulak: Soxhlet extraction is done for the extract of Mulak. 100 gm of Mulak fruit are taken and pound to make course powder and 150 ml of ethanol is added. Ethanolic extract of Mulak has been collected after the drying in water bath.

(c) Rason: 10gm of Rason bulblets are taken and pound to extract the juice.

Physicochemical properties of the herbs.

The foreign matter content, moisture content (loss on drying at 105 °C) and total ash content (at 450 °C) were evaluated [29-30]. The extracts were also evaluated for various phytochemicals such as alkaloid (Mayer test, Wahner's test, Dragendroff's test and Hager's test), carbohydrates (Molisch test, benedict test, Fehling test), glycosides (Modified Borntrager's test, Legal's test), flavonoids (alkaline reagent test, Lead acetate test), tannin (Ferric chloride test, lead acetate test).

In vitro anti-acne studies: Propionibecterium acne is purchased from the MTCC, Chandigarh (Order ID MTCC/SUP.1/201802051020/13251/3128). Bacteria were inoculated in broth cooked meat agar in a test tube for 18 hours and after that streak plating is done of inoculated media. The standardization of propionibacterium acne has been standardizing by using McFarland turbidity standard. The different dilution of BaCl2 and H2SO4 have been made which compared by the bacterial colonies in saline solution and compare the turbidity to the concentration to 0.5, which shows the 1ml of solution contains 1.5 x 108 cells [31].

Anti-acne activity of the Herbs extract.

Cooked meat medium agar for culturing of the bacteria has been taken 12.5 gm and dissolves in 100ml of water and add 1gm agar for solidification of the media and then autoclave the material. There are different dilutions that have been made of the extracts and 40µl is pipette out from the solution for dissolve in disc then check its activity by using disc diffusion method.

Preparation of the polyherbal anti-acne cream.

The concentration of the extracts of herbs is decided according to their activity and base of cream are selected by using the literature references, trials and stability (Table 2).

Anti-acne activity of prepared cream [15, 20, 24].

The concentrations of the extract have been decided and accordingly cream has been prepared and 400mg of the cream from each formulation batch have been taken to test their activity by using disc in cooked meat medium in plates and provide the anaerobic conditions by using the desiccator and candle is used for consuming the presence of oxygen.

Evaluation of the prepared cream: The prepared cream was evaluated for its organoleptic characters (odour, color and form), pH, nature of smear and ease to remove it [31].

In vitro permeation study of cream: Calibration curve of mixture of herbal extract was prepared by dissolving 1:1:1 mixture of drug Rason, Mulak and Marich in phosphate buffer pH 7.4. The above prepared stock solution was diluted by using phosphate buffer pH 7.4 to obtain the concentration range of 1-5 µg/mL. Prepared serial dilutions were analyzed by using UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) at the absorbance maxima of 341nm.

In-vitro drug permeation studies of cream: In-vitro drug permeation studies of the prepared cream were carried out by using cellophane membrane with the aid of Franz Diffusion Cell (FDC). A known amount of cream was applied to the receptor compartment of FDC and study was carried out for 12h in phosphate buffer pH 7.4 medium [32].

In-Vitro permeation study using Franz Diffusion Cell: In-vitro permeation study of herbal cream was carried out using Franz Diffusion Cell (effective surface diameter 1.2cm, volume 25mL, Singh Scientific Pvt. Ltd. India). The donor compartment of FDC was supplied with 250mg of the cream and the receptor compartment was filled with receptor fluid. Temperature of the receptor fluid was maintained up to 37oC and the study was carried out for 12h. The sampling intervals were kept as 0.5, 1, 2, 3, 4, 5, 6 and 12h. The obtained samples were analyzed by using UV-Visible spectrophotometer [33].

Statistical analysis of data: All the data were statistically analyzed by analysis of variance using INSTAT 1 software. Results are quoted as significant where $p < 0.05$. The values of the coefficient of variation were calculated for each reading to have an idea about reproducibility of results.

Table 2. Physicochemical properties of *Mulak* seed, Marich fruit and Rason bulb.

Sr. no.	Parameters	Mulak *(Raphanus sativum)*		Marich *(Piper nigrum)*		Rason (*Allium sativum)*	
		Standard (API vol. III)	Mean	**Standard** (API vol. III)	Mean	**Standard** (API vol. II)	Mean
1.	LOD	-	0.56%	-	1.26%	NLT60%	63.06%
2.	Foreign matter	NMT 2%	0.83%	NMT2%	0.7%	NMT2%	1.1%
3.	Total ash	NMT 5.5%	2.8%	NMT5%	3.6%	NMT4%	3.6%
4.	Acid insoluble ash	NMT 1.5%	0.8%	NMT0.5%	0.5%	NMT1%	0.5%
5.	Water soluble extractive value	NLT 4.5%	6.8%	NLT6%	6.93%	-	10.8%
6.	Alcohol soluble extractive value	NLT 11%	13.06%	NLT6%	10.13%	NLT2.5%	10.5%

❖ **API**- The Ayurvedic Pharmacopoeia of India, **NMT**- Not More Then, **NLT**- Not Less Then, **LOD**- Loss on drying

3. RESULTS

Physicochemical properties of Plant product.

The physicochemical properties for the plant product such as Mulak seed, Maricha fruit and Rason bulb were evaluated for different parameters which are shown in the table 3. These parameters are LOD, Foreign matter, Total ash, Acid insoluble ash, Water soluble extractive value and Alcohol soluble extractive value. The parameters were as per the standards and all within the normal range of standard.

We have also evaluated the biochemical constituents present in the Mulak seed, Maricha fruit and Rason bulb such as flavonoids, alkaloids, tannins, glycosides and fixed oils. These phytoconstituents were present in all the three-plant product (Table 4).

In vitro anti-acne activity of the plant extract.

Anti-acne properties of the methanolic extract of plant product were evaluated through the disc diffusion method.

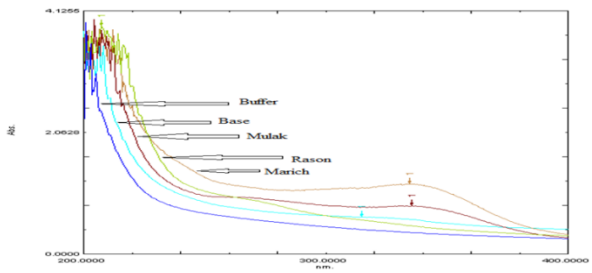

Figure 1. Overlay spectrum of Drugs, Base and Buffer.

Different concentration of the extract has been made with the DMSO and checks its anti-acne activity by using the *in vitro* disc diffusion method. The 40µl of solution is taken out and check the anti-acne activity. The maximum inhibition was shown by Marich extract even at 0.4 mg/ul and the least inhibition was shown by Mulak (Table 5).

Anti-acne activity of the prepared formulation.

There is two distinctive concentration of medicating are chosen based on concentration of the herbal extract which shows highest effect individually, and the 400 mg of the prepared formulation of both samples have been utilized for preparation of the disc. The zone of inhibition shown by formulation 1 (F1) and formulation 4 (F4) was found to be 9 and 10 mm respectively.

In-vitro drug permeation studies of cream.

The permeation study for the formula has been evaluated for 30 minutes to 12 hours at 341 nm. The formula has shown a drug release of 11.31% at 30 minute which increases to 76.65% in 12 hours. The result is shown in table 6. For the interaction among the phytoconstituents from different plants has been also studied by overlaying spectra. The overlay of spectra of specificity study of Mulak, Rason, Marich, Base and Buffer is shown in fig respectively (figure1). It observed from the scan that was no interaction of drugs, excipient and buffer. Hence, the study was specific for the estimation of those drugs.

Table 3. Composition of the formulation.

Sr.no	Ingredients	Formula %W/W (10gm)			
		F1 (g)	F2 (g)	F3 (g)	F4 (g)
1.	Marich	2	2	1.33	1.33
2.	Mulak	1	1	1.33	1.33
3.	Rason	1	1	1.33	1.33
4.	Steric acid	0.22	0.22	0.22	0.22
5.	Glyceryle monosterate	0.3	0.3	0.3	0.3
6.	Isopropyle monosterate	0.12	0.12	0.12	0.12
7.	Propyl perabene	0.018	0.018	0.018	0.018
8.	Triethanolamine	0.12	0.12	0.12	0.12
9.	Glycerin	0.6	0.6	0.6	0.6
10.	Methyl perabene	0.018	0.018	0.018	0.018
11.	Bees wax	0.4	-	-	0.4
12.	Rose oil	0.02	0.02	0.02	0.02
13.	Water	4.9	4.5	4.98	4.94

Table 4. Phytochemical screening of *Mulak* seed, Marich fruit and Rason bulb.

S.No.	Compounds	Chemical Tests	Mulak *(Raphanus sativum)*	Marich *(Piper nigrum)*	Rason (*Allium sativum)*
			Results (Alcoholic Extract)	Results (Alcoholic Extract)	Results (Alcoholic Extract)
1	Flavonoids	Lead acetate tests	+ve (Yellow ppt)	+ve (Yellow ppt)	+ve (Yellow ppt)
		Shinoda test	+ve (Orange colour)	+ve (Orange colour)	+ve (Orange colour)
2	Alkaloids	Mayer's test	+ve (Yellow ppt)	+ve (Yellow ppt)	+ve (Yellow ppt)
		Dragendroff's test	+ve (Reddish brown ppt)	+ve (Reddish brown ppt)	+ve (Reddish brown ppt)
		Wagner test	+ve (Red ppt)	+ve (Red ppt)	+ve (Red ppt)
3	Tannins	Ferric chloride test	+ve (Bluish black ppt)	+ve (Bluish black ppt)	+ve (Bluish black ppt)
4	Glycosides	Kellar Killani's test	-ve (Brown ring at junction absent)	+ve (Brown ring at junction present)	+ve (Brown ring at junction present)
5	Fixed oils	Spot test	+ve (Stain of oil)	+ve (Stain of oil)	+ve (Stain of oil)

Table 5. Zone of inhibition of *Marich, Rason* and *Mulak.*

Sr. no.	Drug concentration (mg/ul)	Zone of inhibition (mm)		
		Marich	*Rason*	*Mulak*
1.	0.4	2	-	-
2.	0.8	3	-	-
3.	1.2	7	3	-
4.	1.6	8	5	2
5.	2.0	8	6	4

Table 6. Mean % release of the cream.

Sr. No.	Sampling time point (h)	Abs (341nm)	Concentration	% DR
1	0.5	0.013	1.103	11.31%
2	1.0	0.027	2.14	21.96%
3	2.0	0.048	3.70	37.95%
4	3.0	0.058	4.15	40.52%
5	4.0	0.067	5.10	52.34%
6	5.0	0.089	6.73	69.05%
7	6.0	0.092	6.95	71.34%
8	12.0	0.099	7.47	76.65%

4. CONCLUSIONS

In this study we had used the three herbs and developed their four different concentrations of cream formulation, out of two formulation are consider best according to their form and the neglected formulation have the presence of bees wax which causes formulation hard so two formulations are considered as the final formulation. *In vitro* study of the Radish (Mulak), Black pepper (Marich) and Garlic (Rason) have been done and the maximum zone of inhibition of these herbs respectively is 4mm, 8mm and 6mm, and the zone of inhibition of the prepared formulation is 9mm and 10mm which are shown in results. The *In-vitro* skin penetration study has been done to calculate the amount of drug release. After the 12 h study the percentage of drug release is calculated 76.65%. Finally, we are concluding that the prepared formulation and the herbs used both have the anti-acne activity and the skin penetration is good.

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