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In vitro anti-inflammatory study, formulation development, characterization of novel medicated ointment containing standardized extract of *Uncaria gambier* and standardized extract of *Valeriana wallichii*

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Abstract
After exploring numerous pharmaceutical databases such as PubMed, Google Scholar, Scopus, etc., it was observed that no combined anti-inflammatory perspectives of *U. gambier* standardized extract (UGSE) with *V. wallichii* standardized extract (VWSE) in an *in vitro* method (human red blood cells method) have been reported yet. Therefore, it was planned to screen both the extracts together to explore the synergistic activity. Also, the goal of the current study was to investigate potential anti-inflammatory applications for ointment formulations containing both UGSE and VWSE. The pharmaceutical properties of the formulations, such as pH, the skin irritancy test, viscosity, appearance, extrudability, spreadability, washability, and swelling index, were evaluated. The number of therapy choices for therapy has favorably expanded as a result of this study. Future uses for the created polyherbal formulations, which also contain extracts from *Uncaria gambier* and *Valeriana wallichii*, are conceivable. This finding brought back the ideas of ethnopharmacology in connection to modern medicine and explained the uses of polyherbal formulations in conventional medicine.

Keywords: ointment, formulation, *Uncaria gambier*, *Valeriana wallichii*, extract, characterization

Introduction

To protect humans from dangerous species like bacteria, white blood cells and a variety of crucial chemical mediators are generated during an inflammation reaction. While the biological mechanism is necessary for life, when it is aggravated, it causes excruciating pain and misery. Inflammatory diseases, like inflammation, make patients feel far worse than they should and assault their own tissue. Anti-inflammatory medications are therefore seldom used to ease pain and subsequent responses [1]. Many biochemical processes in the inflammatory process are catalyzed by enzymes like cyclooxygenase-1/2 (COX-1/2), lipoxygenase (LOX), prostaglandin synthetase (PGS), prostaglandin dehydrogenase (PGDH), and others. This results in the production of leukotrienes, which are crucial in acute inflammation. Numerous anti-inflammatory drugs, both steroidal and non-steroidal, have attracted attention for pharmacotherapeutics but have not been widely used because of issues like impaired pharmacokinetics, unintended side-effects, and so on. Regular efforts are made to address these anti-inflammatory-related issues through a variety of pragmatic methods in an effort to produce an optimal outcome [2].

A climbing shrub that is indigenous to Southeast Asia, mainly Malaysia and Indonesia, is called *Uncaria gambir* (W. Hunter) Roxb. Because of its astringent qualities, *U. gambir* has historically been used by males in Malaysia, Indonesia, and Singapore to prolong sexual encounters, prevent early ejaculation, and lessen toothache owing to the numbing effect of a polymer tanning combination. Its catechin component, which comes from the leaves, has antioxidant properties. Gambir leaves and young shoots can help treat deafness, obesity, atherosclerosis, spongy gums, dysentery, diarrhea, and other conditions. Additionally, chewing gambir combining calcium hydroxide and dried areca nuts may maintain dental hygiene and fortify teeth and gums. This plant is employed as a dark dye and tanning ingredient, was one of numerous classic export commodities in the nineteenth century [3].

Small perennial herb *Valeriana wallichii* grows to a height of 14–45 cm. The herb valerian is a popular herbal remedy. A member of the Valerianaceae family, which includes over 250 species of Valeriana found worldwide. Herbs are typically found to thrive in the Himalayas' mountainous (1,300-3,300 m) landscape. The name Valeriana (Valerianaceae), which is a source of significant phytomedicines, derives from the Latin word "Valere," which means to be in excellent health. Since ancient times, valerian roots have been utilized for their calming and antispasmodic effects. The herb is well recognized for treating hysteria, sleeplessness, epilepsy, and anxiety. It is thought to be helpful as a strong muscle relaxant, antispasmodic, stimulant, and hypotensive, as well as to enhance liver function in digestive diseases. Valerian roots have also been used historically to treat mild kinds of fever. The reputation of *Valeriana* species is mostly for treating nervous stress in contemporary rational phytotherapy. The herb has been used as a relaxant, a carminative, to enhance complexion, to treat epilepsy, insanity, snake poisoning, eye problems, and emotional arrest (as a tranquilizer/sedative). For individuals with mild to severe insomnia, valerian continues to be a safe sedative/hypnotic option. It also has a depressive impact on the central nervous system. A moderate sedative may also be made from the plant [4].

After exploring numerous pharmaceutical databases such as PubMed, Google Scholar, Scopus, etc., it was observed that

no combined anti-inflammatory perspectives of *U. gambier* standardized extract (UGSE) with *V. wallichii* standardized extract (VWSE) in an *in vitro* method (human red blood cells method) have been reported yet. Therefore, it was planned to screen both the extracts together to explore the synergistic activity. Also, the goal of the current study was to investigate potential anti-inflammatory applications for ointment formulations containing both UGSE and VWSE. The pharmaceutical properties of the formulations, such as pH, the skin irritancy test, viscosity, appearance, extrudability, spreadability, washability, and swelling index, were evaluated.

Materials and Methods

Chemicals

Analytical-grade chemicals were bought from SD Fine Chem Ltd., India, including white wax, white petroleum, and other compounds. From HiMedia Chemicals Ltd., India, PEG 300, cetostearyl alcohol, and chloroform were purchased. The suppliers of propyl paraben and methyl paraben were Sigma Aldrich Ltd., Germany. From Dabur India Ltd., India, honey (99.98% pure) was procured. The double distillation process was carried out using Borosil® distilled water equipment.

Instrumentation

For measurements, A double-beam Shimadzu® Ultraviolet-Visible Spectrophotometer (Model UV-1800, Japan) attached to a device with a spectral bandwidth of 1 nm and wavelength precision of 0.3 nm and a pair of 10 mm path duration aligned quartz cells was used for spectroscopic research. Shimadzu® electronic balance was utilized (Model AUW220D, Japan). VSI® (model VSI-1B) used a digital pH meter to measure the pH. To measure the viscosity, a Brookfield Digital DV-II+ (USA) viscometer was utilized (using spindle 6). Accelerated stability study was performed in a stability chamber (Bio-Technics, India).

Plant Extracts

U. gambier standardized extract (60%-90% Catechin) and *V. wallichii* standardized extract (0.8% Valerenic acid) were purchased from S.A. Herbal Bioactives Ltd., Mumbai, India.

In-vitro anti-inflammatory activity

An *in vitro* approach was employed to investigate the anti-inflammatory effects of UGSE and VWSE. This procedure was predicated on the idea that the release of lysosomal enzymes during inflammation causes some disarray. The most frequent disease among them is acute inflammation because of their extracellular action. By preventing these chemical mediators from acting or by maintaining the stability of the lysosomal membrane, experimental substances' potential can be estimated. The prevention of hypotonicity-induced membrane lysis in human red blood cells (HRBC) was employed to evaluate anti-inflammatory characteristics since the membranes of human red blood cells and lysosomal membrane components are similar. For this operation, blood was obtained from a stable person who hadn't taken any inflammatory drugs in the preceding 15 days. Centrifuged at 3000 rpm after being diluted in a quantity that is similar to Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% sodium chloride). Carefully separating and storing plasma. Before being suspended in a 10% solution, the sealed blood corpuscles were cleaned with a 0.9% saline solution. Aliquots of plant extract were made with distilled water for concentrations of 250 µg/mL. For each concentration, 1 mL of phosphate buffer, 2 mL of hyposaline, and 0.5 mL of HRBC solution were added. After being incubated at 37°C±1°C for 30 minutes, the mixture was centrifuged at 3000 rpm for 20 minutes. Using spectrophotometry at 560 nm, the hemoglobin content of the supernatant solution was calculated using diclofenac sodium as a reference standard. In addition to the extract, the control was also ready. The formula was used to calculate the percentage of HRBC membrane stabilization by plant extract, assuming that there was 100% hemolysis in the test group [5]:

$$\% \text{ Protection} = 100 - \frac{\text{OD of Drug treated sample}}{\text{OD of Control}} \times 100$$

Formulation development

Preparation of ointment base

White wax was melted in a hot pan between 70-75°C to create the ointment basis. White petroleum was added when the wax had completely melted, and the mixture was then left on the hot plate until it liquefied. After being heated and allowed to liquefy, the liquid was then taken off the heat and allowed to solidify. The liquid was agitated until it started to solidify (Table 1) [6].

Table 1: Composition of ointment base.

Ingredients	Quantity (in gram)
White wax	1.5
White petroleum	1.5
Cetosteary alcohol	1.5
PEG 300	2.5
Methyl paraben	0.025
Propyl paraben	0.025

Preparation of polyherbal formulation

After being semi-dried, the extracts were utilized to produce ointment. Based on the ointment, the polyherbal formulations (F1 and F2) were created. The usual trituration technique was applied, involving melting and combining of solid fats. The needed quantity of the ointment base was then properly blended with the 40°C melted base. The mixture was gently and consistently agitated until a uniform dispersion was achieved (Table 2) [7].

Table 2: Composition of polyherbal ointments.

Ingredients	Formulation-1 (F1) Quantity (g)	Formulation-2 (F2) Quantity (g)
Uncaria gambier	3	1.5
Valeriana wallichii	1.5	3
Honey	0.5	0.5
Ointment base	5	5

Evaluation of polyherbal formulations

The formulations were characterized as per the protocols given by Yadav *et al*., 2022 [8].

Physical Evaluation

The produced compositions' color, overall look, and application-specific feel were observed, and the outcomes are examined.

pH

A digital pH meter that had been calibrated and again calibrated using buffered solutions at pH-4 and pH-7 before each use was used to determine the pH of the ointment formulations (F1 and F2). The reference electrode and glass electrode were completely dipped in the ointment to measure the pH levels of the formulations.

Spreadability

Using a unique device made of a flat wooden block held by a pulley at one end, the spreadability of the formulas was assessed. Release of 2 g of the polyherbal product onto a ground slide allowed for the evaluation of the formulations' drag and slip resistance. The formulation was sandwiched between two identical-sized slides, and the device was supported by a hook. A unit kilogram weight was placed over the slide in order to release the trapped air in the equations and create a consistent film between the two slides. The borders' protruding extra formulation was taken care of. The length of time required for the top slide to move 7.5 cm after being attached to 50 g of weight to create a pulling force was calculated using the hook. The spreadability of the formulation was calculated using the following formula:

$$\text{Spreadibility} = \frac{M \times L}{T}$$

Where, M = weight tied to the upper slide (50 g); L = length of glass slide (6 cm); T = time taken (sec) to separate the glide slides from each other.

Washability

By putting the ointments to the skin and personally seeing how quickly the polyherbal compositions could be cleansed with distilled water, the washability of the products was evaluated.

Skin irritancy test

0.5 g of the formulation was placed over a 6 cm2 area of skin, followed by a piece of gauze that was loosely held in place by a dressing (semi-occlusive) for an hour. The residual content was eliminated after removing the gauze for an hour without changing the other conditions. A detailed investigation of sensitivity traits and extra rash or reaction symptoms was conducted. After the program was followed for seven days straight, evaluations were completed.

Viscosity

To determine the apparent viscosity values of the ointment compositions, the manufacturer's suggested operating technique was performed using a Brookfield viscometer with spindle number 6 at 50 rpm at room temperature.

Extrudability

The polyherbal ointment formulations were placed in a collapsible aluminum tube, which was then sealed with a regular plastic cap and crimped shut with ointment sealing equipment. Between the two slides, the tubes were inserted and then fastened. The top came off instantly when a weight of 500 g was placed over the slides. In 10 seconds, the mixture was extruded into a ribbon-like structure. The extruded ribbon's length was gauged.

Swelling index

Because the ointment comprises hydrophilic components, the swelling index of the product was ascertained by dissolving 2 grams of the substance in distilled water (10 mL). After the mixture had been in the beaker for an hour, it was placed in a Petri plate. After the content was weighed, the swelling was estimated using the following formula:

$$\text{Swelling index} = \frac{Wt - Wo}{Wo} \times 100$$

Where, Wt = weight of swollen after 1 hr; Wo = original weight of ointment at zero hr.

Accelerated Stability Studies

The stability of F1 and F2 were studied for 90 days in accelerated humidity and temperature conditions (40°C±2°C / 75%±5% RH). The formulas were put in a PVC container that was foil-wrapped. After being removed from the stability chamber for 90 days, the formulations received additional testing for medical features such appearance, pH, spreadability, viscosity, washability, and extrudability.

Statistical analysis

Three times the experiment was run. The findings were displayed using the mean and standard deviation (SD). The computations were performed using Minitab® version 17 software. The unpaired Student t-test (two-tailed) was used to analyze the differences between the monitoring and study groups in relation to pharmaceutical outcomes.

Results and Discussion

***In vitro* anti-inflammatory activity**

In vitro anti-inflammatory activity showed that all extracts, both individually and collectively, had significant anti-inflammatory efficacy compared to the reference medication diclofenac sodium (76.99%). UGSE and VWSE each contributed 63.5% and 57.27% of the membrane stabilization/protection in HRBCs, respectively, at a concentration of 250 µg/mL. The anti-inflammatory impact was raised to 70.25% when the extracts UGSE and VWSE were evaluated combined (at a concentration of 250 µg/mL each) (Table 3). When both extracts were administered at once, the anti-inflammatory effect was observed to be increased by 20%. As measures of anti-inflammatory activity, hypotonicity suppression and heat-induced red blood cell membrane lysis were utilized. The presence of alkaloid, phenolic, and flavonoid components in them, all of which have distinct biological effects, may explain these observations.

Table 3: *In vitro* anti-inflammatory potential of *Uncaria gambier* standardized extract and *Valeriana wallichii* standardized extract combination.

Treatment	Concentration (µg/mL)	Absorbance (560 nm)	% Protection[#]
Control	-	0.578 ± 0.003	-
Uncaria gambier standardized extract	250	0.211 ± 0.002***[a]	63.5
Valeriana wallichii standardized extract	250	0.247 ± 0.003***[a]	57.27
Uncaria gambier seeds standardized extract + *Valeriana wallichii* roots standardized extract	250 + 250	0.172 ± 0.004***[a]	70.25
Diclofenac sodium	100	0.133 ± 0.003***[a]	76.99

All values represent mean ± SD of n = 3;***p<0.001 with respect to the control group. [a]Determined as compared with the control group (solution of 0.9% sodium chloride) using the above formula. [#]% protection offered by the extract or standard refers to the prevention of hypotonicity-induced HRBC membrane lysis.

Organoleptic properties

Both ointments' compositions are rather superb, beautifully colored, exceedingly soft to the touch, grit-free, and non-irritating; neither has any such flaws. Formulation-1 has a yellow-brown hue with a strong herbal scent, as opposed to Formulation-2, which is yellow. Formulation 2 appeared to be more appetizing than Formulation 1.

Skin irritation test

No specific edema or erythema indications were observed following therapy for a continuous seven days, according to the analysis of the skin irritation test. Observation showed that the Formulation-1 was significantly less irritating than the Formulation-2. On the other hand, polyherbal remedies showed enhanced human use compatibility with no local irritation. When use compatibility is considered, several innovative synthetic excipients included in synthetic cosmetics today irritate the skin in sensitive populations.

Viscosity

The formulations' viscosities were discovered to be 4600 cps (Formulation-1) and 5500 cps when the rheological properties of the formulations were examined (Formulation-2). It has been assumed that when torque increases, shear stress also rises, which results in a reduction in viscosity. The Brookfield viscometer spindle was impeded by the honey addition, which caused Formulation-2 to become viscous. Since it is well known that viscosity decreases as emulsifier concentration rises, the lack of a high emulsifier concentration might also be to fault. The chemical was also concentrated to stay on the skin's surface for a longer period of time and to continue acting because of its high viscosity.

pH

The formulations' pH values were discovered to be 6.8 (Formulation-1) and 6.9 (Formulation-2). This shows suitability for dermal application since the pH of the combination roughly resembles the pH of the skin (6.0-7.0).

Swelling index

For both formulations, the swelling indices were hardly detectable. The swelling for Formulation-1 was 1.18%, whereas the swelling for Formulation-2 was 1.23%. The ointment contains hydrophilic excipients, however despite this, there was rarely any swelling because the formulation has a significant amount of extract component, which prevents swelling. Even though swelling can have both beneficial and negative consequences, some degree of occlusive swelling is required for cutaneous applications.

Spreadability

The spreadabilities of the two ointment formulations (F1 and F2) were discovered to be 7.6 g.cm/sec and 7.1 g.cm/sec, respectively. Spreadability of the produced formulation rises as viscosity lowers. Formulation-2's high viscosity causes it to be difficult to spread. Formulation-2 may have a larger effect since the material is concentrated at the desired spot and is more likely to attach to the injured region due to its decreased spreadability and increased likelihood of adhesion.

Extrudability

Both Formulation-1 and Formulation-2 were found to have ++ and ++ extrudability from the collapsible tubes, respectively. Due to Formulation-2's increased viscosity, which prevented free extrusion from the collapsible tube, the extrusion of Formulation-2 was less than that of Formulation-1.

Washability

The formulations' washability was determined to be +++ for Formulation-1 and +++ for Formulation-2. Formulation-2 exhibited reduced washability due to its greater viscosity, improved retention power, and increased stickiness (Table 4).

Table 4: Evaluation parameters.

Parameters	Formulation-1	Formulation-2
Appearance	Yellow-Brown color, characteristic odor	Yellow colored, characteristic odor
Spreadability (g.cm/sec)	7.6	7.1
Extrudability	++	++
Skin irritancy test	Non-irritant	Non-irritant
pH	6.8	6.9
Viscosity (cps)	5600	6400
Washability	+++	+++
Swelling index (%)	1.18	1.23

Short-term accelerated stability conditions

The polyherbal wound healing formulations (F1 and F2) did not show any noticeable changes in terms of washability, physical appearance, spreadability, viscosity, or extrudability under the accelerated stability conditions (40°C±2°C and 75%±5% RH for 90 days). The increased synthesis of a few tiny fragmented acidic components may have contributed to the pH decrease in Formulation-1. Formulation-2, on the other hand, was discovered to be more pH and viscosity resilient (Table 5). The product's use of honey, which stopped the chemicals from rapidly deteriorating, is the most likely cause. As a consequence, it was determined that the ointment compositions were rather stable.

Table 5: Accelerated stability studies.

Parameters	Formulation-1	Formulation-2
Appearance	No change	No change
Spreadability (g.cm/sec)	7.4	6.9
Extrudability	++	++
pH	6.7	6.8
Viscosity (cps)	5200	6000
Washability	+++	+++

Conclusion

The number of therapy choices for therapy has favorably expanded as a result of this study. Future uses for the created polyherbal formulations, which also contain extracts from *Uncaria gambier* and *Valeriana wallichii*, are conceivable. This finding brought back the ideas of ethnopharmacology in connection to modern medicine and explained the uses of polyherbal formulations in conventional medicine.

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Conflict of Interest

Authors state that there is no conflict of interest regarding the publication of this manuscript.

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