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Formulation and evaluation of polyherbal gel containing extracts of *Azadirachta indica, Adhatoda vasica, Piper betle, Ocimum tenuiflorum* and *Pongamia pinnata*

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ABSTRACT: In the Indian system of medicine-Ayurveda, azadirachta indica, adhatoda vasica, piper betle, ocimum tenuiflorum and pongamia pinnata has been mentioned as a remedy for treatment of various infectious diseases and ailments. Based on the folkloric use, the present study was designed to formulate and evaluate polyherbal gel containing extracts of *Azadirachta indica*, *Adhatoda vasica*, *Piper betle*, *Ocimum tenuiflorum* and *Pongamia pinnata*. Gel formulations (Formulation A, B and C) were prepared which comprised of the ethanolic extracts of *Azadirachta indica, Adhatoda vasica, Piper betle, Ocimum tenuiflorum* and *Pongamia pinnata* in a concentration of 0.1, 0.3 and 0.5 %, respectively in a base. The prepared formulations were evaluated for appearance and homogeneity, pH, viscosity and rheological studies, spreadability, skin irritation test (patch test) and washability. The formulations were also screened for their antimicrobial activity by disc plate method against *S. aureus*, *B. subtilis*, *A. niger* and *E. coli*. The results of the studies revealed that all formulations under study viz. A, B and C showed better zone of inhibition as compared with the control. However, formulation C exhibited maximum activity against the selected strains which may be attributed to its greater amount of herbal extracts as compared to formulation A and B. The polyherbal gel formulations were observed to possess antimicrobial action. The effective activity may be attributed to the synergistic action of the plants constituents present in the formulation.

KEYWORDS: Antimicrobial activity; patch test; polyherbal gel; evaluation test.

1. INTRODUCTION

More than 80% of the world's population still greatly depends upon traditional medicines for treatment of various skin diseases [1]. In the recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries, as herbal medicines have been reported to be safe with minimal side effects especially when compared with synthetic drugs [2, 3]. Herbal treatments applied topically have gained considerable attention due to their widespread use and ill-defined benefit/risk ratio [4, 5]. There are numerous medicinal plants which are widely used in the treatment of skin diseases and also known to possess antimicrobial activity [6]. Topical application of gels at pathological sites offer great advantages in a faster release of a drug directly to site of action as compared to cream and ointment [7, 8].

Azadirachta indica (Neem), is phytochemically rich in steroids, alkaloids, tannins, triterpenes, flavonoid and anthraquinone glycosides [9, 10]. It has been known to be used traditionally for their various therapeutic properties like antibacterial, antimicrobial, antioxidant, skin disorder, and wound healing activity [11, 12]. Also it has been reported to possess various therapeutic properties like anti-inflammatory, antipyretic, antimalarial, antiulcer, antidiabetic, neuropharmacological effect, anthelmintic activity, antimicrobial and antibacterial effect [13-20]. *Ocimum tenuiflorum* has been found to exhibit various activities like antioxidant, antidiabetic, chemo preventive effect, anti-ulcer, anticarcinogenic, anti-stress and also useful in modulation of immune response [21-26]. *Adhatoda vasica* has been reported to be used traditionally for their various medicinal

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properties like anthelmintic, antiulcer, anticestodal, antioxidant, antifungal, antimicrobial and antibacterial effect [27-29]. *Piper betle* and *Pongamia pinnata* have been known to possess various therapeutic properties like antioxidant, antifungal, antimicrobial, hypoglycemic and antibacterial effect [30-36]. As per the literature survey the aforesaid plants have been reported for their antibacterial and antimicrobial effect by different researchers in most of the research articles. Based on this information it was decided to develop a herbal gel containing plant extracts which will possess better activity and prove to be effective against microorganisms. Despite of the fact that these plants possess good antimicrobial action, their use and application on the skin surface in the raw form is difficult. Hence, the present investigation was thus undertaken for preparation of polyherbal gel formulation using ethanolic extracts of *Azadirachta indica*, *Adhatoda vasica*, *Piper betle*, *Ocimum tenuiflorum* and *Pongamia pinnata*, so as to facilitate their effective use to exhibit its antimicrobial action. The prepared formulations were thereafter evaluated for their physical appearance, pH, viscosity, spreadability, drug content, skin irritation test, washability and antibacterial activity.

2. RESULTS

2.1. Evaluation of poly herbal gel

2.1.1. Physical analysis of the prepared polyherbal gel

It was observed that the freshly prepared formulations were off white to yellow in color (Table 1). Regarding the base and formulation A, B and C, there was no change in color, odor and appearance up to the observation period of 30 days at 8^0C and 40°C using different storage conditions, also the Formulations A, B and C were stable [6].

2.1.2. pH of the prepared formulations

It was found to be in the range of 6.62 to 7.08, kept at different storage conditions for 30 days. pH of the formulations and base kept at 8°C for one month did not show much change and data were significant over control (base) during one month ($p < 0.05$). Interestingly at 40°C, formulation A exhibited elevated change in pH (7.08), while the others remained slightly stable during one-month study. Data of formulations A, B and C at 40°C were found to be significant Table 2.

2.1.3. Viscosity test

Viscosity and Rheological properties of the formulations were found to be 7869±3.54 to 7968±3.92. The data of viscosity in formulations A, B, C and Control at 8°C and at 40°C were significant as shown in Table 2.

2.1.4. Centrifugation

Centrifugation test for base and formulation kept at different storage conditions were performed for 30 days. No phase separation after centrifugation was found in formulations A, B, C and base at 8 and 40°C during one-month study Table 1.

2.1.5. Spreadability

Spreadability of the base and formulations (A, B and C) were studied and found to in the range of 8.3±0.09 to 10.0±0.01. All the formulations and base were found to possess good spreadability.

2.1.6. Washability

Formulations were applied on the skin and then ease and extent of washing with water were checked manually. All the formulations exhibited good washability and left no traces over the skin on washing with water due to non-greasy properties.

2.2 Acid value, peroxide value and total fatty matter determination

Acid value, peroxide value and total fatty matter for the base and formulations kept at different storage conditions were observed for 30 days and the values for base and formulations A, B, C were found within the range Table 2. Acid value was found to be in the range of 2.30 to 2.91, peroxide value was found to be in the range of 1.64 to 1.88 and total fatty matters were found to be in the range of 15.01 to 15.5 for the formulations and base kept at different storage conditions for 30 days. Data of acid values of the formulations and base were found to be significant ($p < 0.05$) during one month of stability study. Peroxide value data in formulations A,

B, C and control at 8°C and 40°C were found to be significant (p <0.05). Total fatty matter data were found to be significant in all except formulation A, B, C and control at 8 and 40°C Table 2.

Table 1. Physical study of the prepared formulations during one month.

Duration	Storage Conditions					
	7 days		15 days		30 days	
Parameter	8° C	40° C	8° C	40° C	8° C	40° C
Appearance						
Formulation A	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Slightly Liquid
Formulation B	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Formulation C	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Slightly Liquid
Control or Base	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Color						
Formulation A	Yellow	Yellow	Yellow	Yellow	Yellow	Dark Yellow
Formulation B	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Formulation C	Yellow	Yellow	Yellow	Yellow	Yellow	Dark Yellow
Control or Base	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
Odour						
Formulation A	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Bad Smell
Formulation B	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Formulation C	Characteristic	Characteristic	Characteristic	Characteristi	Characteristic	Characteristic
Control or Base	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Centrifugation test						
Formulation A	NSL	NSL	NSL	NSL	NSL	NSL
Formulation B	NSL	NSL	NSL	NSL	NSL	NSL
Formulation C	NSL	NSL	NSL	NSL	NSL	NSL
Control or Base	NSL	NSL	NSL	NSL	NSL	NSL

NSL: No separation of layer; SL: Separation of layer

2.3. Patch test evaluation of volunteers

Patch test was performed to check the safety of the formulation and base on human skin. The prepared formulations and base were applied on the forearms of volunteers for 48 h [6]. The values obtained are shown in Table 3. The data showed that the parameters namely; ease of application was observed to be in the range of 2.4±0.2341 to 3.9±0.3214. The spreadability result of formulations and base were observed to be in the range of 2.5±0.2537 to 3.7±0.2321. Sense just after application of the formulations and base were found to be in the range of 2.5±0.2642 to 4.0 ±0.2775. Irritation as well as sense of softness on application for formulations A, B and C over forearms of volunteers, was recorded in the range of 2.56±0.2212 to 4.4±0.3431. All the results of the patch test were quite good as compared to the base. With paired sample *t-test*, it was evident that the effects of formulations and base were highly significant (p < 0.001) regarding all parameters of patch test. Volunteers reported that there was no irritation, redness after application of the prepared formulations A, B and C. Results of the patch test are shown in Figure 1.

2.4. Stability of polyherbal gel

Stability of the base and formulations (A, B and C) were studied at different storage conditions and assessed for their physical characteristics like color, appearance and odor (for 30 days) [6]. The results are shown in Table 1. Formulation A showed no significant changes in appearance, odor and color after 30 days.

2.5. Antimicrobial activity

It was determined by measuring the diameter of zone of inhibition. The results obtained in the evaluation of the antimicrobial activity of Formulation A, B, C and control(base) against the selected micro-organisms are shown in Table 4 and Figure 2A, 2B, 2C and 2D. Base showed zone of inhibition in the range of 8.98±0.7943 to 9.76±0.8798 against *S. aureus, E.coli B. subtilis* and *A. niger.* Formulation C showed better zone of inhibition in the range of 15.87±0.7804 to 19.01±0.6542 as compared to Formulation A, B and base. Thus, formulation C exhibited maximum activity against selected strains due to high amount of herbal extracts in comparison to others. The results were found to be statistically significant (p < 0.05).

Table 2. Chemical study of the prepared formulations during one month.

Duration	**7 days**		**15 days**		**30 days**	
	Storage Conditions					
Parameter	**8° C**	**40° C**	**8° C**	**40° C**	**8° C**	**40° C**
pH						
Formulation A	6.63±0.22	6.91±0.17	6.87±0.19	6.93±0.23	6.80±0.38	7.08±0.14
Formulation B	6.90±0.17	6.91±0.19	6.80±0.35	6.82±0.54	6.91±0.42	6.81±0.98
Formulation C	6.98±0.20	6.95±0.25	6.90±0.35	7.01±0.44	6.98±0.24	6.71±0.88
Control or Base	6.71±0.25	6.62±0.30	6.78±0.19	6.85±0.54	6.80±0.25	6.85±0.35
Viscosity and Rheological studies (Cps)						
Formulation A	7968±3.92	7962±2.33	7958±3.50	7952±4.01	7945±2.90	7939±3.09
Formulation B	7939±2.67	7934±3.65	7925±4.01	7918±3.65	7910±4.06	7903±3.56
Formulation C	7918±2.65	7910±3.90	7895±2.65	7894±4.11	7885±4.03	7865±3.96
Control or Base	7911±2.12	7898±2.76	7891±3.76	7882±4.04	7876±3.65	7869±3.54
Spreadability (gm-cm^2)						
Formulation A	8.4±0.01	8.4±0.04	8.3±0.09	8.4±0.01	8.3±0.06	8.3±0.10
Formulation B	9.8±0.02	9.8±0.03	9.8±0.08	9.7±0.04	9.6±0.08	9.7±0.09
Formulation C	9.9±0.03	9.9±0.02	9.9±0.06	9.8±0.06	9.8±0.07	9.7±0.09
Control or Base	10.0±0.01	10.0±0.03	10.0±0.04	10.0±0.05	9.5±0.10	9.5±0.07
Acid Value						
Formulation A	2.65±0.12	2.59±0.20	2.63±0.22	2.74±0.16	2.85±0.23	2.91±0.23
Formulation B	2.55±0.15	2.57±0.19	2.57±0.21	2.75±0.21	2.66±0.23	2.79±0.15
Formulation C	2.56±0.10	2.76±0.16	2.54±0.12	2.76±0.21	2.45±0.16	2.76±0.17
Control or Base	2.30±0.06	2.37±0.09	2.45±0.11	2.43±0.05	2.46±0.15	2.50±0.17
Peroxide Value						
Formulation A	1.49±0.23	1.53±0.25	1.55±0.30	1.62±0.25	1.64±0.28	1.67±0.30
Formulation B	1.61±0.11	1.64±0.09	1.70±0.15	1.76±0.22	1.78±0.25	1.85±0.32
Formulation C	1.65±0.10	1.70±0.07	1.72±0.09	1.79±0.20	1.80±0.21	1.88±0.33
Control or Base	1.64±0.09	1.67±0.12	1.70±0.16	1.73±0.12	1.74±0.15	1.79±0.21
Total fatty matters						
Formulation A	15.55±0.23	15.42±0.32	15.34±0.26	15.30±0.15	15.24±0.32	15.18±0.35
Formulation B	15.40±0.12	15.32±0.15	15.25±0.21	15.28±0.18	15.20±0.23	15.10±0.30
Formulation C	15.33±0.19	15.32±0.30	15.20±0.23	15.13±0.28	15.07±0.29	15.00±0.32
Control or Base	15.35±0.10	15.49±0.11	15.31±0.23	15.25±0.21	15.20±0.26	15.01±0.24

Table 3. Evaluation of prepared formulations on skin (Patch test) in human volunteers

Variable	Average point for Control ± SEM	Average points for Formulation A ± SEM	Average points for Formulation B ± SEM	Average points for Formulation C ± SEM
Ease of application	2.4±0.2341	3.9±0.3214	3.8±0.1765	3.8±0.2343
Spread ability	2.5±0.2537	3.4±0.2142	3.6±0.2621	3.7±0.2321
Sense just after application	2.5±0.2642	4.0 ±0.2775	3.5±0.2227	3.8±0.1243
Sense on Long term	2.4±0.2361	3.8±0.2794	3.7±0.2632	3.9±0.1986
Irritation	3.7±0.2735	4.4±0.3431	4.0±0.2741	4.2±0.2012
Sense of softness	2.56±0.2212	3.7±0.2341	3.8±0.2161	3.9±0.2103

Table 4. Antimicrobial sensitivity result of the prepared formulations A, B, C and Control.

Test organism	Zone of inhibition (mm)			
	Control	Formulation A	Formulation B	Formulation C
S. aureus	9.76±0.8798	15.56±0.6012	17.70±0.6609	19.01±0.6542
E.coli	9.65±0.7809	11.45±0.8648	13.76±0.6098	15.87±0.7804
B. subtilis	8.98±1.020	13.33±0.6523	15.65±1.0090	17.68±1.0245
A. niger	8.98±0. 7943	15.64±0.8090	17.79±1.1565	19.22±0.9807

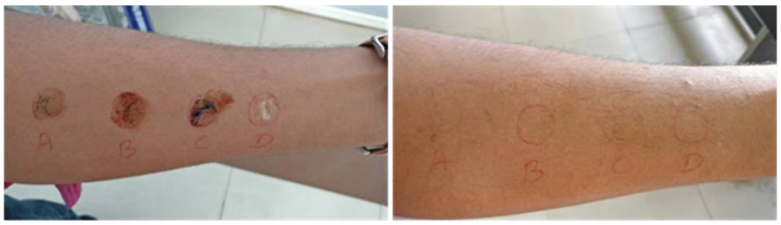

Figure 1. A: Formulation A; B: Formulation B; C: Formulation C and D: Control (Base) (i) Formulation and control (base) application at time = 0 hr (ii) Effect of all formulation and control at time = 48 hr after application on forearms of volunteers.

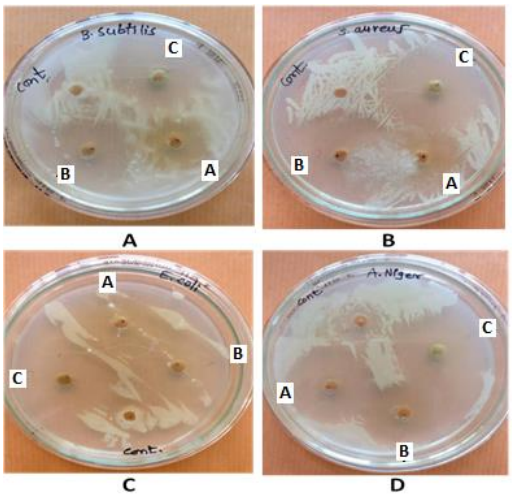

Figure 2. (A) Zone of inhibition of control and herbal formulations A, B, C and control against *B. subtilis* (B) of inhibition of control and herbal formulations v against *S. aureus* (C) Zone of inhibition of control and herbal formulations C and control against *E. Coli* (D) Zone of inhibition of control and herbal formulations C and control against *A. niger*.

3. DISCUSSION

Plants are considered to be a vital source of potentially useful constituents for the development of new therapeutic agents, as most of them are safe with less or no side effect(s). Topical application of gels at pathological sites offer great advantages in a faster release of a drug directly to site of action as compared to cream and ointment [7, 8]. Nowadays, gels have been widely used as a vehicle for topical delivery of drugs. Extracts of plants and herbs with specific medicinal properties can be incorporated in this dosage form as active ingredients in order to additional benefits [6, 37] *S. aureus* [48], *E. coli*, *B. subtilis* [49] and *A. niger* [50] are amongst the commonest pathogens that can cause skin infections. The antimicrobial properties of *Azadirachta indica* [9, 10, 14, 17, 50], *Ocimum sanctum* [25], *Adhatoda* vasica [28, 29], *Piper betle* [30-32] and *Pongamia pinnata* [35-36] plants have been previously investigated on some plant and human pathogens. However, their application and use in the raw form on to the skin surface is difficult therefore the extracts of these plants were developed in the form of gel formulation.

Cosmetically, the chemical constituents of *Azadirachta indica, Adhatoda vasica*, *Piper betle*, *Ocimum tenuiflorum* and *Pongamia pinnata* are considered to be antiseptic and natural preservatives.

Herbal cosmetic products are assumed to be safe for longer periods of time. However, quality control for efficacy and safety of herbal cosmetic products is of paramount importance; and quality control tests must therefore be carried out for these preparations. Stability studies and patch test are well known methods which will prove its efficacy and efficiency of the cosmetic herbal formulations [6, 37] Short term stability studies as per ICH guidelines, revealed that the pH of all the formulations and base indicated variability at different storage conditions. Viscosity, Rheological studies, Spreadability, Acid Value, Peroxide Value and Total fatty matters showed minimal variations in the results which proved that all the prepared formulations are stable for 8 and 40^0 C. Applicability of the herbal formulation was proved to be satisfactory from the results of viscosity and spreadability. In our studies it was observed that the prepared formulations readily spread on application to the skin or affected part and homogeneity confirmed no lumps, respectively. Also, the physicochemical parameters applied in the testing of stability of cosmetics formulations made apparent consequences that formulations C is much better than formulations A, B and base due to its relatively higher concentration of active constituents.

Literature surveys revealed that individually all extracts has potentially been known for their antimicrobial activity. However, no literature is available related to the formulation of a polyherbal formulation containing the extracts of *Azadirachta indica, Adhatoda vasica*, *Piper betle*, *Ocimum tenuiflorum* and *Pongamia pinnata.*

Figure 1 depicts the results of the patch test, which exhibited no irritation, redness on underarm after application of formulation A, B and C as reported by volunteers. Also, the results of washability test proved non-greasy properties of all prepared formulations.

This study clearly indicated that formulation A, B and C which possessed plant extracts were more potent than the base. The possible explanation for this is the presence of active constituents of plants which exhibit antimicrobial activity. But as compared with other formulations the potency of Formulation C was found to be greater.

4. CONCLUSION

Results of the studies revealed that the prepared polyherbal formulations A, B and C which comprised of ethanolic extract of *Azadirachta indica*, *Adhatoda vasica*, *Piper betle*, *Ocimum tenuiflorum* and *Pongamia pinnata* in a concentration of 0.1, 0.3 and 0.5 %, respectively produced no skin irritation after performing patch test on the underarm of human volunteers for 24 hours. Also the physical analysis and stability studies of the prepared polyherbal gel proved potency and efficacy. Thus, these formulations can be used safely on human skin. The effective activity exhibited by the polyherbal formulations may be attributed to the synergistic action of the plants constituents present in the formulation. The high amount of plant extracts (0.5 %) increased the antimicrobial activity of the formulation.

5. MATERIALS AND METHODS

5.1. Chemicals

Analytical grade chemicals were used for the study. The solid media and broth used for microbial culture were procured from Hi-Media Pvt. Limited, Bombay, India. Carbopol 940 (Merck Ltd), propylene

glycol-400 (SD Fine Chemical Ltd), Ethanol (Merck Ltd), methyl paraben (Supreme Chemicals), propyl paraben (Supreme Chemicals), Triethanolamine (SD Fine Chemical Ltd), EDTA (S.D Fine lab India).

5.2. Collection and extraction of plants parts

Fresh leaves of *Azadirachta indica, Ocimum tenuiflorum*, *Adhatoda vasica and* seeds of *Pongamia pinnata* and *Piper betle* were collected from the Western Ghat regions of Maharashtra. The taxonomical identification of the collected plant species was done at Department of Botany, YC College of Science, Karad, Maharashtra, India. The plant material was dried in a hot air oven at 40°C. The plant materials were coarsely powdered separately by using a grinding mill. About 30 g of each powder specimen were defatted with petroleum ether (60-80° C) in a Soxhlet apparatus followed by its extraction with ethanol. The obtained extracts were further concentrated on a rotary evaporator and were kept in a vacuum dryer till further use.

5.3. Preparation of polyherbal gel

Formulation A, B and C were prepared which comprised of ethanolic extract of *Azadirachta indica*, *Adhatoda vasica*, *Piper betle*, *Ocimum tenuiflorum* and *Pongamia pinnata* in a concentration of 0.1, 0.3 and 0.5 %, respectively as shown in Table 5. Carbapol- 940, propylene glycol 400, ethanol, methyl paraben, propyl paraben, EDTA and tri-ethanolamine were used to prepare 100 gm of gel by adding sufficient quantity of distilled water. Water required for these formulations was divided into two parts. In one part the exact amount of extract was dissolved and to this calculated quantity of propylene glycol 400 and ethanol was added and in other part, Carbapol-940 was dissolved and to this solution methyl paraben, propyl paraben and EDTA was added. Both of the solutions were mixed in a beaker and tri-ethanolamine was added to the mixture drop wise to obtain the gel consistency. It was stirred by using propeller for 2 hours at 500 rpm to obtain a homogenous gel, devoid of any entrapped air bubbles. Various formulation batches were prepared according to the Table 5. Plant extracts were excluded to make the Gel base. The prepared gel formulations and base were kept at room temperature for 24 hours.

Table 5. Formulation table of Gel base and Polyherbal gels.

Ingredients	Quantity taken in (%) for Gel base	Quantity taken in (%) for Formulation A	Quantity taken in (%) for Formulation B	Quantity taken in (%) for Formulation C	Role
Carbopol 940	1.0	1.0	1.0	1.0	Gelling agent
Extract of each drug	-----	0.1	0.3	0.5	Antibacterial activity
Propylene glycol 400	4.0	4.0	4.0	4.0	Base
Ethanol	3.0	3.0	3.0	3.0	Solvent
Methyl paraben	0.2	0.2	0.2	0.2	Preservative
Propyl paraben	0.02	0.02	0.02	0.02	Preservative
EDTA	0.03	0.03	0.03	0.03	Chelating agent
Triethanolamine	1.2	1.2	1.2	1.2	pH adjustment
Water	Q.S. 100	Q.S 100	Q.S 100	Q.S 100	Aqueous base

5.4. Evaluation tests for Polyherbal gel

5.4.1. Appearance and homogeneity

The prepared gels and control (base) were tested for physical appearance and homogeneity by visual observation [6].

5.4.2. pH

Digital pH meter (Systronics digital-DI-707) was used to determine pH of the prepared formulations and control (base) [6]. 3 gm of gel was accurately weighed and dispersed in 30 mL of distilled water and stored for two hours, then pH was measured separately [6, 37]. The measurement of pH of each formulation was done in triplicate and the average values have been represented in Table 2.

5.4.3. Viscosity and rheological studies

Brookfield viscometer (DV-III programmable Rheometer) instrument was used to determine the rheological characteristics of control (base) and Formulations at 25° C [6]. The measurement was made over the whole range of speed settings from 10 rpm to 100 rpm with 30 seconds between two successive speeds and then in a descending order [6, 37].

5.4.4. Spreadability

A special apparatus as suggested by Mutimer et al., 1956; was designed for determining spreadability of the prepared gel formulations [6, 38]. An excess of gel sample was placed between the two glass slides and a 1000 g weight was placed in slides for 5 minutes to compress a sample to uniform thickness [6, 37]. Weight (50 gm) was added to the pan. The time required to separate the two slides was taken as a measure of spreadability [6]. Lesser the time taken for separation of two slides, better the spreadability. It was calculated by using the formula:

$$S = M.L/t$$

Where M is the weight (g) tied to the upper glass slide; L is the length (cm) moved on the glass slide and t is time to separate the slide (sec).

5.4.5. Washability

Formulations were applied on the skin and then ease and extent of washing with water were determined manually [6].

5.5. Chemical tests

5.5.1. Acid value

0.5 gm of gel was taken and dissolved in 10 times of absolute alcohol. It was heated on a hot plate for 5 min and 2 to 3 drops of phenolphthalein indicator was added to it and titrated with 0.1 N KOH until faint pink color appeared [39].

Acid Value = 56.1 × Titre value × N of KOH/Weight of sample.

5.5.2. Peroxide value

In a separate 200 mL flask, 5 gm of gel sample of control (base) and formulations, 30 mL of acetic acid and chloroform solution were added and swirled gently [6]. Then 0.5 mL of potassium iodide solution was added with continuous shaking and 30 mL of water was added thereafter. Finally the solution was then titrated with 0.1 M sodium thiosulfate solution with vigorous shaking. End point of titration was noted when yellow color almost disappears. Then 0.5 mL of 1% starch was added and titration was continued with vigorous shaking to release all iodine from chloroform layer, until blue color disappeared [6, 40].

Peroxide value = S × M × 1000/gm sample

Where S = mL of sodium thiosulfate and M = Molarity of sodium thiosulfate solution.

5.5.3. Total fatty matter determination

2 gm of gel sample and 20-25 mL of 1:1 dilute HCl was taken into the 200 mL flask, then the solution was heated on a water bath till the solution becomes clear [6]. The sample (aqueous phase) was drawn in a 250 mL separating funnel and then allowed to cool at room temperature. 50 mL of petroleum ether (organic phase) was then added in the funnel and shaked and left for separation to occur [6]. The organic phase was collected. The above aqueous layer partitioned twice with same quantity of petroleum ether. The organic layers were collectively evaporated to obtain residue which was consequently washed with water. The residue was filtered and sodium sulfate was added to it. The mixture was again filtered, the extract was dried and the content was determined [6, 41].

Total fatty matter (%) by mass = $100 \times M_1/M_2$;

M_1 = mass of residue; M_2 = mass of sample in gram.

5.6. Product evaluation on skin (Patch test)

Twenty healthy human volunteers were selected whose ages were in between (20-35) years. Consent form was filled by each human volunteer prior starting the study [6]. Volunteers having serious skin diseases, asthma were excluded from the study. Patch test was performed on the forearms of each volunteer to determine any possible reaction(s) to the formulation [6, 42]. The prepared formulations A, B and C along with control (base) were applied on the forearms of the volunteers separately. Adhesive tape was used to fix them in place and the test sites were marked. The patches were left in place for 48 h, during which care was taken not to wash the applied area [6]. The patches were removed and observation (redness, itching, or blemishes) with their reading was taken one hour later. These visible signs along with any itchy or irritable sensations indicated that there is something wrong to the product. Clear skin devoid of aforesaid visible signs indicated that the product is safe to use [6, 40, 43].

5.7. Stability of polyherbal gel

Short term stability (for 30 days) of control (base) and formulations (A, B and C) were verified at 8±0.1 and 40±0.1 ^{o}C storage conditions (in incubator) with 75% relative humidity (RH) as mentioned by Pandey et al. by checking for their physical characteristics like color, appearance, odor and centrifugation test [6].

5.8. Antibacterial activity of polyherbal gel

The prepared formulations and control (base) were screened for their antibacterial activity by Disc plate method [6, 44-47]. It was tested on nutrient medium against *S. aureus*, *B. subtilis, A. niger* and *E. coli* which are representative types of Gram positive and Gram negative organisms. The activity was determined by measuring the diameter of zone of inhibition recorded [6]. The test strains of *S. aureus*, *B. subtilis*, *A. niger* and *E. coli* were collected from Department of Microbiology, YC College, Karad (415124), Maharashtra, India. The plates were inoculated with test cultures and were incubated at 37±1°C for 24 h. The next day, the wells (6 mm diameter) were made with help of 6 mm diameter cork borer and the wells were loaded with prepared formulations namely A, B and C along with base as a control. After 24 h of incubation, the test determined the efficacy of the product in terms of zone of inhibition of the organism.

5.9. Statistical analysis

The measured values obtained for different parameters were analyzed using SPSS 20 software and results were further tested by paired sample t test.

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