EVALUATION OF ANTIARTHRITIC POTENTIAL AND PHYTOCHEMICAL ANALYSIS OF DIFFERENT FRACTIONS OF SELECTED MEDICINAL PLANTS

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ABSTRACT

Arthritis is a chronic inflammatory condition that is a major disability throughout the world. For so many years there are several therapies used for the treatment but they have many disadvantages like side effects, poor bioavailability, high cost etc. So, researches are going on for the development of alternative effective therapy for arthritis. The current study explores anti-arthritic activity (by targeting pro-inflammatory cytokines like IL-6) and phytochemical analysis of different fractions of *Capsicum annum*, *Boswellia serrata* and *Lawsonia inermis*. In this study overall seven fractions were prepared with the help of fractionation. They were coded as CO, COHF, COMF, BPS, HMEP, HMAP and HAMF. The anti-inflammatory activity of the fractions was evaluated by using an *in-vitro* IL-6 and TNF- α ELISA study. The non-toxic concentration of the study samples was determined by MTT assay. High-Performance Thin-Layer Chromatography was used for qualitative analysis of phytoconstituents present in the fractions. Liquid Chromatography-Mass Spectroscopy was used for the identification of phytoconstituents present in the fractions of COMF, BPS and HMEP. HPTLC analysis revealed the presence of capsaicin, galactose, arabinose, luteolin, β -sitosterol, LCMS analysis showed the presence of capsaicin, luteolin, lalioside and arabinose.

Keywords: Arthritis, Inflammation, Fractionation, *In-vitro* study, HPTLC, Tumour necrosis

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INTRODUCTION

Arthritis is a combinatorial word originated by the mixing of Latin word “Arthron” which signifies joint and Greek word “Itis” inflammation. It is an incessant, incendiary and systemic autoimmune disease that principally affects the joints of the human body. There are several forms of arthritis are there, they are; Autoimmune (Rheumatoid arthritis (RA), Reiter syndrome etc.), Degenerative like Osteoarthritis (OA), Crystal deposition (Gout) etc. Out of those RA and OA are most commonly affected the human body. The initial symptoms are weakness, musculoskeletal agony and stiffness and it affects human joints after some months. The small joints are affected initially but later bigger joints are likewise influenced, become swollen, warm and agonizing.¹

The prevalence of arthritis is increased worldwide nowadays. Some facts proved that it becomes a major disease and many people are affected by it. From 2013 to 2015 in the United States², individuals matured between 18 to 44 years, 7.1% reported having arthritis. In general, 26% of women and 19% of 1% of men were accounted for to have arthritic the the US from 2013-2015.² In India it is seen that osteoarthritis is prevalent among all forms of arthritis.³

The exact pathophysiology of this disease is yet to be known but hypotheses said that several free radicals name nitrous oxide and superoxide radicals are generated as byproducts due to cellular metabolism. Interleukins and Tumour necrosis factor (TNF- α) which are produced from T-cells may be induced by the release of those free radicals which influences the growth factors production, tissue destruction and inflammation may happen due to the cytokines and adhesive molecules on the immune.⁴ The pathological changes seen in arthritic patients are synovial membrane hyperplasia, cartilage erosion and articular destruction are seen due to the inflammatory cells infiltration and neovascularization.⁵

The prolonged use of the conventional treatment against arthritis leads to adverse reactions, including severe infections. So the research is going on for the development of relatively effective alternatives

but less expensive therapies with minimal or no side effects for treating arthritis.⁶ Here natural plant products become vital and promising resources for developing new therapeutically active pharmaceutical products for the treatment of arthritis. From the knowledge and detailed survey of the medicinal plants, the discovery of new drugs might be possible which are therapeutically effective and also cheaper.⁷

In this study, three medicinal plants were taken as *Capsicum annum*, *Boswellia serrata* and *Lawsonia inermis*.

Capsaicinoids are the major active constituent of *C. annum*, composes of capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin and nonivamide.⁸ The capsaicin is currently used for treating osteoarthritis.⁹ It has a membrane-stabilizing property by the interference of activation of phospholipase A2.¹⁰

Boswellic acids (BAs) are major pharmacologically active constituents of *B. serrata*.¹¹ There are six major BAs were reported, they are α and β -Boswellic Acids (BA, 10–21%), Acetylated α and β -Boswellic Acids (ABA, 0.05–6%), 11-keto- β -Boswellic acid (KBA, 2.5– 7.5%) and 3-O-acetyl-11-keto- β -Boswellic acid (AKBA, 0.1–3%).¹² It also contains polysaccharides which is the combination of arabinose with a minute quantity of xylose and galactose.¹³ It is seen that the population of leucocytes is reduced by the oral administration of BAs, it also inhibits polymorphonuclear leukocytes migration in vitro and electrophoretic pattern of synovial fluid protein was also changed in bovine serum albumin (BSA) induced arthritis.¹⁴

The plant *L. inermis* is widely found in India, contains a natural dye Lawsone¹⁵ Other important compounds present in the leaves are flavonoids (luteolin, apigenin etc.), Coumarins (esculetin, fraxetin, scopletin) and Steroids (β -sitosterol).¹⁶ Two compounds named Isoplumbagin and lawsaritol from the stem bark and root of *L. inermis* showed anti-inflammatory effect against Carrageenan induced paw oedema model. At 100 mg/kg oral dose isoplumbagin and lawsaritol showed 61 and 60% inhibition along with 40% inhibition showed by phenylbutazone in comparison with controls. Isoplumbagin showed potent anti-inflammatory activity.¹⁷ Butanol and chloroform fractions showed more potent anti-inflammatory, pain-relieving and antipyretic effects than aqueous fraction of crude ethanol extract of *L. inermis* in a portion subordinate way.¹⁸

EXPERIMENTAL

Material and Methods

Collection and Authentication of Plant Materials

All the herbs were collected and authenticated by the Pharmacognosy department, The Himalaya Drug Company, Bangalore, Karnataka, India.

General Procedure

Extraction and Fractionation of Plant Materials

Extraction and Fractionation of Capsicum oleo Resin from *C. annum*

Dried 5 kg of Red chilli was coarsely powdered and refluxed with 15 L acetone at 55°C for 1h, keep it for 30 minutes and filter. The extraction process was repeated twice under the same conditions. All three filtrates were concentrated in a vacuum rota evaporator. About 200 g of oleo resin was obtained and decoded as CO (capsicum oleo resin) for further study. The fractionation of CO was done by using different solvents.

Oleo resin of about 200g was fractioned with 4 L hexane. The hexane fraction was concentrated under vacuum and decoded as COHF (hexane fraction). Hexane insoluble portion was dissolved in 200 ml chloroform and partitioned with 2 L of demineralized water. Dried the organic layer separately and discarded the aqueous layer. The organic layer was redissolved with 400 ml of methanol and allowed to stand for overnight. The methanolic layer was gently decanted and partitioned with 10 L hexane. The methanolic layer was collected and concentrated in Rotavapour Buchi and decoded as COMF (methanol fraction).

Extraction of Boswellia and Estimation of Polysaccharides

About 5 kg of Boswellia oleo gum resin was taken to which water was added and heated at 90-100°C under stirring for 3 h. Extraction was repeated thrice under the same conditions and the extracts were filtered through 100 mesh muslin cloth. All filtrates were combined and concentrated using a water bath. A dried extract of about 600g was obtained.

About 10g of the aqueous extract of *B. serrata* was taken and 50 ml of methanol was added, refluxed at 70°C for 25 minutes. The procedure was repeated thrice and discarded the filtrate. The marc obtained was subjected to hydrolysis reaction. Thereafter 50 ml dilute hydrochloric acid was added and refluxed at 100 °C for 3 h, the mixture was allowed to stand for 20 minutes and filtered, pH was adjusted between 6.5-7 using ammonia and the filtrate was evaporated up to 20 ml. Then 200 ml of ethanol was added under magnetic stirrer and allowed it to stand for 1h. The mother liquor was collected and concentrated under rota evaporator and decoded as BPS (Boswellia polysaccharides).

Extraction of *L. inermis* (Henna Leaves)

The henna powder of about 268 g was extracted with methanol. The process was repeated twice. The residue obtained was treated with water at 94 °C for 3 h. The aqueous extract was filtered and concentrated on the water bath till it reaches TS (total solid content) 40%. About 110 ml of final concentrated liquid was obtained. Both methanolic and aqueous extracts were coded as **HM** (henna methanolic extract) and **HA** (henna aqueous extract) taken for further fractionation process.

Fractionation of Aqueous and Methanolic Extract

The aqueous extract was treated with 300 ml methanol under continuous stirring, it was filtered, washed the precipitate with methanol followed by 100 ml acetone. The precipitate was dried at 65 °C which was taken as a study sample and de coded as **HAMF** (methanolic fraction). The methanolic extract of henna was taken to which 1 L of hexane was added. The hexane insoluble portion was separated and redissolved in water. Then it was partitioned with 500 ml hexane. The aqueous layer was separated and partitioned with ethyl acetate. Ethyl acetate was concentrated separately. It was decoded as **HMEP** (ethyl acetate partition) and **HMAP** (aqueous partition).

In-vitro Anti-inflammatory Study of prepared Fractions

IL-6 ELISA and TNF- α ELISA studies were used to determine the anti-inflammatory activity of the prepared fractions.

Cytotoxicity Study by MTT Assay¹⁹

The THP-1 (Human peripheral blood monocytes) cells were grown in 96 well plates in RPMI 1640 with 2% FBS and incubated for the assay. The initial stock solution of the test product (10mg/ml) was prepared and further diluted with 1:10 ratio by dissolving in RPMI 1640 medium with 2% FBS and from that sample, subsequent dilutions were made to obtain the concentration of 1000, 500, 250, 125, 62.5, 31.25 and 15.625 μ g/ml. The dilutions (100 μ l/ well) were added to the THP-1 cells and incubation was done at 37°C with 5% CO₂. Cell control was also maintained. After 24 hrs incubation, the cell viability was determined by MTT assay. In MTT assay, 10 μ l of MTT (5mg/ml) solution was added into each well and incubated for 4 hours at 37°C. The cell supernatant was discarded and the cell bound dye was extracted by adding 100 μ l of dimethyl sulfoxide (DMSO) into each well and the measurement of absorbance was done at 540 nm. The % cytotoxicity was determined from absorbance estimations of treated and control groups. The CTC₅₀ values for the sample were determined.

IL-6 ELISA (Enzyme-linked Immunosorbent Assay) Study¹⁹

The study was carried out in THP-1 cells. To activate the secretion of IL-6 cytokines the cells were exposed to 1 μ g/mL of lipopolysaccharide (LPS) and co-treated with prepared test samples. Incubation was done for 24 h at 37°C and the cell supernatant was separated by centrifugation. The content of IL-6 was quantitatively estimated according to the assay procedure provided in Kit-Krishgen Biosystem ELISA kit catalog: KB1068. Dexamethazone was used as a reference drug. All incubation steps were performed at room temperature. A microplate reader was used to determining the optical density at 450 nm.

TNF- α ELISA Study¹⁹

The study was carried out in THP-1 cells. For the activation of the secretion of TNF- α cytokines the cells were exposed to 1 μ g/ml of lipopolysaccharide (LPS). The cells were co-treated with prepared test samples and incubated for 24 h at 37°C. then, the cell supernatant was separated by centrifugation. TNF- α levels were estimated by ELISA performed according to the assay procedure provided by the manufacturer (Kit- Krishgen Biosystem ELISA kit catalog: TA0119). Dexamethazone was used as a

standard drug in this study. All incubation steps were performed at room temperature. Microplate reader was used to determining the optical density at 450 nm.

From the anti-inflammatory studies, it was found that six fractions showed good anti-inflammatory activity compared with Dexamethazone. Further HPTLC and LCMS analysis was done for the identification of phytoconstituents in the selected fractions.

HPTLC Analysis of Selected Fractions

HPTLC analysis of six fractions named CO, COHF, COMF, BPS, HMEP and HMAP was carried out.

Table-1: Mobile Phases used for the Fractions

Fractions	Mobile Phases Used	Visualizing Agents Used
CO, COHF and COMF	Toluene: Chloroform: Acetone (5.5: 2.6: 1.9)	Vanillin sulphuric acid
BPS	n-propanol: Water (8.5:1.5)	Vanillin sulphuric acid
HMEP	Toluene: Ethyl acetate: Formic acid (10:9:1) (for luteolin) Toluene: Ethyl acetate: Glacial acetic acid (14.5: 4.5: 1) (for β -sitosterol) Chloroform: Methanol (9:1) (for unknown terpenoid)	Vanillin sulphuric acid and Alcl ₃
HMAP	Chloroform: Methanol: Water (6:3.5:0.5)	Vanillin sulphuric acid

LCMS Estimation of COMF, HMEP and BPS

The LCMS estimation was carried out based on the following conditions:

Parameter	Optimization values
Ion source	Electron spray ionization (ESI turbo spray)
Declustering potential	20 V
Focusing table (FP)	400 V
Entrance potential (EP)	10 V
Curtain gas (CUR)	20 psi
Ion spray voltage (IS)	5500 V
Temperature	0°C
Nebulizer gas (GS1)	30 psi
Heater gas (GS2)	40 psi

RESULTS AND DISCUSSION

In-vitro anti-inflammatory Studies of prepared Study Samples

Cytotoxicity Study by MTT assay

Table-2: Quantitative Determination of Non-toxic Concentration of prepared Fractions on THP-1 Cells

S.No.	Name of the Fraction	CTC ₅₀ Values (μ g/ml)	Induction	Concentration of the Test Samples	Solubility
1.	CO	209.74	1 μ g LPS	200 μ g/ml 100 μ g/ml	DMSO
2.	COHF	415.34	1 μ g LPS	200 μ g/ml 100 μ g/ml	DMSO
3.	COMF	96.15	1 μ g LPS	100 μ g/ml 50 μ g/ml	DMSO
4.	BPS	829.68	1 μ g LPS	500 μ g/ml 250 μ g/ml	Aqueous
5.	HMEP	250.94	1 μ g LPS	200 μ g/ml 100 μ g/ml	DMSO
6.	HMAP	>1000	1 μ g LPS	500 μ g/ml 250 μ g/ml	DMSO
7.	HAMF	>1000	1 μ g LPS	500 μ g/ml 250 μ g/ml	Aqueous
8.	LPS Control	1 μ g	LPS	1 μ g	-
9.	Dexamethasone	100 μ M	1 μ g LPS	100 μ M	-

IL-6 ELISA Study

Table-3: Quantitative Determination of IL-6 for the Samples treated with THP-1 Cells

S. No.	Name of the Sample	Concentration Tested ($\mu\text{g/ml}$)	%IL-6 Inhibition
1.	CO	200	86.36
		100	79.03
2.	COHF	200	85.87
		100	62.92
3.	COMF	100	86.23
		50	84.47
4.	BPS	500	82.63
		250	29.66
5.	HMEP	200	82.54
		100	83.21
6.	HMAP	500	79.16
		250	58.69
7.	HAMF	500	52.30
		250	39.96
8.	LPS	1 μg	0.00
9.	Dexamethasone	100 μM	77.05

Fig.-1: Effect of Fractions on LPS (1 $\mu\text{g/ml}$) induced IL-6 production from THP-1 Cells.

TNF- α ELISA Study

Table-4: Quantitative Determination of TNF- α for the Samples treated with THP-1 Cells

S. No.	Name of the Sample	Concentration Tested ($\mu\text{g/ml}$)	%TNF Inhibition
1.	CO	200	24.43
		100	6.45
2.	COHF	200	0.00
		100	0.00
3.	COMF	100	87.09
		50	40.99
4.	BPS	500	0.00
		250	0.00
5.	HMEP	200	89.88
		100	0.00
6.	HMAP	500	0.00
		250	0.00
7.	HAMF	500	0.00
		250	0.00
8.	LPS	1 μg	0.00
9.	Dexamethasone	100 μM	71.17

Fig.-2: Effect of Fractions on LPS (1µg/ml) induced TNF-α production from THP-1 Cells

The % IL-6 inhibition of the fractions obtained were COHF- 85.87 and 62.92, COMF- 86.23 and 84.47, HMEP- 82.54 and 83.21, HAMF- 52.30 and 39.96 and HMAP- 79.16 and 58.69, CO- 86.36 and 79.03 and BPS- 82.63 and 29.66 respectively. Three fractions showed good inhibition of TNF-α; the % TNF- α inhibition of the fractions obtained were COMF- 87.09 and 40.99, HMEP- 89.88 and 0.00 and CO- 24.43 and 6.45 respectively.

HPTLC Estimation of CO, COHF and COMF

Track IDs : T1= CO, T2= COHF, T3= COHP , T4= COMF

Fig.-3: HPTLC Profile of CO, COHF and COMF

The HPTLC profile showed the presence of capsaicinoids (indicated by blue color) after derivatization with vanillin sulphuric acid reagent.

HPTLC Estimation of BPS

T1= Galactose, T2= Sucrose, T3= Arabinose, T4= Glucuronic acid, T5= Crude polysaccharide (After hydrolysis), T6= Galacturonic acid, T7= Glucose, T8= Fructose

Fig.-4: HPTLC Profile of BPS

The profile showed the presence of galactose and arabinose (identified by the colour band) in the water extract after derivatization with vanillin sulphuric acid reagent by comparing with the carbohydrate standards taken.

HPTLC Estimation of HMEP

Fig.-5: HPTLC Profile of HMEP

The HPTLC profile showed the presence of three phytoconstituents (β -sitosterol, Luteolin and a class of unknown terpenoid) in HMEP.

LCMS Estimation of COMF, HMEP and BPS

Fig.-6: LCMS Profile of COMF

The profile showed that protonated molecular ion gave the peak at m/z 306.3. It proved the presence of capsaicin.

Fig.-7: LCMS Profile of HMEP

The profile showed that protonated molecular ions gave the peak at m/z 345.0, 284.9. It proved the presence of two flavonoid group of compounds, they are luteolin and lalioside.

Fig.-8: LCMS Profile of BPS

The profile showed that protonated molecular ion gave the peak at m/z 149.0. It proved the presence of arabinose.

CONCLUSION

From the current research, it was observed that five fractions (CO, COHF, COMF, BOSPL and HMEP) showed good anti inflammatory activity. This activity was shown may be due to the presence of phytoconstituents which are identified by phytochemical analysis. So, these fractions may be used to prepare antiarthritic herbal formulations in the future. However, the *in-vivo* and clinical studies have to be carried out for further research.

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