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Evaluation of Analgesic And Anti-inflammatory Activity of Herbal Formulation Used for Mastitis in Animals

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ABSTRACT

Nature has been the source of medicinal agents for more than thousands of years, interestingly phytotherapy dominates in age old traditional system and also practice of alternative medicine across different cultures as Indian and Chinese system of medicine and Unani literature respectively. More than 70% of world's population is rely on herbal medicines for most part of their primary health care system. Investigations have been carried out from time to time to develop types of herbal formulations either singly or in combinations (Polyherbal formulations) to enhance overall therapeutic potential of the formulation. The present study was carried out to ascertain analgesic and anti-inflammatory activities of methanolic extracts of herbal combination containing equal proportion of *Diploclisia glaucescens* leaf and rhizomes of *Curcuma longa* in Wistar albino rats. The solvent yield of these extract was 16.75% and colour with dark brown to reddish brown with solid to semisolid consistency. The collected extracts were subjected to phytochemical analysis before animal experimentation, revealed the presence of all major phytoconstituents including alkaloids, flavonoids, saponins, glycosides, tannins and terpene compounds. The analgesic activity was assessed by Eddy's hot plate method and Formalin induced rat paw edema model and anti-inflammatory activity by Carrageenan induced paw edema model respectively. Significant analgesic and anti-inflammatory activity was noticed in the herbal formulation treated groups of both 200 and 400 mg/kg body weight. These test drug activity was sustained and comparable to the standard drug, Ibuprofen and Indomethacin. Thus the study can be concluded that the test drug possess significantly higher analgesic and anti-inflammatory activity possibly due to the presence of manifold secondary phytoconstituents.

Keywords: Polyherbal formulation, analgesic, anti-inflammatory activity, *Diploclisia glaucescens*, *Curcuma longa*

India witnessed a remarkable growth in production and consumption of milk and dairy products in recent years, which is emerged as important global food. India is the largest producer of milk with 16% global

production[1]. Bovine mastitis in dairy cattle is the most devastating inflammatory reaction of udder tissues caused by invasion of bacteria. Mastitis affects the entire dairy industries badly throughout the world[2] and is the major cause of economic loss to the dairy industry and its prevalence is aprime factors in determining farm probability. Reduced milk yield due to mastitis has been estimated throughout the literature to be the largest source of loss due to an occurrence of mastitis in dairy cattle[3].The economic losses due to mastitis in United States, UK, India and Worldwide has been estimated at $2 billion[2], $3.71 million, $1.1 billion (4) and $35 billion[5,6] respectively. Among the animal diseases which affect the profitability of rearing, mastitis is considered to be one of theexpensive diseases in terms of production losses[7, 8] and National Mastitis Council, a global organization for mastitis control and milk quality, estimates milk yield loss to be 70 percent of total mastitis costs[9]. Milk loss due to mastitis results in an average milk yield loss per cow of 0-9% in the first lactation and approximately 0-11% in the second lactations and beyond[10]. Regardless of the precision of this estimate, the magnitude of the loss to the industry is staggering. Predisposing factors formastitis incidence highly depend upon type of breed, stage of lactation, management practices and awareness of the dairy farmers[11]. Antibiotics have been used for the treatment of mastitis for more than 50 years and are used at two points, one is to treat outbreaks of mastitis in lactating cows and second is dry cow therapy employed to reduce subclinical infections during dry period and to prevent new infection during early lactation period[12]. In current scenario, antimicrobial investigations revealed the isolated mastitis pathogens were resistant to multifarious antibiotics including pencillin, clindamycin and cefotaxime[13]. Therefore, there is a need to explore alternative approaches for the treatment of mastitis. This research work focus on the use of herbal mixture used in the treatment of bovine mastitis and highlights their importance as an alternative natural product therapeutic resource[14].

Herbal treatments have been found to be as effective as conventional antibiotics in some situations for treating or preventing mastitis in dairy cattle. The herbal remedies contain ingredients that serve as antibacterial and strong anti-inflammatory agents[15]. The present study makes an attempt to unravel the analgesics and anti-inflammatory activity of herbal formulation used for the mastitis in animal by the locals of Wayanad districts of Kerala. The herbal formulation contains a combination of *Diploclisia glaucescens* leaves and rhizomes of *Curcuma longa* at equal ratio.

Diploclisia glaucescens (Blume) Diels is a deciduous climbing shrub belongs to the family *Menispermaceae*, commonly occurs in moist and semi evergreen forests up to 1500 m and distributed in India, Sri Lanka, southern China and Southeast Asia[16]. The leaf extract is given to drink frequently among Tanchangyas for rapid cure from diarrhoea in Rangamati (Bangladesh). Powdered leaf with milk given in biliousness, gonorrhea and syphilis and the stem of this plant used in diabetes, kidney stone and asthma in India. The genus Diploclisia belonging to tribe *Menispermae* contains a good quantity of benzylisoquinolines[17]. The studies on seeds, root and stem of *D. glaucescens* reported isolation of 20- Hydroxyecdysone as major constituent along with proaporphine alkaloid stepharine, triterpinoids serjanic acid, phytolaccagenic acid and their glycosides[18].

Ethno medically, *Diploclisia glaucescens* plant leaves along with gingelly oiland coconut (*Cocos nucifera*) oil 100 ml each is heated and applied externally for sprain. Turmeric (*Curcuma longa* Linn) is extensively used as a spice and grown widely throughout Indian subcontinent. Turmeric plant has been used in traditional medicine as a remedy for various diseases including cough, diabetes and hepatic disorders. For the last few decades, extensive works have been done to establish the pharmacological actions of turmeric and its extracts[19]. Several medical properties have been attributed to *Curcuma longa* Linn. Rhizome

and has been used by medical practitioners as an anti-diabetic[2, 3, 20, 21-23], hypolipidemic[2,3, 20,24, 25-27], anti-inflammatory[2,24, 27], anti-diarrhoeal[24], hepatoprotective[23,24], anti-asthmatic[25,27] and anti-cancerous drug.

MATERIALS AND METHODS

Collection and Identification of Plant materials: The matured fresh leaves of *Diploclisia glaucascens* and rhizomes of *Curcuma longa* were collected from the outfield of Wayanad regions of Kerala situation at latitude DMS 11°37'43.34"N, longitude DMS 76°4'52.5"E and elevation of 773.4 Meters (2537.39 Feet). The plant materials were identified and authenticated taxonomically at MS Swaminathan Research Foundation, Puthoorvayal, Wayanad respectively.

Preparation of Methanolic extract: The plant parts were washed with distilled water to remove dirt and soil and cutted in to small pieces and shade dried. The dried materials were powdered and passed through a 60 mesh sieve. The herbal preparation was formulated by mixing equal quantity of *Curcuma longa* rhizome powder and *Diploclisia glaucascens* leaves powder respectively. The powdered materials were extracted in a Soxhlet apparatus using methanol as solvent by continuous heat extraction for about 70 hrs. The solvent was removed from the extract in a rotary vacuum evaporator and the extract subsequently dried in an oven at 45 ^{o}C to obtain a solid mass of the crude extract which was kept in -20^{o}C prior to use. For experimental purpose the alcohol extract was prepared in distilled water containing 2% v/v Tween 80 (as a suspending agent).

Animals: For all the experimental purpose Wistar albino rats weighing 150-200 gms were used for the study. The animals were maintained under standards environmental conditions of Temperature, illumination, light and dark cycle. The requisite permission for animal experimentation were duly obtained from Institutional Animal Ethical Committee before commencing of the experiment with no. IAEC/COVAS/PKD/4/2018 dated 13.04.2018.

Determination of percent yield of the plant extract: The extract obtained with the solvent was weighed and the percentage yield was calculated in terms of dried weight of the plant material. The colour and consistency of the extract were also noted. Percentage yield of the plant extract was calculated according to the formula[28]. The present study showed an yield of 16.75 %.

$$\% \text{ Yield of the extract} = \frac{\text{Final weight of the extract}}{\text{Initial weight of the powder}} \times 100$$

Phytochemical analysis: The extracts and fractions were subjected to qualitative phytochemical tests for alkaloids,tannins flavonoids, saponins,steroids and triterpenoids, amino acids, proteins and carbohydrates adopting the standard procedures[29,30].

Evaluation of Analgesic activity by Hot Plate Method: The central analgesic activity against thermal stimulus was evaluated in rats following hot plate method. Rats were screened for analgesic activity by placing them on Eddy's hot plate maintained at 55 ± 0.1^{o}C and reaction time were recorded in seconds for forepaw licking or jumping. Only rats which reacted within 15sec and which did not show large variation when tested on four separate occasions, each 20 min apart, were taken for the test. All the test and standard drugs were administered 1 hour prior to placing the animal in hot plate. The response time was noted as the time at which animals reacted to the pain stimulus either by paw licking or jump response,

whichever first appeared recorded by a stop-watch. A cutoff period of 30 sec was maintained to avoid damage to the paw. The control group (I) was orally administered distill water at the rate of 10 ml/kg and standard group (II) receiving 100 mg/kg p.o. Ibuprofen. Similarly group III and IV were given 200 mg/kg and 400 mg/kg of the methanolic extract of herbal formulation consisting of *Diploclisia glaucescens* leaves and *Curcuma longa* rhizomesin equal ratio respectively. The drugs or vehicle were administered orally and the reaction time was observed at 0, 15, 30, 60 and 120 min after drug administration[31-34].

Evaluation of Formalin test to assess the analgesic activity in rats: The formalin-induced paw licking was studied in rats using the method of (39, 40). In this method, 100µl of 3% formalin was injected into the subcutaneous tissue on the plantar surface of the right hind paw of rats one hour after oral administration of the extract @ 200 mg/kg & 400 mg/kg, Indomethacin @ 10mg/kg body wt. and distilled water@ 10 ml/kg. In each rat the pain like behaviour (biting / licking and flinching of the injected paw) were observed as soon as the injection was given (i.e. early phase, 0-5min post-injection) and in the late phase (20-30 min post-injection) after injection. The mean of the time spent on licking the injected paw in each group was recorded[35].

Evaluation of Anti-inflammatory activity:A total of 24 animals were selected randomly and divided in to four groups, each group having six animals. The animals were fasted for 24 h with free access to water prior to experiments. Approximately 100 µl of 1% carrageenan suspension (prepared 1 h before each experiment) was injected into the plantar surface of the left hind paw of all the four groups of rats(36,37) and the site of injection was marked. Rats of group I (positive control group) received only distilled water, similarly Ibuprofen @ 100 mg/kg body wt, herbal formulation low dose @ 200mg/kg and herbal formulation high dose @ 400 mg/kg were given orally to animals 1 h before carrageenan injection as group II, III and IV respectively. The anterio-posterior diameter of the rat paw was measured at 0 – 6 hrs in hourly interval and 24 hrs after carrageenan injection using digital micrometer at the marked site. The difference between the basal value of paw diameter and that measured at different time intervals was noted in millimeters and the average increase in paw volume of each group was calculated and compared with the positive control (distilled water) and the indomethacin (10 mg/kg orally) groups[38-43].

Statistical analysis

All data were expressed as Mean ± SEM. The statistical significance was determined using One way Analysis of Variance (ANOVA) followed by the Duncan's Multiple Range test (DMRT). A $p< 0.05$ was considered as an indication of a significant difference.

Results and Discussion

The world health organization (WHO) have listed more than 21,000 species of plants around the world for medicinal purposes. Among them, over 7500 species of plants were estimated to be used by 4365 ethnic communities for human and animal health care in India[44] and about 2500 species of plants belongs to more than 1000 genera being used by indigenous system of medicines[45]. Among the plant rich countries, India ranks 10th position of the world and fourth among the Asian countries, the Western and Eastern Ghats harbors about 5,332 endemic species of higher plants[46]. There are about twenty five global hotspot has been identified so far, of which Eastern Himalayas and Western Ghats of India have significance[47]. The traditional practices of the present combination was successfully used by locals of

Western Ghats regions of Wayanad district of Kerala for effective treatment of mastitis in animals. The phytochemical analysis of the herbal formulation revealed the presence of all major secondary metabolites viz. alkaloids, flavonoids, glycosides, phenolic compounds, tannins and saponins, the results of the study was supported by others findings[48-53]. The results of the preliminary phytochemical screening provide an empirical basis for the use of medicinal plants in traditional therapy. The phytochemical constituents are responsible for the biological and pharmacological actions of these plants. The detailed secondary metabolites and their medicinal properties has been listed in Table 2.

Table 1: Results of Preliminary phytochemical analysis of Methanolic extract of Herbal formulation containing *Diploclisia glaucescens* leaves and *Curcuma longa* rhizomes

Sl. No.	Phytoconstituents		Crude formulation
1	Alkaloids	Dragendroff's test	Present
		Wagner's test	
		Mayer's test	
		Hager's test	
2	Flavonoids	Lead acetate test	Present
		Sodium hydroxide reagent test	
		Ammonium test	
3	Glycosides	Bromine water test	Present
		Borntrager's test	
		Keller – Killiani test	
4	Steroids	Salkowski test	Present
		Leiberman Burchardt test	
5	Tannins	Lead acetate test	Present
		Ferric chloride test	
		Gelatin test	
6	Phenolic compounds	Gelatin test	Present
		Ferric chloride test	
		Ellagic acid test	
7	Terpenoids	Salkowski test	Present
		Leiberman Burchardt test	
8	Saponins	Foam test	Present
9	Gums and mucilages		Absent
10	Carbohydrates	Fehling's test	Present
		Benedicts test	
11	Oils and Fats		Absent
12	Proteins and amino acids	Biuret test	Absent
		Ninhydrin test	

The hot plate method indicated the central analgesic effect of the methanolic extract of the herbal formulation containing leaves of *Diploclisia glaucescens* and rhizomes of *Curcuma longa* was significant and dose dependent as revealed by the increased reaction time after giving the thermal stimulus to rats. The latency in the reaction time was continued up to 120 minutes after the administration of test drugs

at the dose of 200 and 400 mg/kg body wt orally and the extract revealed sustained and pronounced central analgesic activity. However, the analgesic activity was little lower as compared to standard drug, Ibuprofen respectively. The herbal extracts showed a significant differences on comparision with the control and standard treated groups, but the dosage groups of 200 and 400 mg/kg body wt. does not have any significance, even at the lower dosages the extract exhibited central analgesic activity respectively (Table 3)[68,69].

Table 2: List of secondary metabolites and their medicinal values

Phytoconstituents	Medicinal properties
Alkaloids	Anti-microbial, sedative, relaxant, anti-spasmodic; used to treat tumors, nocturnal leg cramps, diarrhoea, psychiatric and palpitation[54]
Flavonoids	Anti-oxidant, strengthens capillary walls, reduces osteoporosis, improves blood cholesterol levels, and lowers risk of cancer and coronary heart diseases[55-57]
Steroids	Aphrodisiac, reduces cholesterol levels, affects immune system and tumor cells[58-60]
Terpenoids	Anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, anti-cancer; inhibits cholesterol synthesis[61]
Saponins	Anti-inflammatory, anti-hepatotoxic, hypoglycemic, anti-microbial and anti-viral; used in detergents and molluscicides[62,63]
Tannins	Anti-fungal, anti-biotic, anti-inflammatory, analgesic, astringent and wound healing[64, 65]
Phenols	Anti-inflammatory, anti-oxidants, anti-cancer, anti-septic[66, 67]
Glycosides	Sedative, muscle relaxant, diuretic[58]

Table 3: Analgesic activity of Herbal formulation (HF-1) in Eddy's Hot Plate model in *Wistar albino* rats

Sl No.	Groups	Dose (mg/kg)	Reaction time in seconds				
			0 min	30 min	60 min	90 min	120 min
1	Control	10ml/kg DW	6.28 ± 0.10	6.44 ± 0.16^{c}	6.33 ± 0.14^{c}	6.44 ± 0.16^{c}	6.36 ± 0.11^{c}
2	Ibuprofen	100 mg/kg	6.22 ± 0.10^{C}	11.0 ± 0.62^{aB}	18.43 ± 0.93^{aA}	18.1 ± 0.86^{aA}	18.00 ± 0.84^{aA}
3	HF1	200 mg/kg	6.50 ± 0.16^{B}	8.90 ± 0.42^{bAB}	11.80 ± 0.95^{bA}	11.6 ± 0.93^{bA}	11.47 ± 0.92^{bA}
4	HF1	400 mg/kg	6.32 ± 0.18^{C}	8.7 ± 0.21^{bB}	13.61 ± 0.40^{bA}	13.5 ± 0.4^{bA}	11.31 ± 0.4^{bA}

Values are expressed as Mean ± SEM, n=6 in each group, HF- Herbal Formulation

Means with different superscripts ($^{a, b, c}$) are statistically ($p<0.05$) significant between the groups within the reaction time (in seconds) parameter.

Means with different superscripts ($^{A, B, C}$) are statistically ($p<0.05$) significant within the groups between the variable reaction time (in seconds) parameter.

The circumference of paw volume measurement was depicted in table 04 respectively. The development of edema in the paw of the rat after injection of formalin is a biphasic event. Inflammation induced by formaldehyde is biphasic, an early neurogeniccomponent is mediated by substance P and bradykinin followed by a tissue mediated response wherehistamine, 5-HT, prostaglandins and bradykin in are known to be involved[70]. The initial phase of the edema is due to the release of histamine and serotonin and the edema is maintained during the plateau phase by kinin like substance[71] and the second accelerating phase of swelling due to the release of prostaglandin like substances. Hence, it is speculated that apart from inhibition of chemical mediators of inflammation, poly-herbal formulation may also modulate the

pain response in the central nervous system. There was a significant dose-dependent inhibition in both phases of the formalin-induced pain response in rats, with a more potent effect in the second phase. Indomethacin also inhibited pain in both phases significantly compared to control and herbal formulation at low and high doses.

Table 4: Analgesic activity of Herbal formulation (HF-1) in Formalin induced paw edema model in *Wistar albino* rats

Sl No.	Groups	Dose (mg/kg)	No. of Licking / Biting		Duration of Paw licking / Biting	
			0-5 min	20-30 min	0-5 min	20-30 min
1	Control	0.1 ml of 3% Formalin	15.17±0.74^{a}	25.83±0.94^{a}	93.50±1.43^{a}	115.33±2.95^{a}
2	Indomethacin	10 mg/kg	6.83±0.60 ^{d}	14.5±0.92^{d}	22.67±1.49^{d}	57.50±1.31^{d}
3	HF1	200 mg/kg	13.0±0.63^{b}	20.67±0.67^{b}	73.00±1.43^{b}	81.50±1.50^{b}
4	HF1	400 mg/kg	10.67±0.33^{c}	17.17±0.91^{c}	52.00±2.23^{c}	72.50±2.88^{c}

Values are expressed in Mean ± SEM, n=6 in each group, HF- Herbal Formulation

Means with different superscripts ($^{a, b, c}$) are statistically ($p<0.05$) highly significant between the groups in the single reaction time (in seconds) parameter.

Table 5: Anti-inflammatory effect in Carrageenan induced paw edema in rats

Sl No.	Groups	Dose (mg/kg)	Reaction time in seconds							
			0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
1	Control	0.1 ml of 1% Carrageenan	3.20 ± 0.11^{C}	6.67 ± 0.12^{A}	6.79 ± 0.09^{A}	6.90 ± 0.08^{A}	6.80 ± 0.04^{A}	6.72 ± 0.04^{A}	6.61 ± 0.07^{Aa}	6.10 ± 0.07^{Ba}
2	Ibuprofen	100 mg/kg	3.14 ± 0.09^{D}	6.66 ± 0.07^{AB}	6.81 ± 0.05^{AB}	7.00 ± 0.06^{A}	6.65 ± 0.08^{AB}	6.42 ± 0.09^{AB}	6.07 ± 0.11^{Bb}	4.71 ± 0.33^{Cb}
3	HF1	200 mg/kg	3.29 ± 0.06^{C}	6.74 ± 0.15^{A}	6.82 ± 0.13^{A}	6.89 ± 0.09^{A}	6.76 ± 0.07^{A}	6.67 ± 0.07^{A}	6.53 ± 0.08^{Aab}	5.80 ± 0.12^{Ba}
4	HF1	400 mg/kg	3.20 ± 0.10^{E}	6.84 ± 0.06^{AB}	6.91 ± 0.02^{A}	7.02 ± 0.05^{AB}	6.68 ± 0.10^{A}	6.48 ± 0.08^{BC}	6.26 ± 0.08^{Cab}	5.14 ± 0.02^{Db}

Values are expressed in Mean ± SEM, n=6 in each group, HF- Herbal Formulation

Means with different superscripts ($^{a, b, c}$) are statistically ($p<0.05$) significant between the groups within the reaction time (in seconds) parameter.

Means with different superscripts ($^{A, B, C}$) are statistically ($p<0.05$) significant within the groups between the variable reaction time (in seconds) parameter. The inflammation of the paw induced by carrageen an agent, was showed a significant decrease in all the standard and test treated groups respectively. The inhibition of the acute inflammation in rat paws at the doses of 200 and 400 mg/kg body wt. exhibited an anti-inflammatory activity that became significant ($P<0.05$) and was maintained all along the experiment. However, the anti-inflammatory effect of standard drug, Ibuprofen was greater than that of the extract as presented in table 5[72].

CONCLUSION

The detailed scientific evaluation of the pharmacological properties namely analgesic and anti-inflammatory activities of the botanical medicine namely *Diploclisia glaucescens* leaf and rhizomes of *Curcuma longa*

in combination clearly exhibited its therapeutic efficacy which was found to be comparable to that of standard drugs. The test drug was found to be significantly effective and having sustained effects during the study. Many of these effects could be attributed due to the presence of secondary metabolites. However, further detailed study is needed to identify the responsible biochemical marker compound in the test drug

Conflict of Interest: None Declared.

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