Original Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

EX-VIVO ANTI-INFLAMMATORY POTENTIAL OF NATURALLY OCCURRING MOLECULES BIXIN, PURPURIN AND PSORALEN

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ABSTRACT

The present research was accomplished to evaluate the *ex-vivo* anti-inflammatory potential of natural compounds- bixin, purpurin and psoralen by albumin denaturation assay, membrane stabilization assay at different concentrations. Diclofenac sodium was used as standard reference. In human red blood cell (HRBC) membrane stabilization assay, psoralen 57.01±0.65%, bixin 40.78±0.75%, purpurin 28.94±0.57% showed activity at 1 mM. In protein denaturation assay, psoralen 49.49±0.19%, bixin 38.70±1.05% and purpurin 30.51±0.56% showed activity at 1 mM. But the ability of test drugs to protect the erythrocyte membrane lysis and protein from denaturation was clearly seen at 2 mM when compared to diclofenac-sodium which showed highest effect 78.14±0.67% at 1 mM concentration in HRBC membrane lysis assay and 81.79±0.53% at 1 mM concentration in protein denaturation assay. In HRBC membrane assay, Psoralen 99.5±0.46%, bixin 77.37±2.18% and purpurin 60.83±0.75% showed significant activity at 2 mM. Psoralen showed the lowest IC50 value *i.e.* 475.76±0.98 mM whereas standard drug diclofenac sodium IC50 value was 320.67±0.60 mM. In protein denaturation assay Psoralen 96.23±0.29%, bixin 78.57±0.53%and purpurin 63.66±0.10% showed significant activity at 2 mM. Psoralen showed the lowest IC50 value *i.e.* 507.61±1.26 mM whereas standard drug diclofenac sodium IC50 value was 305.81±0.78 mM. The selected test drugs inhibited shrinkage of the cells as well as the series of actions, which activate or increase the release of this intracellular portion. However, test drugs significantly inhibited the lysis of erythrocytes. In addition, test drugs also were effective in preventing heat induced protein denaturation when compared to standard drug diclofenac sodium IC50.

KEYWORDS: ***Bixin, Purpurin, Psoralen, inflammation, human red blood cells membrane lysis, protein denaturation***

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Received on: 28-06-2017
Revised and Accepted on: 09-09-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.4.p77-81

INTRODUCTION

Inflammation is a normal defensive reaction of biological tissue to an injury. It involves a well-programmed cascade of fluid and cellular alter within biological tissue. Staying alive of all organisms requires they remove foreign invaders like viruses, bacteria, fungi. Inflammation is the living tissue attempt to kill invading microorganisms, eliminate irritants, and set the platform for cell repair. An Inflammation is provoked by the release of inflammatory mediators from damaged cells. The mediators are autocoids such as histamine and serotonin, prostaglandins, bradykinin, interleukin-l, vasopressin and angiotensin. The major symptoms of inflammation encompass redness, swelling, and pain, joint stiffness, loss of joint function as a consequence of infection, irritation, or injury.[1] The regularly used NSAIDs for the treatment of inflammatory conditions are as follows: salicylic acids- aspirin, diflunisal, methylsalicylate, Acetic acids- diclofenac, etodolac, indomethacin, nabumetone, sulindac and tolmetin, Propionic acids- fenoprofen, flurbiprofen, ibuprofen, ketoprofen, naproxin, oxaprazin, Enolic acids- phenylbutazone, piroxicam. Fenamates- mefenamic acid and Cox-II inhibitors - celecoxib.[2] Drugs for arthritis are chloroquine, d-penicillamine, gold salts, methotrexate and non-opiod analgesics for gout are acetaminophen, allopurinol, colchicine, phenacetin, probenecid and sulfinpyrazone.[3]

Anti-inflammatory agents such as salicylatespossess a number of adverse effects chiefly epigastric distress, nausea, vomiting and. Regular microscopic GI bleeding. Blood platelet cyclooxygenase enzyme diminish the amount of platelet TXA2, occurring in prevention of platelet aggregation and a prolonged bleeding time. In toxic doses, salicylates induce respiratory depression and a composition of uncompensated respiratory and metabolic acidosis. A metabolic process is disturbed by taking large doses of salicylates. Nearly 15% of patients experienced hypersensitivity reactions by taking aspirin. True allergy is a symptom which involves angioneurotic edema or bronchoconstriction and urticaria.[4-5] The alternative way to minimize the above mentioned adverse effects of synthetic anti-inflammatory agents is to discover and develop natural anti-inflammatory agents. In this work, anti-inflammatory potential of following natural compounds has been evaluated through HRBC membrane stabilization ability; [6] anti denaturation capacity of egg albumin was assessed.[7]

Bixin is a carotenoid isolated from the seeds of *Bixa orellana* (annatto or achiote) used as an FDA approved food colorant and additive, moreover cosmetic and textile colorant. Countless orange or yellow manufactured foods are colored with achiote.[8] Traditionally, annatoo seeds are used as anti inflammatory agent. Previous *ex-vivo* biochemical assays demonstrated that bixin was able to quench singlet oxygen, a ROS implicated in oxidative lung injury.[9] Consistent with its antioxidant properties, and other studies demonstrate that bixin prevents oxidative DNA damage and lipid peroxidation.[10] Anthraquinones such as purpurin (trihydroxy anthraquinone) was the main bioactive compound isolated from the root part of R. cordifolia. Manjistha (*Rubia cordifolia*) also called as Indian madder belongs to Rubiaceae family. Manjistha is an Ayurvedic tonic herb that has been prized for centuries for its long list of benefits and uses.[11] Manjistha is a potent antioxidant, anti-inflammatory and anti-microbial. It enhances circulation, helps to process and remove toxins from the body.[12]Psoralen (also known as psoralene) is the parent compound in a family of natural products known as flurocoumarins. Psoralen is one of the phytoconstituents of anise seeds, caraway seeds, carrots, celeriac, celery, chervil, cilantro, coriander seeds, cumin seeds, dill, fennel seeds, figs, grapefruit, lemons, limes, lovage, mustard seeds, parsley, parsnips and root parsley.[13]

MATERIALS

Diclofenac sodium and Purpurin were procured from Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India. Bixin was procured from Marven Bio Chem, Hyderabad, India. Psoralen was procured from Yucca Enterprises, Mumbai, India. All the other necessitated chemicals were of analytical grade obtained commercially.

METHODS

Evaluation of Ex-vivo Anti-Inflammatory Activity

Human Red Blood Cell Membrane Stabilization Assay

Procedure

The human red blood cells (HRBC) membrane stabilization method had been used to evaluate the anti-inflammatory potential of test drugs. Bixin, purpurin, and psoralen were used for the *ex-vivo* anti-inflammatory activity. Briefly, blood was withdrawn from normal healthy human beings who were not under NSAIDs treatment for 14 days prior to the study. The same volume of sterilized Alsever solution which is composed of 0.5% anhydrous citric acid, 2% dextrose, 0.42% NaCl, 0.8% and sodium citrate was added to the collected blood. The blood was centrifuged at 3000 rpm and packed cells had been washed with isosaline (0.9% or 0.85% w/v NaCl, pH 7.2) and a 10% suspension was made with isosaline. The reaction mixture (4 mL) contains various concentrations (0.5 mL; 0.06255, 0.1250, 0.2500, 0.5000, 1, 2 mM) of the test drugs prepared using dimethyl sulfoxide (DMSO) and 1 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml hyposaline (0.36% NaCl), and 0.5 ml HRBC suspension were added to each test concentration. These were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin content in the supernatant solution was measured at 560 nm in spectrophotometer. Diclofenac (0.5 mL; 0.0625, 0.1250, 0.2500, 0.5000, 1 mM) was used as the reference standard and a similar volume of DMSO served as control. The percentage hemolysis was calculated by assuming the hemolysis produced by the control group as 100%. The percentage of HRBC membrane stabilization or protection was calculated using the formula: [14]

$$\%\ inhibition = \frac{\text{Abs control} - \text{Abs test}}{\text{Abs control}} \times 100$$

Albumin denaturation assay

Procedure

The assay mixture 5 mL was prepared using 0.2 mL of egg albumin from a healthy wild hen, 2.8 mL of phosphate buffered saline (pH 6.4) and also 2 mL of different concentrations of test drug so that final concentrations were 0.0625, 0.1250, 0.2500, 0.5000, 1 and 2 mM. Control was prepared omitting the test drug. Then the assay mixture was incubated at (37°C ± 2) for 15 min and then heated at 70°C for 5 min. After cooling, their turbidity was measured at 660 nm in ultraviolet–visible spectroscopy by taking vehicle as blank. Diclofenac at concentrations of 0.0625, 0.1250, 0.2500, 0.5000 and 1 mM was used as reference standard and treated the similarly as the test solutions. The percentage inhibition of protein denaturation was calculated by using the following formula: [15-16]

$$\% \ inhibition = \frac{\text{Abs control} - \text{Abs test}}{\text{Abs control}} \times 100$$

STATISTICAL ANALYSIS

Graph pad prism software, version 6 and MS excel were used to analyze the data. All results of present study are expressed as mean ± SD (*n* = 3).

RESULT

Human Red Blood Cell Membrane Stabilization Assay

As shown in Table.1, psoralen (57.01±0.65%), bixin (40.78±0.75%) and purpurin (28.94±0.57%) showed activity at 1 mM. The highest effect (57.01±0.65%) was proved by psoralen at 1 mM concentration. The lowest effect (28.94±0.57%) was proved by the purpurin at 1 mM concentration. The ability of test drugs to preserve the HRBC membrane lysis had been clearly found at 2 mM which was compared to diclofenac-sodium (standard reference) showed highest effect at (78.14±0.67%) at 1 mM concentration. Psoralen (99.5±0.46%), bixin (77.37±2.18%) and purpurin (60.83±0.75%) showed significant activity at 2 mM. The highest effect (99.5±0.46%), was proved by psoralen at 2 mM concentration. The lowest effect (60.83±0.75%) was proved by the purpurin at 2 mM concentration. IC50 also was calculated with percentage of inhibition against concentration for test drug as well as standard drug as depicted in table no 1. Psoralen showed the lowest IC50 value *i.e.* 475.76±0.98 mM whereas stanadard drug diclofenac sodium IC50 value was 320.67±0.60 mM.

Table 1

Effect of test drugs on HRBC membrane lysis (inhibition%)

	Conc mM	Bixin (Test) % Inhibition	Purpurin (Test) % Inhibition	Psoralen (Test) % Inhibition	Diclofenac (STD) % Inhibition
Control	0				
	0.0625	2.58±0.35	1.57±0.37	3.52±0.29	6.61±0.33
	0.1250	6.33±0.23	3.47±0.21	7.44±0.30	11.00±0.42
	0.2500	12.03±0.18	7.44±0.50	16.76±0.14	20.35±0.32
	0.5000	22.90±0.46	15.48±0.35	30.38±0.35	38.54±0.14
	1	40.78±0.75	28.94±0.57	57.01±0.65	78.14±0.67
	2	77.37±2.18	60.83±0.75	99.5±0.46	100±0.16
IC50		629.01±2.22	837.05±1.66	475.76±0.98	320.67±0.60

All assays were carried in triplicate and each value is the mean ± SD

Albumin denaturation assay

As shown in Table.2, psoralen (49.49±0.19%), bixin (38.70±1.05%) and purpurin (30.51±0.56%) showed activity at 1 mM. The highest effect (49.49±0.19%) was proved by psoralen at 1 mM concentration. The lowest effect (30.51±0.56%) was proved by the purpurin at 1 mM concentration. Anti protein denaturation ability of test drugs psoralen (96.23±0.29%), bixin (78.57±0.53%) and purpurin (63.66±0.10%) showed significant activity at 2 mM. when compared to diclofenac-sodium (standard reference) which showed highest effect at (81.79±0.53%) at 1 mM concentration. The highest effect (96.23±0.29%) was proved by psoralen at 2 mM concentration. The lowest effect (30.51±0.56%) was proved by the purpurin at 2 mM concentration. IC50 also was calculated with percentage of inhibition against concentration for test drug as well as standard drug as depicted in table no 2. Psoralen showed the lowest IC50 value *i.e.* 507.61±1.26 mM whereas standard drug diclofenac sodium IC50 value was 305.81±0.78 mM.

Table 2

Effect of test drugs on protein denaturation (inhibition%)

	Conc mM	Bixin (Test) % Inhibition	Purpurin (Test) % Inhibition	Psoralen (Test) % Inhibition	Diclofenac (STD) % Inhibition
Control	0				
	0.0625	2.40±0.23	1.94±0.03	3.47±0.13	5.57±0.19
	0.1250	5.29±0.16	4.52±0.14	7.39±0.23	10.55±0.18
	0.2500	10.43±0.12	8.26±0.20	13.72±0.20	21.67±0.15
	0.5	20.76±0.45	17.42±0.13	27.33±0.61	40.81±0.14
	1	38.70±1.05	30.51±0.56	49.49±0.19	81.79±0.53
	2	78.57±0.53	63.66±0.10	96.23±0.29	100±0.23
IC50		635.46±1.34	725.08±0.98	507.61±1.26	305.81±0.78

All assays were carried in triplicate and each value is the mean ± SD

DISCUSSION

The red blood cell membrane is homologous to the lysosomal membrane and its maintainance indicates that the anti-inflammatory agents stop destryoing lysosomal membranes. Maintainance of lysosomal membrane is chief in controlling the inflammatory reaction by inhibiting the liberation of lysosomal constituents such as bactericidal enzymes and proteases from triggered neutrophil, which produce more inflammation and injury to the tissue consequent to extra cellular release.[17-18] Non-steroidal anti inflammatory drugs (NSAIDs) exert their beneficial effects by either inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes. Some NSAIDs showed an anti-inflammatory effect through cell membrane stabilization properties. Hypotonicity induced hemolysis may result from shrinkage of the red blood cells due to osmotic loss of intracellular electrolyte and fluid components.[19] All test drugs, bixin, purpurin and psoralen inhibited the lysis of erythrocytes when compared to the standard drug, diclofenac sodium. However, test drug psoralen significantly inhibited the lysis of erythrocytes. Results (Table 1) reveal that the selected test drugs inhibited shrinkage of the cells as well as the series of actions, which activate or increase the release of intracellular portion. Protein denaturation is an activity where proteins lose their tertiary structure and secondary structure by administration of external stress or chemicals like strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Majority biological proteins lose their biological function when denatured. Proteins denaturation is a well reported cause of inflammation.[20-21] Furthermore, it was already documented that Traditional NSAID's like phenylbutazone and indomethazine prevented denaturation of proteins along with inhibition of endogenous prostaglandins synthesis by blocking COX enzyme.[22-23] Therefore anti- protein denaturation assay is the appropriate method to explore the anti inflammatory activity of test drugs.[24] As a part of the anti-inflammatory activity, potency of test drugs to prevent protein denaturation was studied. Test drugs, bixin and psoralen, prevented heat induced protein denaturation as compared to diclofenac sodium IC50 value. psoralen and bixin was proved in preventing heat induced protein denaturation when compared to standard drug diclofenac sodium IC50. In contrast, purpurin was less effective compared to other test drugs. The percentage inhibition of test drug psoralen is significantly high compared to standard.

CONCLUSION

The results obtained from the present study give scientific information to support the anti-inflammatory potential of naturally occurring molecules bixin, purpurin and psoralen against inflammation. Therefore, form the results of the present study it can be concluded that bixin, purpurin and psoralen possessed marked *in vitro* anti-inflammatory effect against HRBC membrane lysis and the denaturation of protein. Anti-inflammation actions of bixin (carotinoid), purpurin (anthraquinone) and psoralen (flurocoumarin) may be due to its chemical nature. Further investigations are required to explore themolecular mechanisms behind its anti-inflammatory actions of bixin, purpurin and psoralen against inflammation.

ACKNOWLEDGEMENT

The present research was supported by JSS College of Pharmacy and JSS medical college, Mysuru, Karnataka, India. The authors are sincerely thankful to the Dr. G. Parthasarathi, Principal of JSS College of Pharmacy, Mysuru, Karnataka, India for providing all sorts of facilities to carry out this work.

CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript