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IN-VIVO ANTI-INFLAMMATORY ACTIVITY OF DEVELOPED POLYHERBAL GEL FORMULATION

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ABSTRACT:

Inflammation is the condition associated with many of the disease states and this review elaborate the medicinal plants, their parts used in the effective management of Inflammation and its associated conditions. Currently there is a greater global interest in non synthetic, natural derived from plant or herbal sources due to better tolerance and decreased adverse drug reaction. Coetaneous use of NSAIDs may result in skin rashes. Another meta-analysis concluded that even though the classical GI or renal adverse effects may be greatly reduced for topical NSAIDs. Although several agents are known to treat these types' disorders, prolonged use should be avoided due to several side effects. Since; herbal remedies are more accepted in world for their fewer side effects and lower costs. Hence present work aimed to evaluate *in-vivo* anti-inflammatory activity of optimized developed polyherbal gel formulation comprising Ethanolic extract of White turmeric (*Curcuma amada*) Rhizome (EECA), Methanolic extract of Black pepper (*Piper nigrum*) fruits (MEPN) and Methanolic extract Lodhra (*Symplocos racemosa*) Bark (MESR), Menthol and Capsaicin. *In-vivo* anti-inflammatory activity polyherbal gel formulation were carried out using Carrageenan induced Paw edema in rats, Formalin induced Paw edema and Randall-Selitto Test in rats. Results showed that, polyherbal gel formulation has excellent anti-inflammatory activity as compared to marketed Diclofenac sodium gel. Hence such formulation has scope of commercial manufacturing after successful clinical trials.

Keywords: *Curcuma amada*, *Piper nigrum*, *Symplocos racemosa*, Capsaicin, Menthol, Polyherbal gel, in-vivo anti-inflammatory activity

INTRODUCTION:

Medicinal plants have occupied an important position in the socio-cultural, development of rural people of India. Crude drugs are usually dried parts of the medicinal plants that form an essential raw material for the production of traditional remedies of Ayurveda, Siddha, Unani, Homeopathy etc. It has been estimated by WHO that 80% of the people living in the developing countries rely upon the traditional health practices for their primary health care needs. [1, 2]

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. When cells in the body are damaged by microbes, physical agents or chemical agents, the injury is in the form of stress. Inflammation of tissue is due to response to stress. It is defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to response that results from other type of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion.[3] Inflammation dilutes, destroys, or walls off harmful agents that have entered the body. It activates a sequence of biological events to heal the damage. The most common causes of inflammation are infections, burns and trauma, and many types of immune reactions.[4] These traditional medicines take part in an significant position in health services around the world. The opoids or non-steroidal anti-inflammatory drugs, widely used to reduce the inflammation of various types, suffer from severe side effects like redness, itching etc. As a result, a search for other alternatives seems to be necessary which would be more beneficial. Gel formulations are used to deliver the drug topically because of easy application, increase contact time and minimum side effects as compare to other topical preparation and oral administration.[5]

Many plants from Zingiberaceae family is used in traditional system of medicine. *Curcuma amada* (White turmeric) is one member of this family which is traditionally used as carminative and stomachic [6]. Literature survey indicates the presence of manifold chemical constituents in these rhizomes. The rhizomes are used for the treatment of inflammatory conditions as a domestic remedy on experiential basis [7, 8]. *Piper nigrum* commonly known as black pepper, it belongs to the family piperaceae. The plants are native and cultivated in hot and humid parts of India [9]. Black pepper is used as spice as well as medicine by itself or as a part of some herbal remedies in combination with other well known herbs and spices [10]. Pungent alkaloid piperine is the main therapeutically active constituents of pipper nigrum [11]. *Symplocos racemosa* (Symplocaceae) commonly known as "Lodhra" in Sanskrit or "Rodhra, is a small, evergreen tree upto 6 m tall. It is found in the plains and lower hills throughout North and East India [12]. The bark is dark grey and rough; and is useful in diarrhea, dysentery, eye diseases, fever, ulcer, scorpion sting, diabetes, and liver disorders [13]. It has been systematically reported as an antimicrobial, anticancer and has useful effects in gynaecological disorders [14]. Menthol (also "mint camphor"), is a volatile oil extract derived from the genus Mentha (mint), is extensively accessible in natural and synthetic forms. Menthol has been used as a topical pain reliever since ancient times [15]. Capsaicin is a compound found in chili peppers and responsible for their burning and irritant effect. In addition to the sensation of heat, capsaicin produces pain and, for this reason, is an important tool in the study of pain. Capsaicin, a major ingredient of hot pepper, was considered to exhibit an anti-inflammatory property [16].

Gel formulations are used to deliver the drug topically because of easy application, increase contact time and minimum side effects as compare to other topical preparation and oral administration [17].

MATERIALS AND METHODS:

Materials:

Dried rhizomes of *Curcuma amada*, dried fruits of *Piper nigrum* and Lodhra (*Symplocos racemosa*) bark were purchased from local market of Nashik. The plant materials were authenticated by Prof. Manohar Gulab Gavit, Department of Botany, MVPS KAANMS Arts, commerce and Science College, Dist. Nashik (Maharashtra). (Authentication No. KAANMS/2020-21/56/Herbarium 3). Capsaicin was provided as a gift sample by Naturite Agro Products Limited Hyderabad, India. Menthol was purchased from S. D. Fine Chemicals, Mumbai. Carbopol 934. All other chemicals used were of analytical grade.

Animals:

The Wister rats weighing between 150-200 gm were procured from Animal house of KBHSS Trust Institute of Pharmacy, Malegaon and maintained under constant conditions (temperature 25± 2C, Humidity 40-60%, 12 h light/ 12 h dark cycle). During maintenance the animals received a diet of food pellet supplied from animal house and water ad libitum. These experiments were approved by the Institutional Animal Ethics Committee, KBHSS Trust Institute of Pharmacy, Malegaon (IAEC registration no. 1566/PO/Re/S/11/CPCSEA)

Methods:

Preparation of Plant Extract:

Ethanolic extract of White turmeric (*Curcuma amada*) Rhizome (EECA), Methanolic extract of Black pepper (*Piper nigrum*) fruits (MEPN) and Methanolic extract Lodhra (*Symplocos racemosa*) Bark (MESR) was carried out and evaluated for various phytochemical screening. These extracts were used for preparation of polyherbal gel using Carbopol 934 as gelling agent.

Formulation of Polyherbal gel:

Preparation of gel base

Gels base were prepared by cold mechanical method described by Schmolka *et al.* Carbopol 934 was dissolved slowly with stirring in 60 mL of demineralized water for 1 h to avoid agglomeration Then disodium edetate and triethanolamine were dissolved in 10 mL of demineralized water separately and stirred for 10 min. Mixed 4.83 mL of propylene glycol in 12 mL of demineralized water with stirring for 10 min. Disodium edetate and triethanolamine solution were added to Carbopol 934 solution and the pH was then adjusted to 7.4 by stirring the solution for 10 min. Then propylene glycol solution was added with stirring for 10 min until a clear consistent gel base was obtained [18].

Preparation of gel formulation

As per previous study of formulation of polyherbal gel F2 formulation containing 2.67% concentrations of all three extracts i.e. EECA, MEPN, and 1 % of Carbopol 934 was used as gelling agent. (Table 1) EECA, MEPN, MESR, Menthol and Capsaicin were added as a drug to gel base with continuous stirring till drug get dispersed completely. The prepared gel was filled and sealed in the aluminium collapsible tube. A similar procedure was followed for base control gel without the extract and other active ingredient [19].

Table 1: Composition of Polyherbal topical gel (Formulation F2)

Sr.	Ingredient	Quantity (g)	Use

No.			
1.	EECA	2.67	Anti-inflammatory
2.	MEPN	2.67	Anti-inflammatory
3.	MESR	2.67	Anti-inflammatory
4.	Menthol	1	Anti-inflammatory
5.	Capsaicin	1	Anti-inflammatory
6.	Carbopol 934	1	Gelling agent
7.	TEA	1.5	Adjust the pH of the formulation
8.	Disodium EDTA	0.005	Stabilizer, pH adjustment, Chelating agent
9.	Propylene Glycol	5	Permeation enhancer
10.	Methylparaben	0.1	Preservative
11.	Propyl paraben	0.05	Preservative
12.	Water (100 g)	q.s.	Vehicle

EECA: Ethanolic extract of White turmeric (*Curcuma amada*) Rhizome, **MEPN:** Methanolic extract of Black pepper (*Piper nigrum*) fruits, **MESR:** Methanolic extract Lodhra (*Symplocos racemosa*) Bark, **TEA:** Triethanolamine, q.s.: Quantity sufficient

In-vivo anti-inflammatory activity polyherbal gel formulation:

In-vivo anti-inflammatory activity polyherbal gel formulation were carried out using Carrageenan induced Paw edema in rats, Formalin induced Paw edema and Randall-Selitto Test in rats.

Carrageenan induced rat paw edema

Pedal inflammation in animal was produced according to the method described by Winter et al (1962). Rats were divided in 3 groups of six rats in each.

Group I: was applied with gel base and served as **control**.

Group II: standard (Diclofenac sodium Gel 0.5%) and served as reference.

Group III: Application of 1 gm of polyherbal gel **formulation (F2).**

The edema was induced by injecting 0.1 ml of carrageenan (1% w/v) in normal saline into the sub planter region of the left hind paw, after 1 hour of drug application. Paw thickness was measured with the help of Digital Vernier caliper at 0, 30, 60, 120, 180, 240 and 300 min after administration of carrageenan. The data was analyzed using one way ANOVA followed by Dunnett's test and $p<0.01$ was considered significant. [20, 21, 22 and 23]

Formalin induced Paw edema

The formalin-induced rat paw edema model was used for acute as well as chronic inflammation on the basis of formalin concentration. For chronic model 2% of formalin in saline was used. Formalin-induced edema is biphasic, an early neurogenic component is mediated by substance P and bradykinin followed by a tissue mediated response where histamine, 5-HT, prostaglandin are known to be involved. [21, 22 and 23]

The % inhibition of edema was calculated by formula:

% Inhibition = 1- {a-x/b-y} x 100

Where,

a = paw thickness of test animal after treatment

x = initial paw thickness of test animal

b = paw thickness of control animal after treatment

y = initial paw thickness of control animal.

Randall-Selitto Test in rats

The Randall-Selitto or paw pressure test was developed as a tool to assess response thresholds to mechanical pressure stimulation and is often considered a measure of mechanical hyperalgesia. This test involved application of an increasing mechanical force to the surface of the paw or tail until withdrawal or vocalization occurs. In practice, this test is useful for assessment of nociceptive thresholds in rats rather than mice as animals need to be heavily physically restrained with the tested paw held out, and mice rarely tolerate such handling. The exception is use of the test on the tail of mice (Minett et al., 2014), although this may not be useful to assess nociceptive behaviors in commonly used models that are localized to the hind paw. The Randall-Selitto test can be performed using bench-top or hand-held devices with animals either restrained in a hammock that provides access to the hind paws, a towel, or in a plastic cone or cylinder. To obtain reliable data, animals need to be habituated to the restraint method and experimental apparatus, which can become very time-intensive. Mechanical pressure is applied focally to the dorsal or plantar surface of the hind paw or tail, which is placed between a pointed probe tip and a flat surface. The pressure is then increased at a constant rate until a nociceptive behavioral response is observed. [24, 25, 26, 27 and 28]

Rats were divided in 3 groups of six rats in each.

Group I: was applied with gel base and served as **control**.

Group II: standard (Diclofenac sodium Gel 0.5%) and served as reference.

Group III: Application of 1 gm of polyherbal gel **formulation (F2).**

The paw pressure test was carried out by using the Randall and Selitto paw withdrawal method. Pressure was measured in grams force using an Ugo Basile Analgesy Meter by applying an increasing force to the left hind paw of rats until they reacted either by paw withdrawal or squealing. A threshold for nociception was determined for each animal at the start of the experiment to obtain baseline reading. Under light anesthesia intra-articular injection of 0.1ml of Complete Freund's Adjuvant was given in left knee joint. All formulations were applied on the left Knee joint with at least 50 times gently rubbing in a circular manner. All the animals treated with drugs as per the groups and control animals were received gel base application on the same day and for 72 hours. After 72 hours, nociceptive threshold (in gm) was estimated. Each animal served as its own control. [24, 29, 30]

RESULT AND DISCUSSION:

Preliminary physicochemical characteristics of *Curcuma amada* Rhizome

Results obtained for quantitative determination of proximate analysis and qualitative screening of phytochemicals in rhizome of *C. amada* is presented in Table 2 & 3. Total thirteen phytochemicals were screened in which ten were found present in different solvent extracts. They are cardiac glycosides, flavonoids, phenols, carbohydrates, saponins, tannins, alkaloids, sterols, quinones and terpenoids. Remarkably, carbohydrate, flavonoids, phenols, saponins, tannin, quinones, alkaloids and terpenoids were present in the rhizome of these plants. This suggests that the rhizomes have extensive potentials of phytochemicals. Physiochemical parameters of the rhizome of *Curcuma amada* Roxb are tabulated in Table 2. Different extracts of the powdered rhizome were prepared for the study of extractive values. Percentage of extractive values was calculated with reference to the air dried drug. The results are shown in Table 1. The loss on drying at 105°C in rhizome was found to be 12.49 %. Total ash value of plant material indicated the amount of minerals and earthy materials attached to the plant material. Analytical results showed total cash value content was 6.85 %. The negligible amount of acid insoluble siliceous matter present in the plant was 5.22 %. [31, 32]

Table 2: Physicochemical characteristics of *Curcuma amada* Rhizome

Parameters	Result (%w/w)
Total Ash	6.85±0.92
Acid insoluble ash	5.22±0.21
Water insoluble ash	2.62±0.08
Water soluble extractive value	15.37±0.19
Alcohol soluble extractive value	4.96±0.32
Loss on Drying	12.49±0.17

*All values represent mean ± standard deviation (n=3)

The result indicates that *Curcuma amada* rhizome hold promises as source of pharmaceutically important phytochemicals. Flavonoids present in non-areal parts like rhizomes play some metabolic role and control development in living system. Tannins are known to inhibit pathogenic fungi. The flavonoids and phenolic compounds in plant have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, antiinflammatory, anti-carcinogenic, astringent, anti-diabetic, anti-tubercular, antipyretic effects etc. [33, 34]

Phytochemical Screening of *Curcuma amada* Rhizome:

Table 3: Phytochemical Screening of *Curcuma amada* Rhizome

Phytoconstituents	Solvent Extract		
	Aqueous	Chloroform	Ethanol
Alkaloid	+	+	+

Cardiac glycoside	-	-	+
Carbohydrate	+	+	+
Flavonoids	-	-	+
Phenols	-	-	+
Phlobatannins	-	-	-
Protein	-	-	-
Saponin	+	+	+
Sterol	-	+	+
Tannin	+	+	+
Terpenoid	-	+	+
Quinone	+	-	+
Oxalate	-	-	-

'+': Present, '-': Absent

The alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids. flavonoids, phenols, saponins, proteins, alkaloids and terpenoids. The methanol extract had the presence of cardiac glycosides, carbohydrate, alkaloids, flavonoids, phenol, tannins, saponins and terpenoids. The medicinal value of plants means definite physiological action on the human body due to presence chemical substances. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against diseases. Alkaloids protect against chronic diseases. Saponins protect against hypercholesterolemia and antibiotic properties. Steroids and triterpenoids show the analgesic for central nervous system activities. [31, 32]

The main active ingredient in turmeric is known as curcumin. It has better antioxidant and anti-inflammatory properties. Curcuminoids are known as polyphenolic a pigment which includes curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcumin is the primary curcuminoid in turmeric and the compound for which most studies have been done. The result indicates that *Curcuma amada* rhizome hold promises as source of pharmaceutically important phytochemicals. Flavonoids present in non-areal parts like rhizomes play some metabolic role and control development in living system. Tannins are known to inhibit pathogenic fungi. The flavonoids and phenolic compounds in plant have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti- inflammatory, anti-carcinogenic, astringent, anti-diabetic, anti-tubercular, antipyretic effects etc. [33, 34]

Physicochemical characteristics of Black pepper (*Piper nigrum*) fruits:

Table 4: Physicochemical characteristics of *Black pepper (Piper nigrum) fruits* extract

Parameters	**Result (%w/w)**
Extractive value Petroleum ether Methanol	 11.32±0.66 14.29±0.75
Total ash value	5.12±0.21
Acid insoluble ash value	0.43±0.09
Water soluble ash value	4.01±0.28
Loss on Drying	9.63±0.31
Moisture content	0.52±0.07
pH of 1% solution	6.54±0.83

*All values represent mean ± standard deviation (n=3)

The purity of the drug was checked by determining different physicochemical parameters which included extractive values, total ash value, acid insoluble ash value, water soluble ash value, moisture content, LOD, pH values of 1% and 10% solutions. The results of physicochemical parameters are summarized in Table 4. The moisture content in the fruits of *P. nigrum* was found to be 0.48% which indicated that the drug was properly dried and well stored. LOD of the fruits of *P. nigrum* was found to be 10.23%±1.43%. The pH values of the *P. nigrum* fruits extracts (1% and 10% solutions) were also evaluated with the help of digital pH meter. The pH of the 1% and 10% solutions of drug was found to be 6.23±0.43 and 8.18±0.44 respectively.

Phytochemical Screening of *Black pepper (Piper nigrum) fruits* extract:

The results of preliminary qualitative phytochemical screening revealed the presence of alkaloids, carbohydrates, phenolic compounds, flavonoids, proteins, saponins, lipids, tannins and steroids. All these phytochemicals except lipid were found to present in the methanolic extracts. The results of phytochemicals analysis are presented in Table 5.

Table 5: Phytochemical Screening of *Black pepper (Piper nigrum) fruits extract*

Phytoconstituents	**Solvent Extract**	
	Petroleum ether	**Methanol**
Alkaloid	-	+
Carbohydrate	-	+
Phenolic compounds	-	+

Flavonoids	-	+
Proteins and amino acid	-	+
Saponin	-	+
Lipids/Fats	+	-
Tannin	-	+
Sterols		+

'+': Present, '-': Absent

The phytochemical screening showed that the fruit were rich in alkaloids, flavonoids, tannins and saponins. They were known to show medicinal activity as well as exhibiting physiological activity. *Piper nigrum* (L.) has many favourable chemical properties and beneficial effects. Besides, this review presents a summary of the data on the chemical composition of black pepper, including minerals, vitamins, carotenoids and flavonoids, and various therapeutic benefits.

Physicochemical characteristics of Lodhra (*Symplocos racemosa*) Bark:

Determination of physicochemical parameters such as Total ash value, Acid insoluble ash value, Water soluble ash value, Alcohol soluble extractive value, Water soluble extractive value and Moisture content (LOD) indicated that parameters were found below the limits as per Pharmacopoeial requirement.

Table 6: Physicochemical characteristics of *Lodhra (Symplocos racemosa)* Bark

Parameters	Value obtained on dry weight basis (% w/w)*	Value described in API (% w/w)
Total ash value	9.26 ± 0.63	NMT 12 %
Acid insoluble ash value	0.38 ± 0.06	NMT 1 %
Water soluble ash value	5.73 ± 0.57	NMT 10 %
Alcohol soluble extractive value	8.69 ± 0.83	NLT 9 %
Water soluble extractive value	13.43 ± 1.61	NLT 15 %
Moisture content (LOD)	4.84 ± 0.96	-

*All values represent mean ± standard deviation (n=3)

Phytochemical Screening of Lodhra *(Symplocos racemosa)* Bark Extract:

Phytochemical screening of the methanol extract and water extract of bark of *Symplocos racemosa* showed the presence of various phytoconstituents. Methanol extract showed presence of Carbohydrates, Saponin, Tannins, Alkaloids, Glycoside, Flavonoids, Steroids/Triterpenes and Resins and water extract showed presence of Carbohydrates, Saponin, Tannins, Alkaloids, Glycoside and Resins. Results are shown in Table 7.

Table 7: Phytochemical Screening of *Lodhra (Symplocos racemosa)* Bark extract

Chemical tests	Methanol extract	Water extract
Carbohydrates	+	+
Proteins	-	-
Saponin	+	+
Tannins	+	+
Alkaloids	+	+
Glycoside	+	+
Flavonoids	+	-
Steroids/Triterpenes	+	-
Resins	+	+

'+': Present, '-': Absent

Formulation of Polyherbal Topical Gel

Formulation F2 were prepared using Carbopol 934 as gelling agent successfully.

***In-vivo* anti-inflammatory activity polyherbal gel formulation:**

Carrageenan induced rat paw edema:

The development of carrageenan-induced edema is biphasic, the 1st phase is mediated through the release of histamine and serotonin, with a peak value at 1h; whereas the 2nd phase is related to the release of prostaglandins with a peak value at 3 h. Formulation F2 significantly ($P < 0.01$) inhibited the mean paw volume at 3 h and 5 h after carrageenan injection. The results showed that the anti-inflammatory effect of the formulation F2 better than the effect of standard gel formulation. The highest inhibition was found at 5 h post carrageenan injection, which is supposed to be due to inhibition of late phase mediators, arachidonic acid product and prostaglandins, of acute inflammation induced by carrageenan. (Figure 1 and 2)[35, 36, 37]

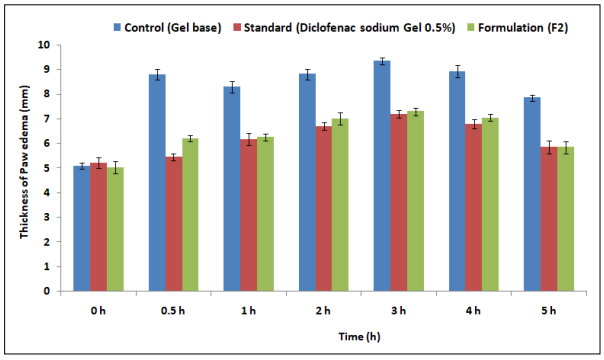

Figure 1: Anti-inflammatory activity of different gel formulations by Carrageenan induced rat paw edema

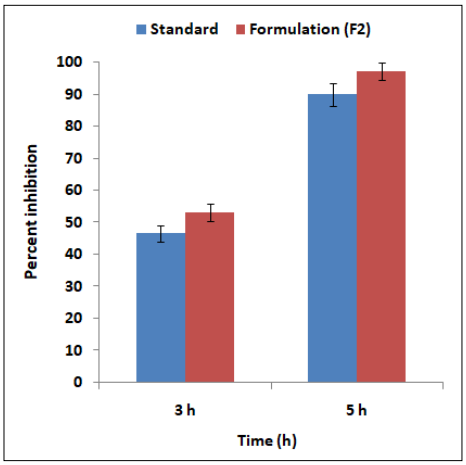

Figure 2: Percent inhibition for anti-inflammatory activity by Carrageenan induced rat paw edema

Formalin induced Paw edema

Formalin-induced rat paw edema model was used for acute as well as chronic inflammation on the basis of formalin concentration. For chronic model, 2% of formalin in saline is used. Statistical analysis showed that the edema inhibition by Formulation F2 was significantly differing from control group. The results showed that the anti-inflammatory effect of the formulation F2 was better than the effect of other gel formulation. (Figure 3)

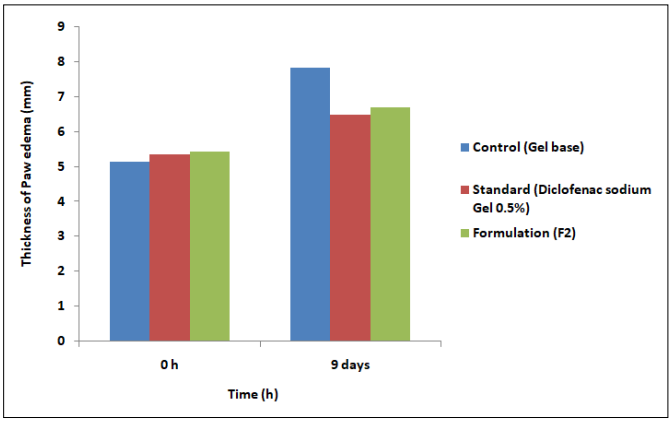

Figure 3: Anti-inflammatory activity of different gel formulations by Formalin induced rat paw edema

Randall-Selitto Test in rats

Analgesic effect of topical application of formulation F2 and standard preparations i.e. Diclofenac sodium Gel by means of Randall and Selitto's method in rats. In animals treated with formulation F2 gait was minimally affected and locomotor activity was near to normal. In control group all the animals were limping and inactive. Pain threshold in control group was significantly decreased at 72 hours. In comparison to control, there was significant increase in the pain threshold in the F2 formulation treated group. (Figure 4) Randall & Selitto who made use of the knowledge that inflammation increases sensitivity to pain and that this increased sensitivity is susceptible to modification by analgesics.

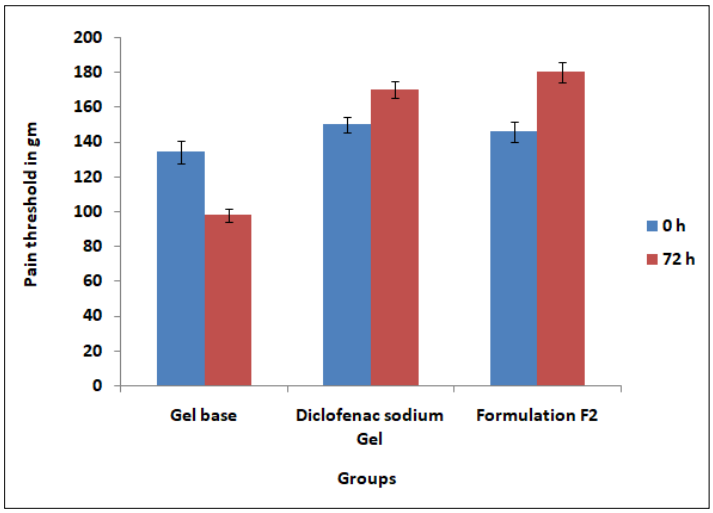

Figure 4: Comparison of Pain Threshold at 0 and 72 Hours

CONCLUSION:

Phytochemical investigation of Methanolic extract of Black pepper (*Piper nigrum*) fruits showed presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids. flavonoids, phenols, saponins, proteins, alkaloids and terpenoids. Physicochemical characteristics of Black pepper (*Piper nigrum*) fruits showed presence of alkaloids, carbohydrates, phenolic compounds, flavonoids, proteins, saponins, lipids, tannins and steroids. Physicochemical characteristics of Lodhra (*Symplocos racemosa*) Bark showed presence of Carbohydrates, Saponin, Tannins, Alkaloids, Glycoside, Flavonoids, Steroids/Triterpenes. Polyherbal anti-inflammatory gel formulation (F2) were prepared using 1 % of Carbopol 934 as a gelling agent. In-vivo pharmacological activity using Carrageenan and Formalin induced Paw edema in rats, Randall-Selitto Test in rats and in-vitro anti-inflammatory activity using inhibition of albumin denaturation showed that, F2 formulation has excellent anti-inflammatory activity as compared to marketed Diclofenac sodium gel. Hence study concluded that developed formulation can be commercialized after clinical study and successful scale-up.

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