RESEARCH PAPER

Standardized and Pharmacological Evaluation of Herbal Gel for Anti-Inflammatory Activity

***[1]Arpan Debnath, [2]Debjit Teri, [3]Ayush Mohanty, [4]Aishik Paul, [5]Prasiddha Pal, [6]Debkantha Gope**

[1]Assistant Professor, Department of Pharmacology, B.R Nahata College of Pharmacy, faculty of pharmacy, Mandsaur University, 458001, M.P, India.
Email: arpandebnath333@gmail.com

[2]Assistant Professor, Department of Pharmacology, Mazidia Academy of Pharmacy, Singur, W.B ,712409, India.
Email: debjitteri095@gmail.com

[3]Assistant Professor, Department of pharmaceutical analysis, Indira Gandhi Institute of Pharmaceutical Sciences, Bhubaneswar, 751015, India.
Email: mohanty.ayush79@gmail.com

[4]Assistant Professor, Department of Pharmacology, APC College of Pharmacy, Singur, W.B, 712409, India.
Email: aishikpaul59@gmail.com

[5]Assistant Professor, Department of Pharmacology, Bengal School of Technology, Sugandha, 712102, W.B, India.
Email: prasiddhapal6@gmail.com

[6]Assistant Professor, Department of Pharmacy, Suresh Gyan vihar University, Jaipur, Rajasthan, 302017, India.
Email: debkantha99@gmail.com

Corresponding author : *Arpan Debnath

Abstract

Objectives: - The present study was carried out to standardized and pharmacological evaluation of herbal gel for anti-inflammatory activity.

Material and Methods: - Herbal extract of plants which contains curcumin, Plumbago zeylanica, Terminalia Billerica and linoleic acid are the active constituents responsible for Anti-inflammatory effect. Extraction of the plant material was done by different solvents. TLC was done to confirm the active constituents present in polyherbal. The in-vivo anti-inflammatory activity of polyherbal extract showing promising COX-2 inhibition was assessed using carrageenan and Phorbol Myristate Acetate (PMA) induced mice oedema animal model.

Results: - The percentage yield of extracts were found to 68.9, 75.4, 65.8. The Rf value of polyherbal extract was found to be 0.94. In vivo anti-inflammatory study shows herbal extract had a significant impact on inhibition of oedema formation. The cytotoxicity evaluation study of ethanolic fraction of herbal extract have no effect on cell viability. HPTLC fingerprint of flavonoids of the herbal extract was also prepared as a measure of quality control. Moreover, the herbal extract can be considered as a resource for searching novel anti-inflammatory agents possessing COX-2 inhibition.

Conclusion: - The herbal extract showed high extraction yield, confirmed flavonoid fingerprinting, significant in vivo anti-inflammatory activity, no cytotoxicity, and promising potential as a safe COX-2 inhibitory anti-inflammatory agent.

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1. INTRODUCTION

Ancient medicinal sciences like Ayurveda and Siddha are said to have originated in traditional Indian civilization. The market for natural medicines is expanding daily in India and other Asian nations due to the high level of confidence that Indian culture has in natural medicine.[1,2] Major goods derived from natural sources include Ayurvedic formulations, nutraceuticals, cosmeceuticals, health beverages, nutritional supplements, crude extracts, and poly-herbal formulations, among others. Since many natural medications have a preventative impact against a variety of pathological disorders, these items are used for therapeutic, rejuvenating, and health restoration purposes.

[3,4] Herbal products have a wide range of uses and a rich history, but there is still work to be done to standardize their quality. Standardization is the phrase used to describe the identity, quality, and purity of pharmacological compounds, as well as their stability, toxicity profiles, and shelf life. The absence of rules and practical, affordable standardization approaches makes the standardization of herbal formulation a difficult problem.[5,6] The qualitative, quantitative, and fingerprint profiling of herbal medicines were given a lot of attention by the regulatory bodies. The primary active ingredient in herbal formulations can be used for standardization, and when the active ingredient is unknown, a marker material can be used for analysis.[7,8]

When referring to natural substances, the word "standardization" refers to the use of contemporary standardization procedures for organoleptic and pharmacogenetic assessment, ash and extractive value evaluation, phytochemical evaluation, microbial load testing, and phytochemical profiling.[9,10] To standardize raw materials from natural sources, tests are also conducted to measure chemical groups, determine moisture content, detect heavy metals, detect pesticide residues, and detect radioactive contamination. The current situation necessitates the development of contemporary methods for estimating the quality of herbal products in order to guarantee the safety and quality of herbal medicines as well as to improve the adoption of natural medicines worldwide through standardization of herbal drugs.[11,12]

The immune system's reaction to infections, damaged cells, poisonous substances, or radiation is inflammation, which eliminates the damaging stimulus and starts the healing process. Thus, inflammation is an essential defensive mechanism for good health. Cellular and molecular processes and interactions often effectively reduce the risk of infection or harm during acute inflammatory reactions.[13,14] This mitigation step helps to resolve the acute inflammation and restore tissue homeostasis. Unchecked acute inflammation, however, can develop into chronic inflammation, which can lead to a number of chronic inflammatory disorders. Redness, swelling, heat, discomfort, and loss of tissue function are all signs of inflammation at the tissue level. These symptoms are brought on by the local immunological, vascular, and inflammatory cell reactions to infection or damage.[15,16] Changes in vascular permeability, leukocyte recruitment and accumulation, and the release of inflammatory mediators are significant microcirculatory processes that take place throughout the inflammatory phase. Inflammation can be brought on by a number of pathogenic reasons, including infection, tissue damage, and myocardial infarction. Inflammation can have infectious or non-infectious causes.[17,18] The body starts a chemical signalling cascade in reaction to tissue damage, which triggers reactions meant to repair damaged tissues. Leukocyte chemo taxis are activated by these signals and transported from the general circulation to the damage areas. The cytokines produced by these activated leukocytes cause inflammation. There are two forms of inflammation: acute and chronic.[19,20]

The intricate biological reaction of bodily tissues to damaging stimuli, such infections, damaged cells, or irritants, is called inflammation. Inflammation serves the main functions of removing the original source of cell damage, removing damaged cells, and starting the healing process. It is an essential immune system defensive mechanism.[21,22] However, the term "inflammation" can have two meanings. While chronic inflammation can result in a number of illnesses and ailments, such as autoimmune disorders, cardiovascular diseases, cancer, and neurological disorders, acute inflammation is essential for healing. The mechanics of inflammation, the forms of inflammation, important mediators, the immune system's function, the causes of chronic inflammation, the effects on health, and different treatment modalities will all be covered in this thorough discussion.[23,24]

With more traditional medicine research, plant-based medications that are eco-friendly, bio-friendly, affordable, and generally safe have emerged from the fringe to the mainstream in recent decades. According to the World Health Organization, 21,000 plants are used medicinally worldwide.[25,26] India is home to 2500 of these species. Known as the Botanical Garden of the World, India is the world's biggest producer of medicinal plants. The plants have been categorized by the review based on their botanical name, country of origin, parts utilized, and active agent type. Several writers have reviewed the literature on herbal anti-inflammatory medicines.[27,28]

In order to improve therapeutic action and reduce adverse effects, two or more medicinal plants are combined in certain ratios to create polyherbal compositions. The foundation of ancient medical systems like Ayurveda, Siddha, and Unani is the idea of synergism, which is the cornerstone of the polyherbal approach. This notion involves diverse bioactive components working on distinct targets simultaneously.[29,30] This multi-component strategy

improves the pharmacological activity, absorption, and safety compared to single-herb formulations. With its antioxidant, anti-inflammatory, immunomodulatory, and antibacterial qualities, polyherbal medicine has been thoroughly studied for the treatment of chronic illnesses like diabetes, cancer, inflammation, and skin disorders. Polyherbal formulations are becoming widely recognized as safe, affordable, and efficient alternatives to traditional synthetic medications as more scientists continue to validate, standardize, and have better control over them.[31,32]

2. MATERIAL AND METHODS

2.1 Preparation of methanolic extracts

The Botanical Survey of India, Pune documented Curcumin, Plumbago zeylanica and Terminalia Billirica, and linoleic acid which were available in the Rahata region in Ahmednagar in Maharashtra. The dry powdered plant specimens (10g) were extracted gradually over a maximum time of 8 hours using Soxhlet apparatus in hexane, ethanol and water. Evaporation of the extracted samples was done at ambient temperature and reduced pressure. The dried extracts were kept in refrigerator at 40 o C and later analysed.

2.2 HPTLC analysis

Thin layer chromatography was done using CAMAG (Germany) equipment. TLC plates (Merck silica gel 60 f254, 20 cm x 10 cm) had been moistened with methanol. Activation of the plate was done in an oven at 1000o C with 10 minutes. The application of 10ml of individual plant extracts (1 mg/ml) on precoated plates was done using Linomat 5 application method. Rutin hydrate (5 and 10mg/ml) was used as a marker flavonoid. The flavonoids were extracted with a mobile phase of ethyl acetate /formic acid/ glacial acetic acid/ water in 100:11:11:27. A natural product (NP) reagent was used as a flavonoid derivatizing reagent with the spots formed being observed using a CAMAG UV cabinet (366 nm) and recorded digitally using a CAMAG photo documentation system.

2.3 COX inhibition assay

A Colorimetric COX Inhibitor Screening Assay Kit was used to do the assay. The reaction set up will include assay buffer (150 ml), heme (10 ml), enzyme (COX-1 or COX-2, 10 ml) and plant sample (1 mg/ml, 10 ml). The peroxidase component of the COX catalytic domain is used in the assay. The colorimetric evaluation of peroxidase was done by looking at the rise of oxidized N, N, N, N'-tetramethyl-pphenylene diamine (TMPD) at 590 nm. Aspirin (1 mM acetylsalicylic acid) was used as the reference drug. The percent COX inhibition was calculated using following equation:

COX inhibition activity (%) = $1-\frac{T}{C}\times 100$

Where T = Absorbance of the inhibitor well at 590 nm.

C =Absorbance of the 100% initial activity without inhibitor well at 590 nm.

2.4 Formulation of topical gel

Herbal gel was made using a gelling material known as Carbopol 934 with a concentration of 1% w/w in deionized water using a mechanical stirrer. The skin pH (6.8 -7) was maintained at a steady rate of triethanolamine and agitation. The gel contained different per cent amounts of 5, 10, 15, 20 and 25 w/w of the two extracts to which an appropriate period of agitation was given to bring even dispersion of the extract in the gel matrix. The prepared gel was filled using collapsible tubes. The formulations were kept in a dry and cold place. The following criteria were evaluated in the formulation.

Ingredient	**Quantity in %**				
	F1	**F2**	**F3**	**F4**	**F5**
Methanolic extract of Curcumin, Plumbago zeylanica, Terminalia bellarica and linoleic acid	15	20	25	30	35
Carbopol 934	2	2	2	2	2
Methylparaben (0.5%)	0.5	0.5	0.5	0.5	0.5
Propylparaben (0.2%)	0.3	0.3	0.3	0.3	0.3
Propylene glycol 400 (5%)	6	6	6	6	6
Triethanolamine	q.s	q.s	q.s	q.s	q.s

Table 1. Formulation of gel

2.4.1 Organoleptic evaluation

Physical parameters such as color and appearance were recorded.

2.4.2 Viscosity

Gel viscosity was measured through Brookfield viscometer (RVT model) and a spindle number of 7.

2.4.3 Extrudability

The gel mixtures were enclosed in commercial capped collapsible aluminum tubes and crimped at the end. The weights of the tubes were recorded. Two glass

slides were used to clamp the tubes. A total of five hundred grams was added on the slides and then the cap was removed. The polyacrylamide gel was extruded and weighted. It was determined what percent of the gel was extruded (>90% extrudability: Excellent, >80% extrudability: Good, and >70% extrudability: Fair).

2.4.4 Spreadability

The assessment of the spread ability was done with the help of the equipment which consisted of a wooden block with a pulley at one end. The method was used to determine the spread ability properties of gels based on the slip and drag properties of gels. The gel (approximately 2 g) that was under consideration was applied in excess on the ground slide. This slide was then placed between the gel and another glass slide which had an identical size as that of the fixed ground slide and was fitted with a hook. The 1 kg mass was left to rest on top of the two slides during 5 minutes so that the air could be removed and uniform coating of gel could be achieved between the two slides. The excess gel was scraped off on margins. The top plate was then pulled with 80 g using a thread attached to the hook and measured the time (in seconds) which the top slide used to move a distance of 7.5 cm. A smaller interval indicated an improved spread ability. Spread ability was calculated using the following formula

$S = M \times L / T$

Where,

S = Spreadability

M = Weight in the pan (tied to the upper slide) L = Length moved by the glass slide

T = Time (in sec.) taken to separate the upper slide from the ground slide.

2.4.5 Measurement of pH

The digital pH meter was used to measure the pH of the formulated gels. The sample was evaluated after 1, 30, 60 and 90 days in preparation to determine whether there are any temporal differences. 1 gram of gel had to be dissolved in 100 milliliters of distilled water and left to stand during 2 hours. Measuring of the pH of the formulation was done three times, and the mean results were obtained.

2.4.6 Homogeneity

All developed gels were packed in containers and then tested for homogeneity by visual inspection. They were tested for their appearance and presence of any aggregates.

2.4.7 Grittiness

Each of the formulations was also observed microscopically with respect to any detectable particulate matter that was observed under light microscope. Therefore, the gel preparation obviously satisfies the demand of the absence of specific matter and form grittiness desired in any topical preparation.

2.5 Stability study

Stability study was done using ICH guidelines. The prepared gel was filled in collapsible tubes and kept under various temperature conditions and humidity conditions, i.e. 25+2 0 C/ 60 +5% RH, 30+2 0 C/ 65 +5% RH and 40 +2 0 C/ 75 +5% RH over a span of 3 months and examined in terms of appearance, pH and spreadability.

2.6 Skin irritation test

The average weight of the intact skin of the Wistar rats of both sexes was 150 200 g. The rat was shaved 3 days prior to the experiment. On the test animal and gel base on the control group prepared gel formulations were applied. The animals were put through 7 days of treatment and erythema and edema of the treated skin studied.

2.7 Evaluation of anti-inflammatory activity

Albino Wistar rats weighing 150–200g on average, of either sex, were used. All of the study's mice were housed in typical settings, eaten typical rodent chow, and given unlimited access to water. In the control, test, and standard groups, each consisting of six animals, all animal protocols were followed. Rat paw edema caused by carrageenan Prior to the experiment, the animals were given unlimited water and fasted for twenty-four hours.One hour before each experiment started, 0.1 ml of 1% w/v carrageenan in saline was injected into the plantar area of the rat's right hind paw to induce edema. 0.2 g of herbal gel was applied to the plantar side of the hind paw and gently rubbed there for 50 times. The rat in the control group received 0.2 g of the simple gel base in the same way as a standard. One hour before the carrageenan injection, a placebo or medication was administered. With the use of a plethysmometer, the volume of the paws was measured as soon as the carrageenan was injected and at 1, 2, 3, and 4 ad hoc intervals following the administration of the toxic substance. The following formula is used to calculate the percentage inhibition of volume in the paw.

%Inhibition = [Paw volume [Control] - Paw volume [Test] ×100÷ Paw volume (Control)

3.RESULTS

3.1 HPTLC profiling

The HPTLC analysis was conducted as one of the quality control of the chosen samples of plants. A marker flavonoid compound rutin was used in preparing HPTLC finger print of the ethanol soluble

flavonoids. The HPTLC analysis results indicate the variation of flavonoid content in T. bellarica, in addition, it is the only sample that had rutin content, and all other samples did not have any rutin content.

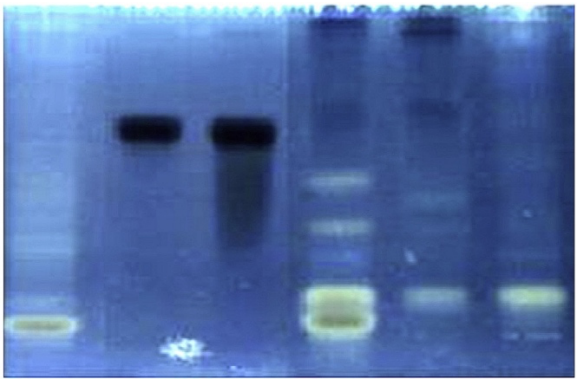

Figure 1. HPTLC profile of methanol extract of selected medicinal plants

3.2 Effect of plant samples on COX inhibition

The findings of the COX inhibition by various fractions of the selected plants are summarized. The mean COX-1 and COX-2 were determined by dividing the COX inhibition activity of three solvent extracts. In general, it was discovered that the fraction of T. bellarica (mean activity COX-1, 62.78 % and COX-2, 74.78 %) and curcumin (mean activity COX-1, 54.89 % and COX-2, 78.74 %) were observed to be significant COX-1 and 2 inhibitor when compared to other plant sample. Linoleic acid demonstrated the minimum COX-1 inhibition (29.78 percent), and P. zeylanica demonstrated the lowest COX-2 inhibition (mean activity 43.79 percent). A superficial examination on the COX inhibitory profile of the selected plants further reveals that the ethanol fractions were discovered to be stronger COX inhibitory solutions compared to water and hexane extracts, which evinced moderate or no COX inhibitory actions. The results have been compared to Aspirin (1 mM) with a COX-1 (09.55 ± 0.49 %) and COX-2(13.75 ± 0.19 %) level of COX-2.

Plant name	**COX-1(%) inhibition**			
	Water	**Ethanol**	**Hexane**	**Mean activity**
Curcumin	29.18±0.75	85.49±0.18	47.58±0.86	54.29
Plumbago zeylanica	NR	45.29±0.52	39.58±0.65	26.58
Terminalia bellarica	78.19±0.58	63.29±048	49.58±0.47	63.79
linoleic acid	75.19±0.85	88.29±1.55	NR	54.28
Aspirin	9.28±0.59	NR	NR	NR

Table 2. Effect of different fractions of selected medicinal plants on COX-1 activity

Plant name	**COX-2 (%) inhibition**			
	Water	**Ethanol**	**Hexane**	**Mean activity**
Curcumin	27.29±0.65	82.19±0.63	49.78±0.46	55.49
Plumbago zeylanica	34.29±0.67	65.78±0.64	34.59±0.73	45.79
Terminalia bellarica	89.28±0.73	72.19±0.92	62.49±0.48	74.58
linoleic acid	87.59±0.71	86.49±1.98	55.49±0.75	75.19
Aspirin	12.59±0.16	NR	NR	NR

Table 3. Effect of different fractions of selected medicinal plants on COX-2 activity

3.3 Physical evaluations of gel

All gels are pale green in color with a clear look and smooth touch on the skin and which stood the same on the stability testing period. All these formulations have displayed optimum viscosity. The PH of all the formulations prepared were in the range of 6-7 that is believed to be acceptable to prevent chances of irritation upon application to the skin. After 3 months, all formulations that are prepared and without the presence of gritty particle are homogeneous. In addition, the stability study results indicated that the preparation was stable at the normal management storage.

Formulation	**Appearance**	**Viscosity**	**Spreadability**	**pH**	**Homogeneity**
F1	Pale green	4629	25.29	6.4	Homogeneous
F2	Pale green	4359	23.49	6.6	Homogeneous
F3	Pale green	4348	25.95	6.9	Homogeneous
F4	Pale green	4639	18.49	7.0	Homogeneous
F5	Pale green	4654	18.92	6.8	Homogeneous

Table 4. Physical evaluation of gel formulations

3.4 Extrusion of the gel

Aiding in the use of the gel is the extrusion of the gel out of the tube. High consistency gels might not flow out of tube but low viscous gels can flow fast and therefore, appropriate consistency is needed to be able to push the gel out of the tube. All gel formulations were found to be good in terms of extrudability.

Formulation	**Weight of formulation**	**Weight of gel extruded**	**Extradibility amount (%)**	**Grade**
F1	15.2	13.1	86.18	Good
F2	15.64	12.9	82.48	Good
F3	15.95	13.42	84.13	Good
F4	15.26	13.15	86.17	Good
F5	15.23	12.7	83.38	Good

Table 5. Extrudability study of gel formulations

3.5 Investigation of anti-inflammatory activity of gel formulations

Carrageenan-induced paw edema method was used to determine the anti-inflammatory activity of gel formulations. The carrageenan caused edema on the rat paw and was inhibited by formulations and standard 1 percent valdecoxib. The 5% and 10 percent extract formulations have not indicated significant percent inhibition of the rat paw edema and the 15 percent extract formulations of 20 percent extracts and 25 percent extract formulations have indicated significant percent inhibition. Formulations F4 and F5 had a significant inhibitory effect of the inflammations up to 57.25, 62.85 at 3h and 58.74, 64.28 at 4h respectively, whereas the reference drug suppressed the inflammations to 68.49 at 3h and 78.49 at 4h. The anti-inflammatory effect of F4 and F5 was the same as that of valdecoxib at respective time point.

Treatment	**Paw volume (ml) at various time intervals after carrageenan administration**			
	1h (%inhibition)	2h (%inhibition)	3h (%inhibition)	4h (%inhibition)
Control	0	0	0	0
F1	12.49	13.8	14.85	7.51
F2	15.67	11.48	24.95	10.28
F3	19.67	43.19	46.88	49.62
F4	19.78	55.49	57.25	58.74
F5	24.89	58.67	62.85	64.28
Standard	45.99	56.22	68.49	78.49

Table 6. Effect of various formulations on carrageenan-induced paw edema in rats

4. SUMMARY AND CONCLUSION

HPTLC fingerprinting showed that the plant samples used had methanol-soluble flavonoids which were diverse in nature with only rutin detected in T. bellarica, a quality control marker in its own right. Investigations on the COX inhibition showed that COX-1 (T. bellarica fractions, COX-1: 62.78; COX-2: 74.78) and COX-2 (T. bellarica fractions, COX-1: 54.89; COX-2: 78.74) were significantly inhibitory, and more selective to COX-2 as compared to other samples. Extracts made with ethanol had greater inhibitory properties than water and hexane extracts. Linoleic acid exhibited little COX-1 inhibition with P. zeylanica exhibiting relatively low COX-2 inhibition.

All gel formulations exhibited satisfactory physicochemical characteristics, such as, pale-green, translucent, smooth texture, optimum viscosity, pH of 6-7, excellent extrudability as well as shelf life of three months without any phase change or grittiness.

Dose-dependent activity was shown in vivo with the use of the carrageenan-induced paw edema model as an anti-inflammatory activity. Extracts of 15-25% exhibited a great edema inhibitory effect. It is interesting to note that F4 and F5 achieved an inhibition of 57.25% and 62.85% respectively at 3 hours and 4 hours respectively which was similar to that of valdecoxib.

All in all, the present study validates the hypothesis that standardized plant extracts, especially T. bellarica, when made into stable topical gels, have potential to be used in the treatment of the COX-2 selective and anti-inflammatory agent, which will justify its therapeutic use.

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