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Formulation and Topical Safety Evaluation of *Zanthoxylum rhetsa* Spray Containing Essential Oil from Pericarp: A Phase 1, Double-blind, Non-Randomized, Single-Arm Patch Test Trial

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ABSTRACT

Our previous study demonstrated that essential oil from the pericarp of *Zanthoxylum rhetsa* (ESO) has a higher yield and greater potency in inhibiting prostaglandin E_2 than essential oil from the fruit. This study aimed to formulate a topical spray containing ESO and to evaluate its safety through patch testing for irritation and allergy. A hydroalcoholic base spray (BS) was formulated and evaluated for appearance, skin feel, pH, viscosity, volume per spray, and evaporation time. Among BS F1–F5, F4 met all criteria and remained stable after six cooling– heating cycles. ESO was incorporated into F4 at 3–9% (v/v) to produce sprays, which passed stability tests. A non-randomized, double-blind, single-arm patch test was conducted in 12 healthy volunteers. Eighteen samples, including BS F4, spray products with 3–9% ESO, ESO alone at 3–9%, and controls, were applied to the upper back for 48 hours and evaluated at 30 minutes and 24 hours post-removal using the International Contact Dermatitis Research Group criteria. All concentrations showed acceptable characteristics; the 9% spray had optimal viscosity and slower evaporation. Clinically (10 completed), five showed no reaction at 30 minutes. One volunteer showed weak positive responses to 8% and 9% ESO and 2% methyl salicylate (score 0.42, slight irritation), and five showed angry back reactions. By 24 hours, all had resolved except one. Therefore, BS F4, sprays with 3–9% ESO, and ESO alone at 3–7% were safe, with the 9% formulation suitable for further clinical evaluation in pain management.

Keywords: *Zanthoxylum*, Essential oils, Formulation, Dermal safety, Phase 1 clinical trial, Irritation, Allergy.

Introduction

Zanthoxylum rhetsa (Roxb.) DC., a member of the Rutaceae family, is a native plant distributed throughout Eastern and Southeast Asia.[1] It has long been used in traditional medicine for its analgesic and anti-inflammatory properties.[1] Our previous study demonstrated that the pericarp of *Z. rhetsa* is a rich source of essential oil, yielding 14.30% w/w, approximately 2.67 times higher than the whole fruit.[2] It also showed stronger inhibition of prostaglandin E_2 (PGE_2) production in RAW264.7 macrophages, with an IC_{50} of 24.13 µg/mL, about 1.70 times more potent than the whole fruit.[2] The major chemical constituents of the essential oils from both the pericarp and fruit include limonene, sabinene, and terpinen-4-ol.[3] These compounds have been shown to inhibit the production of PGE_2 and nitric oxide, and have also been reported to exhibit analgesic and anti-inflammatory effects, which are considered beneficial in the management of knee osteoarthritis (OA).[4,5] The prevalence of this condition is increasing significantly in East and Southeast Asia.[6] Current topical herbal products are used as a treatment for knee OA in complementary and alternative medicine to avoid the side effects associated with oral medications.

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However, topical herbal products may still cause adverse effects such as skinirritation and a burning sensation.[7] Topical herbal products are commonly formulated as creams, emulgels, balms, ointments, liniments, and sprays. Spray formulations are particularly suitable when essential oils serve as the active ingredients, as they avoid thermal processes that may cause degradation of heat-sensitive constituents.[8] A spray is a solution-based formulation consisting of a solute and a solvent or co-solvent, which enhances the solubility of poorly soluble pharmaceutical ingredients.[9] It is easy to spread and is not greasy, viscous, or oily when applied to the skin. However, the safety of topical herbal products on the skin should be a primary concern. Therefore, irritation and allergy testing are essential as part of the clinical trial process prior to application or future efficacy trials. Patch testing is considered the gold standard protocol and is essential for identifying irritant and allergenic agents that cause irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD), respectively.[10] ICD is the most common type, accounting for approximately 80% of cases, while ACD accounts for about 20%.[11] ICD is a non-immunological inflammatory skin response that occurs upon exposure to irritant agents.[12] In contrast, ACD is an immunological inflammatory response classified as a delayed-type hypersensitivity (Type IV) reaction that occurs when the skin is exposed to an allergen in a sensitized individual.[13] A previous study has demonstrated that massage oil containing essential oil from the fruit of *Z. rhetsa* significantly relieved calf muscle pain.[3] However, there have been no reports on the development of topical formulations using essential oil from the pericarp of *Z. rhetsa*, despite it being a richer source of essential oil and exhibiting greater potency in inhibiting PGE_2 production compared to the essential oil from the fruit.[2] Therefore, this study aimed to formulate a topical spray containing essential oil from the pericarp of *Z. rhetsa* and to evaluate its safety through irritation and allergy testing using a patch test on normal skin in volunteers. The findings from this Phase 1 clinical trial support further investigation in subsequent clinical studies.

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Materials and Methods

Our study involves 2 major processes: 1) the formulation of *Z. rhetsa* pericarp (ZRP) spray, and 2) safety evaluation of irritation and allergy testing for the ZRP spray on normal skin in volunteers during clinical trial phase 1

Formulation process of Zanthoxylum rhetsa pericarp spray
Plant material authentication

Z. rhetsa was collected between November and December 2020 in Chiang Rai Province, Thailand. The dry whole fruit was authenticated by a botanist from the Department of National Parks, Wildlife, and Plant Conservation by comparison with the voucher specimen (BKF 193835) at the Forest Herbarium, Bangkok, Thailand.

Plant material preparation and distillation

The ZRP was separated from the dry whole fruit and then sun-dried. The sun-dried pericarp was ground into a coarse powder. The coarse powder (24 kg) was subjected to water distillation using a Clevenger apparatus for 24 hours (h). The essential oil obtained from ZRP (ESO) is yellow and clear, with a percentage yield of 13.33% (v/w). The ESO was stored in an amber glass bottle in a refrigerator at 4°C.

Zanthoxylum rhetsa pericarp spray formulation

The ZRP spray formulation process began with the preparation of the base spray (BS) formula (Table 1) by dissolving the lipophilic components in 95% ethyl alcohol (EtOH) and the hydrophilic components in deionized water (DI water). The hydrophilic solution was then mixed into the lipophilic solution with a solubilizing agent and stirred until homogeneous. The BS formulations were evaluated based on specified criteria, and the best formula underwent a stability test involving a cooling-heating cycle (4°C for 48 h and 45°C for 48 h), repeated for 6 cycles.[14] The selected BS formula was then used to prepare the ZRP spray by adding ESO as the active ingredient at concentrations of 3% (v/v), 4% (v/v), 5% (v/v), and up to 9% (v/v).[3] These formulations also underwent the cooling-heating stability test and were evaluated before and after testing.

The evaluation criteria for the formulation

Both the BS formula and the ZRP spray formula were evaluated according to the specified 6 criteria:

1) Physical appearance: Measured by visual inspection by the principal investigator and co-investigator, including solution clarity, color, and homogeneity.[15,16]
2) Skin feel: Assessed by the sensory perception of the principal investigator and co-investigator when spraying onto the forearm with 10 sprays and gently rubbing it in. Skin feel was evaluated for dry, moist, and viscous.[17]
3) pH value: Measured using a pH meter (Mettler Toledo, New Zealand).
4) Viscosity: Measured using a Brookfield DV2T viscometer (Ametek Brookfield, USA) with Spindle No. 2, set at a rotation speed of 200 revolutions per minute (rpm) at room temperature. The viscosity value was recorded in milliPascal-second (mPa.s) after rotating for 2 minutes (mins).
5) Volume per spray: Measured by spraying into a beaker for 10 sprays, then calculating the average volume per spray in grams (g).[15]
6) Evaporation time: Measured by spraying onto filter paper (Filter Paper No.1 (Ø 125 mm)) (Whatman, USA) at room temperature. The total time (mins) taken for evaporation was recorded from the completion of spraying until the substance had fully evaporated.[15]

Safety evaluation of irritation and allergy testing for the Zanthoxylum rhetsa pericarp spray on normal skin in volunteers during clinical trial phase 1
Trial design

A non-randomized, double-blind, single-arm phase 1 clinical trial was conducted to evaluate skin irritation and allergic reactions to ZRP spray in volunteers with normal skin, using the patch test method.[10] DI water, which was used to fill the patch test chamber, served as the negative control. The study received approval from the Mae Fah Luang University Ethics Committee on Human Research (COA: 072/2021, EC 20216-25) before enrolling volunteers and was registered with the Thai Clinical Trials Registry (TCTR20240903002).

Table 1: Base spray formula

Ingredients	Percentage (% w/w)					Function
	F1	F2	F3	F4	F5	
Lipophilic substance						
Methyl salicylate	2	2	2	2	2	Enhancement agent
Borneol	2	2	2	2	2	Fragrance additive
Camphor	2	2	2	2	2	Fragrance additive
Dimethicone	2	2	2	2	2	Anti-foaming agent
Butylated hydroxytoluene	1	1	1	1	1	Antioxidant
Paraben concentration	1	1	1	1	1	Preservative
95% Ethyl alcohol	80	60	40	60	40	Solvent
Solubilizing agent						
PEG-40 hydrogenated castor oil	0	5	5	5	5	Surfactant
Hydrophilic substance						
Propylene glycol	8	8	8	10	10	Humectant
Glycerol	2	4	4	0	0	Humectant
Deionized water	0	13	33	15	35	Co-solvent

Sample size calculation

The sample size estimation was calculated using the formula: N1 = n / (1 - d),[18] where n is the predetermined sample size based on similar previous research,[19] which specified that 10 volunteers (n = 10) were considered sufficient for the study. The dropout rate (d) in our study was estimated to be 10%, and N1 represents the adjusted sample size. Therefore, the adjusted sample size (N1) in our study was 12 volunteers.

Selection of volunteers

The population in this study consisted of individuals from 3 sub-districts (Thasud, Nang Lae, and Mae Kao Tom) in Muang District, Chiang Rai Province, Thailand. They were invited via a recruitment notice, and the sample consisted of volunteers with normal skin, selected by the principal investigator and co-investigator according to the inclusion, exclusion, and dropout criteria.

Inclusion, exclusion, and dropout criteria

The volunteers were enrolled based on the following inclusion criteria: 1) both genders, aged 18 to 60 years, 2) willing to cooperate and adhere to the research protocol, 3) willing to attend appointments throughout the research period, and 4) willing to participate in the study and sign

the informed consent form. The exclusion criteria were: 1) having skin diseases or skin infections, including tattoos on the upper back, which was used as the test area, 2) a history of allergy, hypersensitivity, or irritation to essential oils, perfumes, herbs, drugs, cosmetic products, and adhesives such as patches, tape, plaster, micropore, or microfilm, 3) pregnancy, breastfeeding, or disabilities, and 4) taking antihistamines or corticosteroids within 2 weeks prior to the study. The dropout criteria included: 1) experiencing adverse events such as swelling of the face, lips, tongue, or throat, difficulty breathing or shortness of breath, rapid or weak pulse, chest tightness or pain, nausea, and vomiting, or 2) choosing to withdraw during the study period.

Study setting

Mae Fah Luang University Hospital, Thailand was the study setting, and the principal investigator contacted all volunteers, explained the research protocol according to the information sheet, and all volunteers willingly signed the informed consent form, with the option to withdraw during the study period.

Intervention

Irritation and allergy testing of the ZRP spray on normal skin in volunteers was conducted using the patch test method, following the standard protocol that was published.[10] The volunteers were not randomly assigned but received equal amounts of each test substance (20 µl per 1 square chamber),[20] as shown in the patch test chambers listed in Figure 1, which consist of 18 test samples, including DI water as the negative control. The volunteers, principal investigator, co-investigator, and medical doctor were blinded to the list of test substances. Clinical assistants were assigned to prepare the patch test chambers (AllergEAZE Clear®, SmartPractice, Canada) and apply the patches to the volunteers' skin. The upper backs of the volunteers were designated as the test area because this region provides sufficient space for testing various substances[10,20] and allows avoidance of the midline and scapula, which can cause patch dislocation.[20] The test area was cleaned with a normal saline solution (0.9% w/v sterile sodium chloride solution) (Klean & Kare®, A.N.B. Laboratories Co., Ltd., Thailand) before the patches were applied. Then, the volunteers underwent the intervention by having the patch test chambers applied to the skin on their upper backs. The patches were covered with a waterproof sheet (Tegaderm®, 3M, USA). The patches remained on the volunteers' skin for 48 h, and they were instructed to follow the given guidelines. Volunteers were scheduled for the first follow-up (1st follow-up) 48 h after the patch was applied. At this time, the patches were removed, and the skin was allowed to rest for 30 mins because erythema and edema occur immediately after removal. This resting period was necessary before the evaluation,[10] which was then conducted as the 1st evaluation. A second follow-up (2nd follow-up) was conducted 24 h after patch removal, followed by the 2nd evaluation. Irritation and allergy testing during the 1st and 2nd evaluations were diagnosed by a medical doctor, while adverse reactions were monitored by the principal investigator and co-investigator.

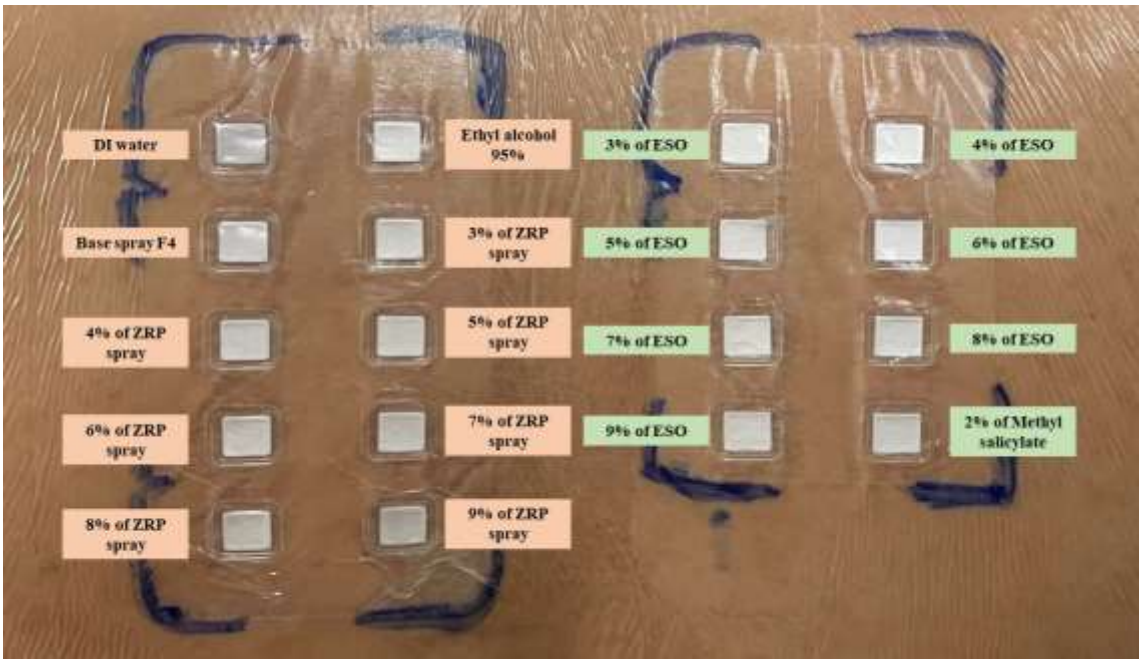

Figure 1: List of substance tests filled in the patch test chamber: DI water (deionized water) was used as a negative control; ZRP spray refers to *Zanthoxylum rhetsa* pericarp spray; ESO refers to the essential oil obtained from *Zanthoxylum rhetsa* pericarp.

Outcome evaluation

All volunteers were evaluated at 3 time points: 1) at baseline (visit 0), 2) at the 1st follow-up, and 3) at the 2nd follow-up. The demographic characteristics of the volunteers were recorded at baseline. The outcome evaluation included the assessment of irritation and allergic reactions based on skin reaction grading after the patch test, following the criteria of the International Contact Dermatitis Research Group (ICDRG). Reactions were classified as 1+ (weak positive: non-vesicular erythema, infiltration, and possible papules), 2+ (strong positive: vesicular erythema, infiltration, and papules), and 3+ (extreme positive: intense erythema, infiltration, and coalescing vesicles). Non-graded reactions included ?+ (doubtful: faint erythema only), IR (irritant reaction), and (–) negative reaction, according to the same criteria.[10] The grading criteria were defined according to ICDRG and were compared against representative clinical images (30 mins and 24 h after patch removal), as shown in the referenced publication.[21] These results were recorded at both the 1st and 2nd follow-ups, along with any adverse reactions.

Statistical analysis

The results of nominal data were presented as numbers (n) and percentages (%), while continuous data were presented as mean ± standard deviation (SD). Statistical analysis for within-group comparisons was conducted using a paired samples t-test, with a significance level set at p-value < 0.05. The skin reaction grading results after the patch test, according to the ICDRG, were presented as the frequency of responders. The grading of skin reactions was calculated as a score using the following formula: $[(\sum (\text{grade} \times n))/(4 \times \text{maximum grade} \times N)] \times 100 \times 0.50$, where n is the number of responders and N is the total number of volunteers.[22] The obtained score was interpreted according to the Human Primary Irritation Index for cosmetic products,

based on the patch test. The index classifies responses as slight (R = 0.00 to < 0.87), mild (R = 0.87 to < 2.42), moderate (R = 2.42 to < 3.44), and severe (R ≥ 3.44), where R refers to the score obtained from grading the skin reaction according to the ICDRG criteria.[22]

Results and Discussion

Zanthoxylum rhetsa pericarp spray formulation

Preliminary selection of the base spray formula prior to stability testing

A spray is a solution formulation, which is a type of liquid pharmaceutical dosage form composed of a solute and a solvent or co-solvent that enhances the solubility of poorly soluble pharmaceutical ingredients.[9,17] Solutions are categorized into aqueous, non-aqueous, or hydro-alcoholic solutions.[9] In our study, BS formulations (F1–F5) were prepared as hydro-alcoholic solutions because the pharmaceutical ingredients in BS consist of both lipophilic and hydrophilic substances (Table 1). Therefore, the use of EtOH as a solvent, along with DI water as a co-solvent, helps to enhance solubility, which is a key consideration in solution formulation.[9] BS was evaluated based on specific criteria, including physical appearance, skin feel, pH, viscosity, volume per spray, and evaporation time (Table 2), to select a preliminary formula before conducting stability testing. The BS formulations F1 to F5 exhibited clear, colorless, and homogeneous solutions (Figure 2), confirming the complete solubility of the pharmaceutical ingredients. Based on physical appearance, all spray solutions were clear, indicating that the ingredients were fully dissolved and the formulations were homogeneous.

The colorlessness matched that of the added pharmaceutical ingredients, similar to a previous study.[15,16] Therefore, the BS formulations F1 to F5 were deemed acceptable based on physical appearance criteria as spray solutions. After applying 10 sprays to the forearm and gently rubbing, F4 and F5 produced a moist sensation, while F2 and F3 felt both moist and viscous. These sensations were due to the humectant propylene glycol in F4 and F5, and both propylene glycol and glycerol in F2 and F3, along with the occlusive dimethicone in F2 to F5, which help retain moisture and prevent water loss from the skin, resulting in a moist sensory perception.[17] However, the viscosity of propylene glycol is 0.58 mPa.s at 20°C,[23] much lower than glycerol's viscosity of 954 mPa.s at 25°C,[24] possibly contributing to the more viscous sensation in F2 and F3 after the evaporation of solvents and co-solvents. Although F1 contains the same humectants (propylene glycol and glycerol) and occlusive dimethicone as F2 and F3, it produced a dry sensation due to its higher EtOH content (80% w/w) compared to 60% in F2 and 40% in F3.

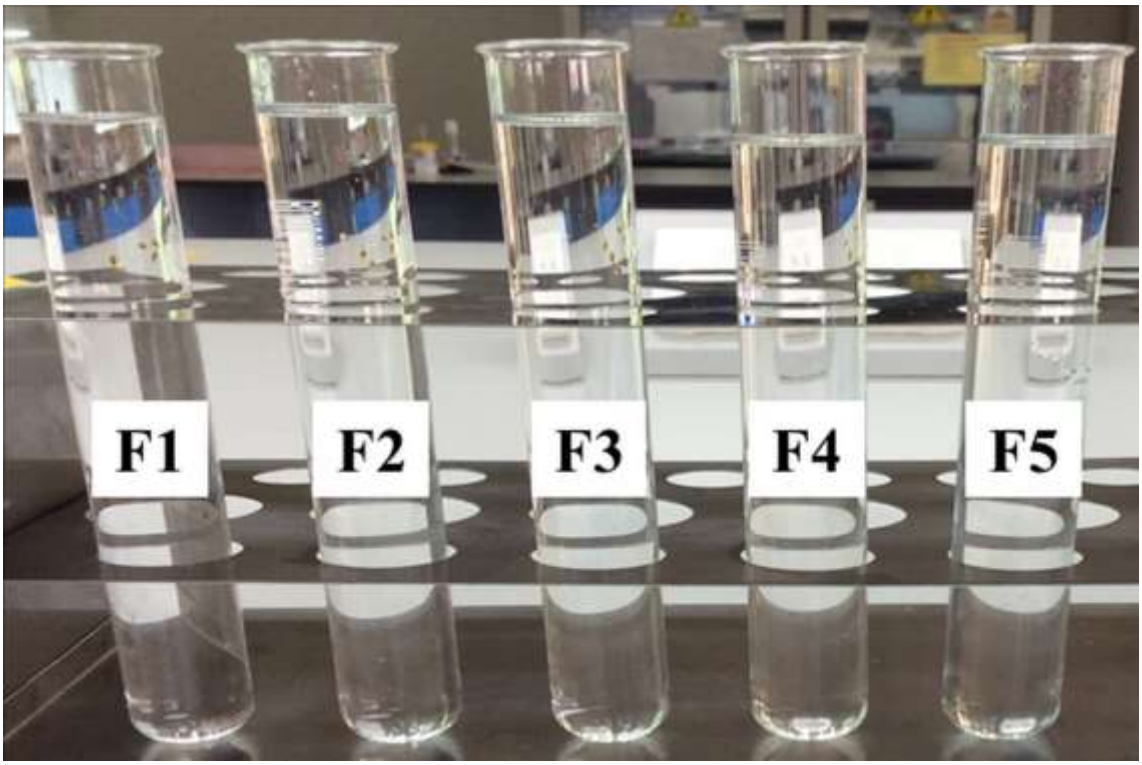

Figure 2: Physical appearance of the base spray formulations (F1–F5), all exhibiting clear, colorless, and homogeneous solutions prior to selecting the optimal formulation for cooling–heating stability testing.

The dry sensation from F1 may increase the risk of skin inflammation and itching, potentially leading to a pruritic effect.[17] Therefore, the BS formulations F4 and F5 were deemed acceptable based on skin feel criteria as spray solutions. The pH range of BS formulations F1 to F5 was 6.5–7.6, which is higher than the natural skin pH range of 4.93–5.12 when no products have been applied for 24 h.[25] However, it has been reported that pH influences the differences in the major chemical compounds in *Z. rhetsa*.[26] Additionally, a similar study found that strong alkaline conditions lead to a reduction in *in vitro* anti-inflammatory activity, while strong acidic conditions result in inactivity in *Z. rhetsa*.[27] Our study demonstrated that F1 to F5 have pH values close to the neutral range. Therefore, the near-neutral conditions of BS formulations F1 to F5 are suitable for ESO as the active ingredient in the formulation process of ZRP spray, helping to avoid the degradation of potency and the major chemical compounds of ESO due to strong acidic and alkaline conditions

The BS formulations F1 to F5 were deemed acceptable based on pH criteria for spray solutions. Viscosity measurements showed that BS formulation F1 exhibited the lowest viscosity at 19.13 ± 0.12 mPa.s. In contrast, BS formulations F2 and F4 had higher viscosities of 32.07 ± 0.31 mPa.s and 30.07 ± 0.23 mPa.s, respectively. BS formulations F3 and F5 exhibited the highest viscosities at 43.53 ± 0.31 mPa.s and 41.40 ± 0.00 mPa.s, respectively.

The viscosity of a spray solution indicates its sprayability,[28] which relates to its flowability on the skin.[16] Previous studies reported that lower viscosity in spray solutions facilitates spraying and spreading over the application area, while higher viscosity limits sprayability and prevents spreading, resulting in the solution remaining in place over time.[16]

Table 2: Characteristics of the base spray formula

Characteristic	F1	F2	F3	F4	F5
Physical appearance	Clear, colorless and homogeneous	Clear, colorless and homogeneous	Clear, colorless and homogeneous	Clear, colorless and homogeneous	Clear, colorless and homogeneous
Skin feel	Dry	Moist and viscous	Moist and viscous	Moist	Moist
pH	7.59 ± 0.07	7.57 ± 0.11	6.52 ± 0.08	7.64 ± 0.11	6.63 ± 0.03
Viscosity (mPa.s)	19.13 ± 0.12	32.07 ± 0.31	43.53 ± 0.31	30.07 ± 0.23	41.40 ± 0.00
Volume per spray (g)	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.00	0.08 ± 0.00
Evaporation time (mins)	2.16 ± 0.12	2.29 ± 0.13	7.44 ± 1.51	2.10 ± 0.54	6.15 ± 0.92

The results were presented as the mean ± standard deviation (SD) with three replications (n = 3)

The viscosity ranges of BS formulations F1 to F5 was 11 to 50 mPa.s, which is the acceptable range for sprayability, ensuring effective flow and coverage without excessive spreading.[16] Therefore, the viscosity ranges of BS formulations F1 to F5 was acceptable for spraying based on viscosity criteria. Volume per spray measurement is the determined average weight per dose for each spray, which is an important quantitative parameter in spray formulation.[15,28] BS formulations F1 to F5 demonstrated a volume per spray ranging between 0.07 g and 0.08 g, which represents the average weight per dose in the spray application. Evaporation time was measured to assess the rapid evaporation ability of the solvent and co-solvent system in the spray formulation.[15,28] BS formulations F1, F2, and F4 showed evaporation times between 2.10 and 2.29 mins, demonstrating faster evaporation compared to F3 (7.44 ± 1.51 mins) and F5 (6.15 ± 0.92 mins). Our results indicated that the slower evaporation times of F3 and F5 led to prolonged application times when gently rubbing the spray onto the skin, compared to F1, F2, and F4. However, the evaporation times of F1, F2, and F4 were slower than those reported in a previous study, which found evaporation times of less than 30 seconds for a spray formulation containing *Piper nigrum* L..[15] Nonetheless, the evaporation times of F1, F2, and F4 were acceptable for spray formulations in our study. In summary, our evaluation demonstrated that BS formulation F4 met specific criteria, including physical appearance, skin feel, pH, viscosity, volume per spray, and evaporation time (Table 2), and was selected for stability testing.

The stability characteristics of the base spray formulation F4 were selected for stability testing

The BS formulation, being a liquid solution, requires the solubility of the pharmaceutical ingredients as a key factor indicating physical stability.[29] The pH of BS also affects the chemical compound of the final product once the active ingredient is added.[30] Additionally, viscosity, evaporation time, and skin feel are crucial for the usability of the spray. Thus, ensuring optimal stability of the BS formulation is essential before moving to the final product development stage. Our BS formula F4 was selected in stability testing because it exhibited the best specific criteria, including physical appearance (clear, colorless, and homogeneous), skin feel (moist), pH (7.64 ± 0.11), viscosity (30.07 ± 0.23 mPa.s), volume per spray (0.07 ± 0.00 g), and evaporation time (2.10 ± 0.54 mins). After stability testing, BS formula F4 also demonstrated that its specific criteria (clear, colorless, and homogeneous for physical appearance; moist for skin feel; 7.46 ± 0.10 for pH; 0.07 ± 0.00 g for volume per spray; and 2.13 ± 1.04 mins for evaporation time) did not show significant differences compared to before testing, except for the viscosity (Table 3). After stability testing, BS formula F4 exhibited a viscosity decrease to 27.93 ± 0.12 mPa·s from 30.07 ± 0.23 mPa·s before testing, showing a significant difference with a p-value < 0.01. The viscosity decreased after stability testing due to the rise in temperature during the cooling (4°C for 48 h) and heating (45°C for 48 h) phases.

This can be explained by a model describing the behavior of a base fluid in relation to temperature and viscosity. The model shows that an increase in temperature leads to increased kinetic energy in particles and decreased intermolecular forces between particles and between particles and the base fluid, resulting in a decrease in viscosity in the base fluid.[31] Our result is similar to that of a previous study on the base cream formulation with longan seed extract, where the viscosity decreased from 5.56 mPa·s to 3.94 mPa·s after freeze (4°C for 48 h) and thaw (40°C for 48 h) stability testing.[32] Although the BS formula F4 exhibited instability in terms of viscosity, the viscosity range of BS formula F4, both before and after stability testing, remained between 11 and 50 mPa·s, which is within the acceptable range for sprayability properties.[16] In summary, our evaluation demonstrated that BS formulation F4 exhibited stability both before and after stability testing in terms of physical appearance, skin feel, pH, volume per spray, and evaporation time. Additionally, its viscosity remained within the acceptable range for sprayability properties throughout the testing period (Table 3).

Characteristics of the Zanthoxylum rhetsa pericarp spray formula at various concentrations after stability testing

After confirming the stability of BS formula F4, it was prepared as ZRP spray by adding ESO as the active ingredient at concentrations of 3% (v/v), 4% (v/v), 5% (v/v), and up to 9% (v/v). All ZRP spray formulations underwent cooling-heating stability tests. The cooling (4°C for 48 h) and heating (45°C for 48 h) conditions were chosen for the stability tests to reflect the real-life application of the spray in Thailand, a country with a tropical climate where the spray can be stored in a refrigerator or kept at ambient temperature. Therefore, these conditions were considered suitable for the stability testing of the spray. All concentrations of ZRP spray were evaluated based on specific criteria, including physical appearance, skin feel, pH, viscosity, volume per spray, and evaporation time (Table 4).

Our findings demonstrated that all concentrations of the ZRP spray exhibited a clear, colorless, and homogeneous appearance, with no differences observed before and after stability tests. This confirms the stability of the physical appearance and complete solubility of ESO as the active ingredient in the BS formula F4. According to the physical appearance criteria, the ingredients were fully dissolved, and the formulations were homogeneous.[15,16] After applying 10 sprays to the forearm and gently rubbing, the ZRP spray at all concentrations, both before and after the stability test, produced a moist sensation, without creating a viscous feel. The pH values of the ZRP spray at all concentrations decreased as the concentration level increased, due to the effect of the pH value in ESO.

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Table 3: Characteristics of the base spray formula during stability testing

Characteristic	Stability testing	F4
Physical appearance	Before	Clear, colorless and homogeneous
	After	Clear, colorless and homogeneous
Skin feel	Before	Moist
	After	Moist
pH	Before	7.64 ± 0.11
	After	7.46 ± 0.10
Viscosity (mPa.s)	Before	30.07 ± 0.23
	After	27.93 ± 0.12**
Volume per spray (g)	Before	0.07 ± 0.00
	After	0.07 ± 0.00
Evaporation time (mins)	Before	2.10 ± 0.54
	After	2.13 ± 1.04

The results were presented as the mean ± standard deviation (SD) with three replications (n = 3). A significantly different p-value was observed before and after testing within the group by using a paired-samples t-test; ** p-value < 0.01.

Table 4: Characteristics of *Zanthoxylum rhetsa* pericarp spray at various concentrations during stability testing

Concentration (% v/v)	Stability testing	Physical appearance	Skin feel	pH	Viscosity (mPa.s)	Volume per spray (g)	Evaporation time (mins)
3% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.97 ± 0.08	28.47 ± 0.23	0.08 ± 0.00	2.44 ± 0.05
	After	Clear, colorless and homogeneous	Moist	6.94 ± 0.05	30.80 ± 0.20*	0.08 ± 0.00	2.37 ± 0.95
4% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.93± 0.03	28.67 ± 0.12	0.08 ± 0.00	4.40 ± 0.10
	After	Clear, colorless and homogeneous	Moist	6.84 ± 0.04	31.10 ± 0.10***	0.08 ± 0.00	4.46 ± 1.02
5% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.79 ± 0.04	28.73 ± 0.12	0.09 ± 0.00	5.56 ± 0.48
	After	Clear, colorless and homogeneous	Moist	6.80 ± 0.01	31.07 ± 0.12**	0.08 ± 0.00	5.20 ± 0.17
6% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.75 ± 0.03	29.00 ± 0.20	0.09 ± 0.00	6.22 ± 0.28
	After	Clear, colorless and homogeneous	Moist	6.72 ± 0.02	31.60 ± 0.20**	0.08 ± 0.00	6.01 ± 0.44
7% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.70 ± 0.02	29.47 ± 0.12	0.09 ± 0.00	6.23 ± 0.30
	After	Clear, colorless and homogeneous	Moist	6.64 ± 0.04	31.57 ± 0.56**	0.08 ± 0.00	6.09 ± 0.99
8% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.68 ± 0.02	30.07 ± 0.23	0.08 ± 0.00	6.22 ± 0.75
	After	Clear, colorless and homogeneous	Moist	6.56 ± 0.04	31.67 ± 0.12*	0.08 ± 0.00	6.32 ± 0.80
9% of ZRP spray	Before	Clear, colorless and homogeneous	Moist	6.57 ± 0.02	30.43 ± 0.06	0.09 ± 0.00	7.96 ± 0.41
	After	Clear, colorless and homogeneous	Moist	6.54 ± 0.02	32.53 ± 0.81	0.09 ± 0.00	7.01 ± 0.65

The results were presented as the mean ± standard deviation (SD) with three replications (n = 3). ZRP spray refers to *Zanthoxylum rhetsa* pericarp spray. A significantly different p-value was observed before and after testing within the group by using a paired-samples t-test; * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

The pH values of the ZRP spray at all concentrations ranged from 6.97 ± 0.08 to 6.57 ± 0.02 before the stability test, and there was no significant difference after the stability test, with pH values ranging from 6.94 ± 0.05 to 6.54 ± 0.02. These findings confirm the stability of the pH value of the ZRP spray at all concentrations. However, the ZRP spray at a 9% (v/v) concentration demonstrated a pH value of 6.57 ± 0.02 before the stability test and 6.54 ± 0.02 after the stability test, which is closest to the pH of 6 found in topical skin products suitable for natural human skin.[33] Additionally, a review article reports that the products with a pH above 6.9 cause pH shifts of more than +0.5 units on skin surface, resulting in skin irritation by reducing epidermal barrier function.[34] In contrast, the pH of the 9% (v/v) ZRP spray, approximately 6.5, is expected to cause only a slight pH shift of less than 0.5 units on the skin surface, thereby reducing the risk of skin irritation. All concentrations of ZRP spray showed increased viscosity as concentration levels rose, likely due to higher ESO concentration. After stability testing, ZRP spray at 3% (v/v), 4% (v/v), 5% (v/v), and up to 8% (v/v) exhibited viscosities between 30.80 ± 0.20 mPa·s and 31.67 ±

0.12 mPa·s, significantly higher than the pre-test range of 28.47 ± 0.23 mPa·s to 30.07 ± 0.23 mPa·s. This increase may result from EtOH solvent evaporation due to elevated temperatures during the cooling (4°C for 48 h) and heating (45°C for 48 h) phases, which intensified viscosity across all concentrations post-stability testing compared to pre-stability testing. These results align with a model that describes the relationship between concentration and viscosity, where higher concentration strengthens particle interactions and intermolecular forces, preventing the typical elevated temperature-induced decrease in viscosity during stability testing.[31] This model is particularly consistent with the ZRP spray at 9% (v/v), where post-stability viscosity (32.53 ± 0.81 mPa·s) was not significantly different from pre-stability viscosity (30.43 ± 0.06 mPa·s), suggesting that the enhanced particle interactions at this concentration counteracted temperature effects, thereby maintaining viscosity.

However, the viscosity of all concentrations of ZRP spray at 3% (v/v), 4% (v/v), 5% (v/v), and up to 9% (v/v), both before and after stability testing, remained between 11 and 50 mPa·s, which is within the acceptable range for sprayability and ensures effective spreading over the application area without excessive residue.[16] Volume per spray measurement is the determined average weight per dose for each ZRP spray concentration.[15,28] All concentrations of ZRP spray, both before and after stability testing, demonstrated a spray volume ranging between 0.08 g and 0.09 g, with no significant differences observed. This indicates stability in the average weight per dose in spray application. Evaporation time was measured to assess the solvent and co-solvent evaporation ability in the spray formulation.[15,28] Our results indicated that the evaporation times of the ZRP spray remained stable across all concentrations, with no significant differences before and after stability testing. Additionally, slower evaporation times were observed as the concentration of the ZRP spray increased. Although the slower evaporation time of the ZRP spray at a 9% (v/v) concentration led to prolonged application times when gently rubbing the spray onto the skin compared to the 3% (v/v) concentration, this prolonged application provided a beneficial effect. Specifically, it contributed to the dilation of superficial microcirculatory blood flow due to the heat sensation induced by the 9% (v/v) concentration of ESO in the ZRP spray. The vasodilation of superficial microcirculation in the local skin region may help reduce pain. A previous study supports this benefit, showing that topical menthol application can relieve pain by increasing skin blood flow in a dose-dependent manner over the treated area.[35]

In summary, our evaluation demonstrated that all concentrations of the ZRP spray, 3% (v/v), 4% (v/v), 5% (v/v), and up to 9% (v/v), exhibited stability both before and after stability testing in terms of physical appearance, skin feel, pH, volume per spray, and evaporation time. However, only the 9% (v/v) ZRP spray maintained stable viscosity throughout the testing period. Nonetheless, the viscosity of all concentrations remained within the acceptable range for sprayability properties (Table 4).

Safety evaluation of irritation and allergy testing for the Zanthoxylum rhetsa pericarp spray on normal skin in volunteers during clinical trial phase 1

The volunteers were enrolled in September 2021. Thirteen volunteers were screened for eligibility, and one was excluded due to having a full-back tattoo. The remaining 12 volunteers were not randomized but received controlled amounts of each test substance (20 µl per 1-square chamber). They were assigned to receive 18 test samples, including DI water as the negative control (Figure 1). The remaining 12 volunteers received the allocated intervention via a closed skin patch test for 48 h. Demographic characteristics were assessed at baseline (Visit 0). At the 1st follow-up (48 h after the closed patch test and 30 mins after removing the patch, followed by the 1st evaluation), 2 volunteers were lost to follow-up as they withdrew from the intervention due to discomfort at the test site after 1 day of the closed patch test. At the 2nd follow-up (24 h after patch removal, followed by the 2nd evaluation), no volunteers were lost to follow-up. Therefore, the remaining 10 volunteers were assessed for outcomes related to skin irritation and allergic reactions at both follow-up visits (Figure 3).[36]

Baseline demographic characteristics of volunteers prior to the intervention for irritation and allergy testing

The baseline demographic characteristics of volunteers prior to irritation and allergy testing of the ZRP spray on normal skin are presented in Table 5. Most volunteers were female (66.67%), while the remaining 33.33% were male. The mean age of the volunteers was 34.00 ± 12.82 years, ranging from 21 to 54 years. Gender and age distribution in our study were similar to a previous study in China that used healthy volunteers for irritation and allergy testing of a cosmetic product via the patch test method.[37] Most volunteers in our study were classified body mass index (BMI) in normal (41.67%), followed by obese (33.33%), and the means of BMI in volunteers was 23.89 ± 5.39 kg/m^2. The assessment of volunteers' vital signs included body temperature, pulse rate, respiratory rate, and blood pressure. The mean body temperature was 36.76 ± 0.27 °C, pulse rate was 80.33 ± 11.06 beats per minute, respiratory rate was 17.83 ± 1.53 breaths per minute, and blood pressure was 127.58 ± 15.89 mmHg (systolic) and 78.83 ± 10.73 mmHg (diastolic). The volunteers' vital signs were within the normal range.[38] Vital signs are essential for assessing physiological function and serve as the 1st step in clinical evaluation to ensure normal health status.[38] Therefore, the normal vital signs in our study confirmed that volunteers were in good health before undergoing irritation and allergy testing of the ZRP spray in this phase 1 clinical trial.

Irritation and allergy testing for the Zanthoxylum rhetsa pericarp spray on normal skin in volunteers

According to well-known, the patch test method is a well-established scientific technique and is widely recognized as the gold standard protocol for identifying the etiologic agents of ICD and ACD.[10] Irritant and allergen agents are tested in clinical experiments using patch testing and applied to the test area for 48 h.[10] After that, the patch is removed, and the results are read 30 mins to 24 or 48 h after removal.[10] ICD and ACD reactions are evaluated, with a grade of + or higher considered a positive reaction, based on the ICDRG criteria.[10] ICD may appear immediately or upon patch removal at 48 h.[39] ACD appears 24 h later, while ICD returns to a negative response at this time.[10] Our results on irritation and allergy testing of the ZRP spray on normal skin in 10 volunteers are presented in Table 6.

After 48 h of exposure to 18 test substances, the 1st evaluation was conducted 30 mins after patch removal. Five volunteers exhibited erythema across the entire back area covered by the patch, including the waterproof sheet, which was classified as an angry back reaction. Angry back refers to an excited skin syndrome characterized by false positive reactions due to nonspecific hyperreactivity of the skin to multiple substances, commonly occurring at patch test sites.[40] Therefore, the irritation observed in these 5 volunteers could not be included in the calculation of the irritation response score according to the human primary irritation index for cosmetic products by patch testing,[22] due to nonspecific skin hyperreactivity. Their skin reacted to multiple substances, including DI water (used as the negative control), the patch material itself, and the waterproof sheet. This phenomenon, known as the angry back response, is consistent with a previous report in which volunteers developed widespread rashes or showed reactions to both white petrolatum (used as the negative control) and the test samples. In one such study, this response was observed in 9 volunteers.[41] Substance test No. 1 (DI water, used as a negative control and co-solvent in BS formula F4), No. 2 (EtOH as the solvent in BS formula F4), No. 3 (BS formula F4), and No. 4 to No. 10 (ZRP spray at concentrations of 3% to 9% v/v) showed negative reactions in 5 volunteers. These results indicate that all ingredients in the ZRP spray, at all tested concentrations (3%–9% v/v), did not induce ICD. Therefore, the ZRP spray and its components were not considered irritant agents on normal skin in these volunteers. Similarly, substance tests No. 11 to No. 15, which contained ESO at concentrations of 3% to 7% v/v, showed negative reactions in 5 volunteers. In contrast, tests No. 16 and No. 17 (ESO at 8% and 9% v/v) and test No. 18 (methyl salicylate at 2% v/v) caused weak positive reactions in 1 volunteer, while the other 4 showed no reaction. These results suggest that ESO at 8% and 9% v/v and methyl salicylate at 2% v/v may cause irritation in some individuals.

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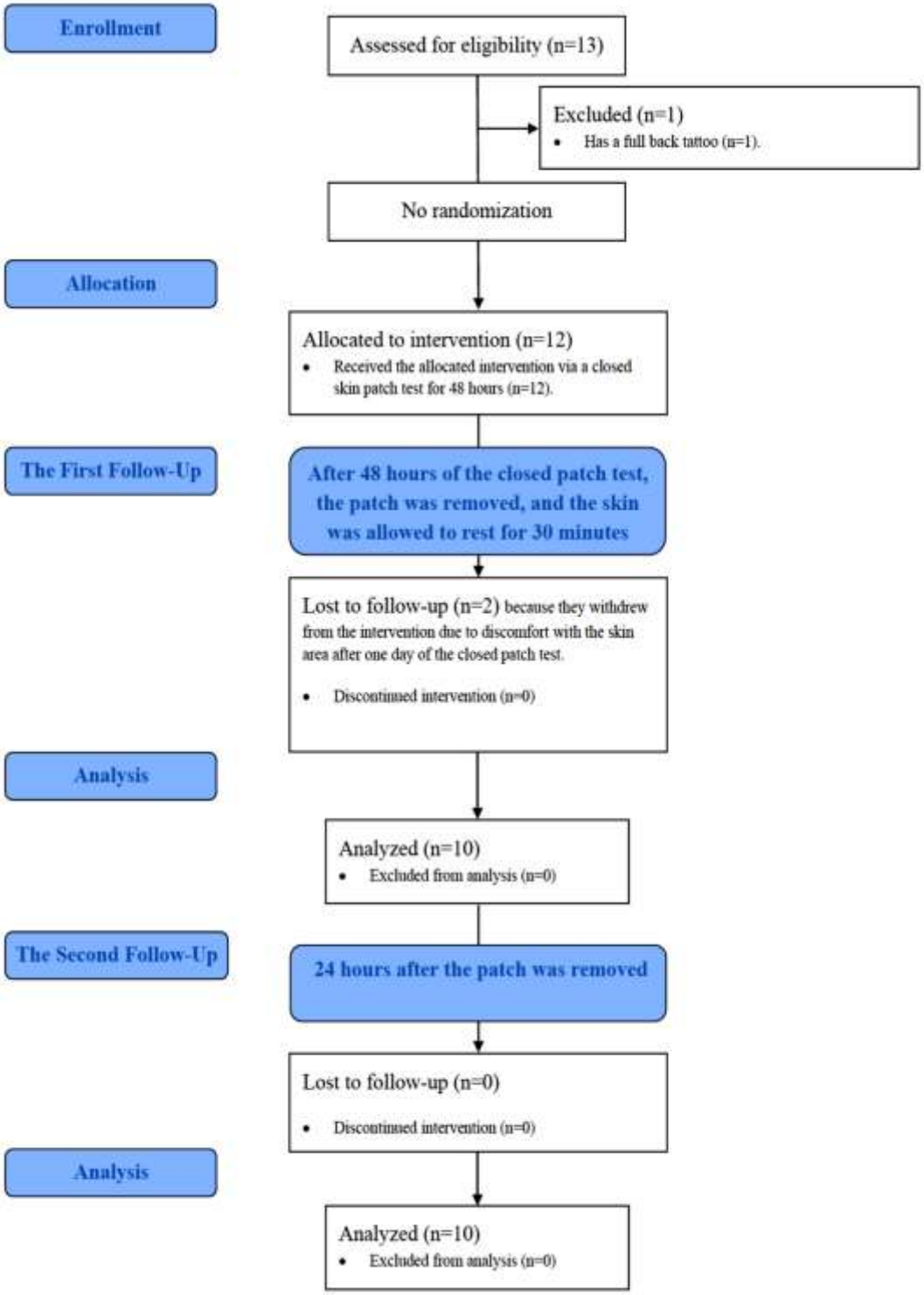

Figure 3: The CONSORT flow diagram of participant enrollment, allocation, and follow-up in the phase 1, double-blind, non-randomized, single-arm patch test trial, modified from the CONSORT 2025 guidelines (originally designed for two-arm randomized trials).[36]

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Table 5: Baseline demographic characteristics of volunteers (n = 12) prior to irritation and allergy testing

General characteristic	Results
Gender	
Male, n (%)	4 (33.33%)
Female, n (%)	8 (66.67%)
Age (Year), mean ± SD	34.00 ± 12.82
Maximum = 54 / Minimum = 21	
Body mass index (kg/m^2)	
Underweight (< 18.50 kg/m^2), n (%)	1 (8.33%)
Normal (18.50 – 22.99 kg/m^2), n (%)	5 (41.67%)
Overweight (23.00 – 24.99 kg/m^2), n (%)	2 (16.67%)
Obese (≥ 25.00 kg/m^2), n (%)	4 (33.33%)
Mean ± SD	23.89 ± 5.39
Vital signs	
Body temperature (°C), mean ± SD	36.76 ± 0.27
Pulse rate (beats per minute), mean ± SD	80.33 ± 11.06
Respiratory rate (breaths per minute), mean ± SD	17.83 ± 1.53
Systolic blood pressure (mmHg), mean ± SD	127.58 ± 15.89
Diastolic blood pressure (mmHg), mean ± SD	78.83 ± 10.73

The grading of skin reactions was calculated as a score of 0.42 which falls within the slight irritation category according to the human primary irritation index for cosmetic products by patch testing.[22] Therefore, ESO at 8% and 9% v/v and methyl salicylate at 2% v/v were classified as slight irritants. However, methyl salicylate is extensively used as a counterirritant in topical analgesics and may cause slight irritation,[42] which is consistent with our findings. Our findings also suggest that when formulating topical products containing ESO or methyl salicylate, the inclusion of a humectant or the use of DI water as a co-solvent may help reduce the potential for skin irritation. The 2nd evaluation was conducted 24 h after patch removal. One volunteer also exhibited erythema across the entire back area covered by the patch, including the waterproof sheet, which was classified as an angry back reaction. However, the skin of this volunteer returned to normal within 48 h following the application of a 0.1% w/w topical steroid. The remaining 4 volunteers showed no reaction and had returned to a negative reaction, with all 5 volunteers also showing negative reactions to the tested substances: No. 1 (DI water, used as a negative control and co-solvent in BS formula F4), No. 2 (EtOH as the solvent in BS formula F4), No. 3 (BS formula F4), and No. 4 to No. 10 (ZRP spray at concentrations of 3% to 9% v/v). These findings indicate that the ZRP spray and its components, at all tested concentrations, did not induce ACD and are not considered allergens on normal skin in the 9 volunteers. This is consistent with the understanding that ACD is a type IV delayed hypersensitivity response, which typically occurs 24 h after patch removal, whereas ICD returns to a negative response at this time due to the removal of the irritant agents.[10] Similarly, our findings showed negative reactions in all 9 volunteers for substance tests No. 11 to No. 17, which contained ESO at concentrations of 3% to 9% v/v, and test No. 18 (methyl salicylate at 2% v/v). Notably, 1 volunteer initially showed weak positive reactions to tests No. 16 and No. 17 (ESO at 8% and 9% v/v) and test No. 18 (methyl salicylate at 2% v/v) during the 1st evaluation (30 mins after patch removal), but these reactions had resolved and returned to negative by the 2nd evaluation (24 h after patch removal). Therefore, these findings indicate that ESO at all tested concentrations and methyl salicylate at 2% v/v did not induce ACD and are not considered allergens on normal skin in the 9 volunteers. This is consistent with the mechanism of ACD responses and also suggests that tests No. 16 and No. 17 (ESO at 8% and 9% v/v) and test No. 18 (methyl salicylate at 2% v/v) may cause slight irritation in some individuals.

In summary, the ZRP spray at all tested concentrations (3% to 9% v/v) was safe for use on normal skin in volunteers, as it caused no adverse effects, including irritation or allergic reactions. However, our findings raise concerns about the use of ESO alone at concentrations of 8% or higher and methyl salicylate at 2% or higher, which may potentially cause slight irritation. The addition of a humectant or the use of DI water as a co-solvent in topical formulations may help reduce this risk. Nonetheless, this study has some limitations, including a small sample size for irritation and allergy testing, and the need for further evaluation of the active compound stability in the ZRP spray in a Phase 2 clinical trial.

However, our results represent the first findings identifying a safe concentration of ESO that does not cause skin irritation, as well as a safe concentration of ZRP spray suitable for supporting a Phase 2 clinical trial.

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Table 6: Skin irritation and allergy testing of ingredients in *Zanthoxylum rhetsa* pericarp spray using patch tests (n = 10)

No.	Substance test	After 48 hours of the closed patch test and 30 minutes after removing the patch							Score	24 hours after removing the patch						
		ICDRG grade								ICDRG grade						
		-	1+	2+	3+	?+	IR	Angry back		-	1+	2+	3+	?+	IR	Angry back
1	DI water	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
2	EtOH	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
3	BS F4	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
4	3% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
5	4% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
6	5% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
7	6% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
8	7% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
9	8% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
10	9% of ZRP spray	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
11	3% of ESO	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
12	4% of ESO	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
13	5% of ESO	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
14	6% of ESO	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
15	7% of ESO	5	0	0	0	0	0	5	0	9	0	0	0	0	0	1
16	8% of ESO	4	1	0	0	0	0	5	0.42	9	0	0	0	0	0	1
17	9% of ESO	4	1	0	0	0	0	5	0.42	9	0	0	0	0	0	1
18	2% of Methyl	4	1	0	0	0	0	5	0.42	9	0	0	0	0	0	1

The results were presented as the number of volunteers (n). (-): the International Contact Dermatitis Research Group (ICDRG) grade indicates a negative reaction; 1+, 2+, 3+: Weak, strong, or extreme positive reaction; IR: Irritant reaction; (?+): Doubtful reaction. DI water refers to deionized water was used as a negative control; EtOH refers to 95% ethyl alcohol was used as a solvent; BS F4 refers to base spray formula F4; ZRP spray refers to *Zanthoxylum rhetsa* pericarp spray; ESO refers to the essential oil obtained from *Zanthoxylum rhetsa* pericarp; Methyl refers to methyl salicylate.

Conclusion

Formulation and topical safety evaluation of ZRP spray containing essential oil from the pericarp: A phase 1, double-blind, non-randomized, single-arm patch test trial demonstrated that the ZRP spray was both physically stable and topically safe at all tested concentrations (3–9% v/v). Among these, the 9% (v/v) formulation showed the most favorable profile, with superior viscosity retention, slower evaporation time, and prolonged skin contact that may enhance microcirculatory blood flow and support pain-relieving effects. In the clinical phase, patch testing on normal skin revealed that ZRP spray at all tested concentrations (3–9% v/v) caused no signs of irritation or allergic reaction in volunteers, confirming the formulation's safety and highlighting the 9% (v/v) ZRP spray as warranting further clinical investigation in a Phase 2 trial to evaluate its therapeutic potential, particularly in pain management applications.

Conflict of interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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