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In-vitro anti-inflammatory activity of oral poly herbal formulations

Rashmi Yadav and Dr. Vijender Singh Mahalwal

Abstract
Aim: To evaluate the *In-vitro* anti-inflammatory activity of Oral poly herbal formulations.
Methodology: The *In-vitro* anti-inflammatory activity was investigated by protein denaturation method using Egg's albumin and Bovine serum albumin. The Hydro-alcoholic extracts of the plants used for the preparation of six poly herbal formulations. *In-vitro* anti-inflammatory activity of all poly herbal formulations were estimated by protein denaturation method using Egg's albumin and Bovine serum albumin at 50 - 250 µg/ml concentrations. The result was assessed UV spectrophotometer at 660nm and compared with the diclofenac sodium as standard drug.
Result: The result revealed that the all six oral poly herbal formulations possessed significant anti-inflammatory activity. But the formulations F5 and F6 exhibited the maximum percentage inhibition of Protein denaturation at 200µg/ml concentration 86.07% (using Egg's albumin) and 85.14% (using Bovine serum albumin) as compared to others formulations. The standard drug diclofenac sod. showed 98.06, 97.91% inhibition for Bovine serum and Egg's albumin methods, respectively.
Conclusion: The study concluded that the formulations is an effective inhibitor of protein denaturation and showed potent anti- inflammatory activity.

Keywords: *In-vitro* anti-inflammatory activity, Protein denaturation, UV spectrophotometer, Egg's albumin, Bovine serum albumin.

Introduction
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 (Amoolya *et al.*, 2017; jeena *et al.*, 2013) [2, 5]. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of protein is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was studied (Reshmaeta *et al.*, 2014; Sundar *et al.*, 2015) [8, 10]. The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role. It can be metabolized by the Cyclooxygenase (COX) pathway to prostaglandins and thromboxane A2, or by the 5-lipoxygenase (5-LOX) pathway to hydroperoxy-eicosatetraenoic acids (HPETE's) and leukotrienes (LT's), which are important biologically active mediators in a variety of inflammatory events. Upon appropriate stimulation of neutrophils, arachidonic acid is cleaved from membrane phospholipids and can be converted to leukotrienes and prostaglandins through 5-LOX or COX pathways respectively. Inhibition of 5-LOX and COX-leads to decreased production of LTs and PGs, such a drug would have the potential to provide anti-inflammatory and analgesic effects with a reduction in the GI side effects (Sunder *et al.*, 2015; Mizushima *et al.*, 1968) [7, 10].
Inflammation is a complex biological response of vascular tissue to harmful stimuli, pathogens, irritants characterized by redness, warmth, swelling and pain. Prolonged inflammation leads to the rheumatoid arthritis.
Anti-inflammatory drugs like NSAIDs used to reduce the swelling and pain of inflammation. But these agents carry the risk of gastro-intestinal toxicity, cardiovascular and other side effects for prolonged use. Therefore to overcome this problem, there is a need for prepared safe and potent therapeutic activity herbal drug.
The objective of present work is to evaluate the *In-vitro* anti-inflammatory activity of oral poly herbal formulations.

Collection and authentication
The plants material were collected from local market (karibavali) of New Delhi. All plants were identified as aerial part of *Vitex negundo* Linn., *Withania somnifera* Dunal., *Ficus rosamosa* Linn., *Berberis aristata* DC., *Tinospora cordifolia* Miers.,by Dr. H.B Singh Ex. chief Taxonomist, National Institute of Science Communication and Information Resources (NISCAIR) New Delhi and in present chief scientist Herbology, Amil pharmaceutical Pvt. Ltd.

Prepare the extracts and oral poly herbal formulations
The individual coarsed powdered of crude drugs were taken and extracted with Hydro-alcoholic (1:1) solvent by the help of Soxhlet apparatus for 72 hrs. The whole extract of individual drugs were collected in conical flasks, filtered and the solvents were evaporated to dryness under reduced pressure.
Six Poly-herbal formulations were prepared by taken the accurate weighted amounts of plants extracts presents in composition table no. 1. All oral poly herbal formulation stored in a well close containers in cool and dark place.

***In-vitro* Anti-inflammatory activity (Protein denaturation) of Oral Poly Herbal Formulations.**

Chemicals and Instruments
Bovine serum, Diclofenac sodium, Phosphate buffer, UV Spectrophotometer.

1. Anti-inflammatory activity (Protein denaturation) using Bovine serum albumin
- **0.5% Bovine Serum Albumin (BSA):** Dissolved 500mg of BSA in 100 ml of water.
- **Phosphate Buffer Saline PH 6.3:** Dissolved 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate, 0.24 g of potassium dihydrogen phosphate in 800 ml distilled water. The pH was adjusted to 6.3 using 1N HCl and make up the volume to 1000 ml with distilled water.
- **Test solution** (0.5 ml) consists of 0.45ml of Bovine serum albumin (0.5% w/v aqueous solution) and 0.05 ml of test solution of various concentrations.
- **Standard solution** (0.5 ml) consists of 0.45ml of Bovine serum albumin (0.5% w/v aqueous solution) and 0.05 ml of Diclofenac sodium of various concentrations.

Procedure
0.05 ml various concentrations (50, 100, 150, 200, 250 µg/ml) of test formulations and standard drug diclofenac sodium (50, 100, 150, 200, 250 µg/ml) were taken, respectively. Then 0.45 ml (0.5% w/v) BSA mixed in all tubes. The samples were incubated at 37 °C for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 660 nm. The control represents 100% protein denaturation. The results were compared with Diclofenac sodium (Sakat *et al.,* 2010; Leelaprakhash *et al.,* 2010; Anoop *et al.,* 2015) [3, 6, 9].
The percentage inhibition of protein denaturation can be calculated.

$$\text{Percent inhibition} = \frac{\text{Abs Control} - \text{Abs Test}}{\text{Abs Control}} \times 100$$

Where,
Abs test = Absorbance of the test sample,
Abs control = Absorbance of control.

2. Anti-inflammatory activity (Protein denaturation) using Egg's albumin
- **Test solution:** The reaction mixture (5 ml) consisted of 0.2 ml of egg's albumin (from fresh hen's egg), 2.8 ml of phosphate-buffered saline (PBS, pH 6.3) and 2 ml of varying concentrations (50,100,150,200 and 250 µg/ml) of the test sample.
- **Control solution:** 5ml consists of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate-buffered saline (PBS, pH 6.3) and distilled water volume upto 5ml.
- **Standard solution** (5ml) consists of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate-buffered saline (PBS, pH 6.3) and 2 ml of varying concentrations (50,100, 150, 200 and 250 µg/ml) of diclofenac sodium drug.

Procedure
All samples tubes were incubated at 37±2°C in a BOD incubator for 15minutes and then extend to 70°C for five minutes. After cooling, their absorbance was measured at 660 nm by using the vehicle (Godhandaraman *et al.,* 2016; Aishwarya *et al.,* 2017) [1, 4].
The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ Inhibition} = 100 \times [V_t / V_c - 1]$$

Where,
V t = Absorbance of the test sample,
V c = Absorbance of control.

Results and observations

Table 1: Oral Poly-herbal formulations compositions for 600 ml.

Plants dried extracts	Oral Formulations					
	F1	F2	F3	F4	F5	F6
Berberis aristata (EtOH: Aq extract) g	40	45	50	60	70	75
Vitex negundo (EtOH: Aq extract) g	20	25	30	32	36	38
Withania somnifera (EtOH: Aq extract) g	40	45	50	55	50	64
Tinospora cordifolia (EtOH: Aq extract) g	40	45	50	55	64	66
Ficus racemosa (EtOH: Aq extract) g	40	45	50	60	70	75
CMC (1% w/v) g	6	6	6	6	6	6
Preservative sodium benzoate (2% w/v) g	12	12	12	12	12	12

- **Protein denaturation using bovine serum albumin**

Table 2: *In-vitro* protein denaturation (Bovine serum albumin) of oral formulations

Absorbance at 660nm						
Formulations	**Concentrations µg/ml**					
	50	**100**	**150**	**200**	**250**	**Control**
F1	0.458± 0.012	0.423± 0.008*	0.392± 0.003**	0.32± 0.005**	0.263± 0.0033**	1.12±0.0166
F2	0.462± 0.0166	0.416± 0.016*	0.367± 0.013**	0.288± 0.013***	0.204± 0.0033***	
F3	0.463± 0.01	0.353± 0.008**	0.293± 0.01**	0.286± 0.01**	0.246± 0.016***	
F4	0.456± 0.02	0.353± 0.006*	0.278± 0.003**	0.246± 0.003**	0.201± 0.005**	
F5	0.401± 0.014	0.286± 0.006**	0.250± 0.01**	0.201± 0.002**	0.166± 0.006***	
F6	0.401± 0.014	0.286± 0.006**	0.250± 0.01**	0.201± 0.002**	0.166± 0.006***	
Diclofenac sod.	0.384± 0.012	0.278± 0.01	0.206 ± 0.002	0.131± 0.0033	0.021± 0.0033	

- Values are expressed in Mean ±SEM (n=3). The *P* values <0.05*, *P*<0.01**, *P*<0.001*** statistical significant when compared with Diclofenac sodium using one way ANOVA test followed by Dunnett's t- test

Table 3: % Inhibition of protein denaturation (Bovine serum albumin) of oral formulations

% Inhibition							
Concentrations µg/ml	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**Diclofenac sod.**
50	59.07	58.69	58.63	59.25	64.13	64.13	65.71
100	62.17	62.79	68.45	68.42	74.40	74.40	75.14
150	64.94	67.20	73.80	75.14	77.64	77.64	81.54
200	71.30	74.22	74.43	78.00	82.02	82.02	88.27
250	76.45	74.58	78.00	81.99	85.14	85.14	98.06

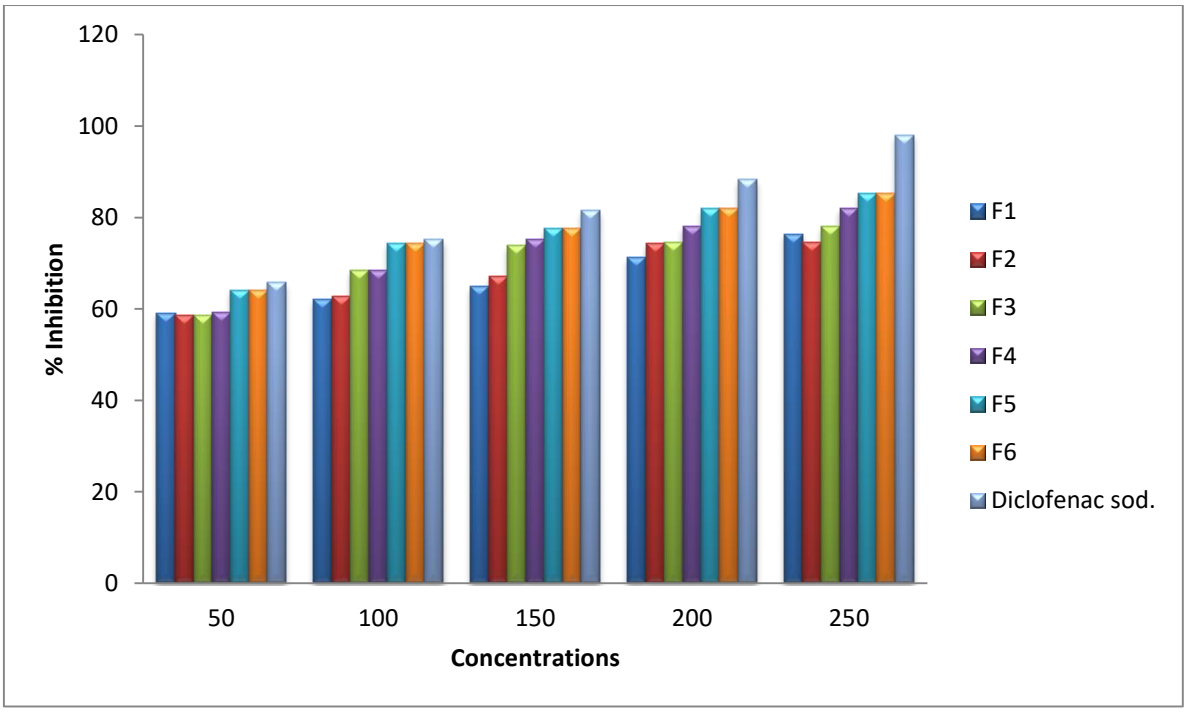

Fig 1: % Inhibition Vs. concentration of protein denaturation (Bovine serum albumin) of oral formulations

- **Egg's albumin protein denaturation method**

Table 4: In- vitro protein denaturation (Egg's albumin) of oral formulations

Absorbance at 660nm						
Formulations	**Concentrations µg/ml**					
	50	**100**	**150**	**200**	**250**	**Control**
F1	0.403± 0.0066	0.368± 0.0033*	0.349± 0.0066*	0.274± 0.0033**	0.258± 0.0033**	1.18± 0.00088
F2	0.405± 0.0033	0.355± 0.0033*	0.327± 0.0066**	0.276± 0.0033**	0.265± 0.0033**	
F3	0.385± 0.0033	0.316± 0.0033*	0.254±3 0.0066**	0.212± 0.0033**	0.191± 0.0033**	
F4	0.356± 0.0033	0.293± 0.00566*	0.253± 0.0057**	0.224± 0.0088**	0.193± 0.00577***	
F5	0.355± 0.0033	0.276± 0.0057*	0.231± 0.0088**	0.193± 0.00133**	0.164± 0.0066***	
F6	0.355± 0.0033	0.276± 0.0057*	0.231± 0.0088**	0.193± 0.00133**	0.164± 0.0066***	
Diclofenac sod.	0.344± 0.00333	0.24± 0.00333	0.200± 0.00667	0.171± 0.001	0.02± 0.00333	

- Values are expressed in Mean ±SEM (n=3). The P values <0.05*, *P*<0.01**, *P*<0.001*** statistical significant when compared with Diclofenac sodium using one way ANOVA test followed by Dunnett's t- test.

Table 5: % Inhibition of protein denaturation (Egg's albumin) of oral formulations

Concentrations µg/ml	**% inhibition**						
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**Diclofenac sod.**
50	65.81	65.64	67.31	69.77	69.85	69.85	70.81
100	68.75	70.22	73.16	75.16	76.55	76.55	79.66
150	70.42	72.28	78.41	78.53	80.42	80.42	82.99
200	76.75	76.55	81.97	81.01	83.61	83.61	85.45
250	78.07	77.48	83.78	83.61	86.07	86.07	97.91

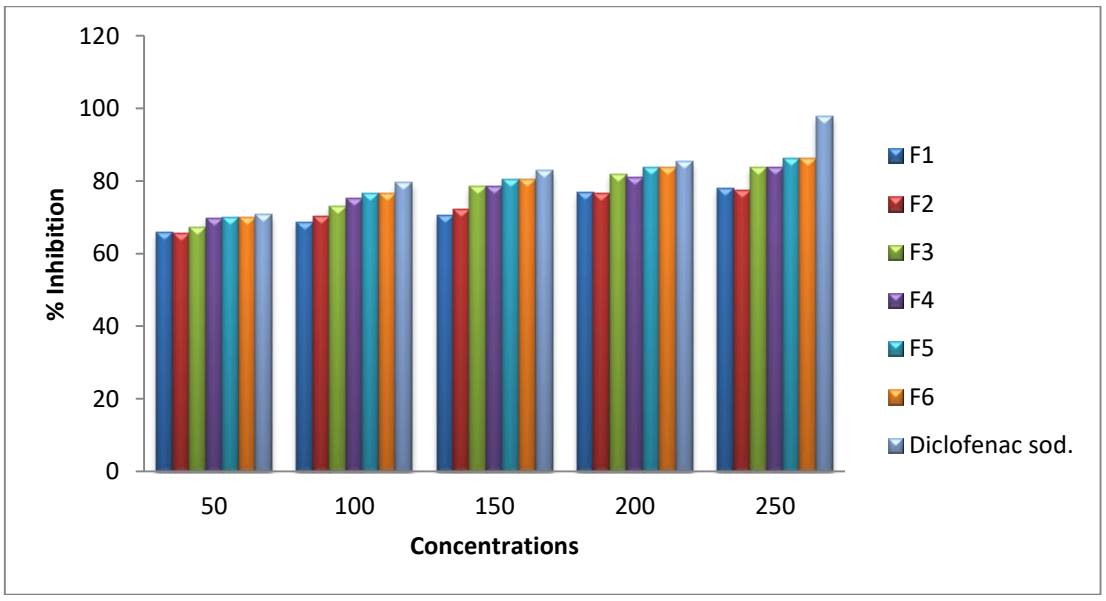

Fig 2: % Inhibition Vs. concentration protein denaturation (Egg's albumin) of oral formulations

Discussion

***In-vitro* protein denaturation (Bovine serum albumin) of oral formulations.**

In this study all oral poly herbal formulations were exhibited significant anti-inflammatory activity by protein denaturation inhibition method (using Bovine serum albumin) at concentration of 50, 100,150, 200 and 250 µg/ml.

The percentage inhibition of protein denaturation of oral formulations F1, F2, F3, F4, F5 and F6 at 250 µg/ml showed 76.45, 74.58, 78.0, 81.99, 85.14 and 85.14%, respectively, whereas standard diclofenac at 250 µg/ml showed 98.06%. The formulations F5 and F6 exhibited maximum % inhibition 85.14% as compare to other formulations.

***In-vitro* protein denaturation (Egg's albumin) of oral formulations.**

In this study all oral poly herbal formulations were exhibited significant anti-inflammatory activity by protein denaturation

inhibition method (using Egg's albumin) at concentration of 50, 100,150, 200 and 250 µg/ml.

The percentage inhibition of protein denaturation of oral formulations F1, F2, F3, F4, F5 and F6 at 250 µg/ml showed 78.07, 77.48, 83.78, 83.61, 86.07 and 86.07%, respectively, whereas standard diclofenac at 250 µg/ml showed 97.91%. The formulations F5 and F6 exhibited maximum % inhibition 86.07% as compare to other formulations.

Conclusion

In the in- vitro anti-inflammatory study conducted on the oral poly-herbal formulations, results indicate that the all oral poly herbal formulations possess anti-inflammatory properties. These activities may be due to the strong occurrence of compounds such as alkaloids, flavonoids, tannins, steroids, and phenols in the composition's plants extracts. But the poly herbal formulation F5 and F6 showed the more potent and significant anti-inflammatory activity as compared to others formulations. Hence it can be used effectively in the management of Rheumatoid arthritis after the further In-vivo evaluation and clinical studies.

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