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Optimized Methods for *In Vitro* and *In Vivo* Anti-Inflammatory Assays and Its Applications in Herbal and Synthetic Drug Analysis

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Abstract: Inflammatory diseases including, different types of rheumatic diseases are the major problems associated with the presently available non-steroidal anti-inflammatory agents. The numbers of plant derived drugs have been screened for their anti-inflammatory and anti-arthritic activity. Drug development in the recent times often relies on use of natural and synthetic drugs, which are promising candidates as therapeutic agents for prevention of diseases and disorders. These drugs possess different chemical structures, with wide range of therapeutic activities. The mechanism of Inflammation mainly involve in development of serious diseases, such as cancer, rheumatoid arthritis, sprains, bronchitis, muscle pains, chronic inflammatory bowel disease, persistent asthma, and liver fibrosis. Development of inflammatory events basically related to various chemicals, such as glucocorticoids (GCs) and mometasone furoate (MF); endogenous factors such as tumor necrosis factor alpha (TNF-α); enzymes and proteins such as copper and zinc-superoxide dismutase (SOD), pro-inflammatory peptide substance (PPS), RGD peptides, interleukin-4 (IL-4), IL-10, interferon- γ (IFN- γ), COX, LOX, cytokines such as interleukin-1 (IL-1); reactive oxygen species (ROS), nitric oxide (NO) and prostaglandin E2; as well as pro-inflammatory cells such as T and NK cells are well known to have an important role. Based on these correlations, numerous assays were used for inflammatory mechanism research, which was described in this paper.

Keywords: Herbal drugs, Synthetic drugs, Anti-Inflammatory assays.

INTRODUCTION

Natural and synthetic drugs are widely used as a source of therapeutic tools for the prevention or treatment of many diseases. These drugs are mainly, polyphenols, chalcones, vitamins, and proteins in nature. Many experimental and epidemiological studies have shown that; numbers of natural and synthetic drugs are involved in reduction of oxidative stress developed due to free radicals [1]. Free radicals are the types of Reactive Oxygen Species (ROS) which includes all highly reactive oxygen containing molecules; these are mainly hydroxyl radicals, peroxy radicals, super oxide radicals, hypochlorite radicals, hydrogen peroxides, singlet oxygen, nitric oxygen radicals, and various lipid peroxides [2, 3]. ROS are also capable of reacting with membrane lipids, proteins, nucleic acids, various metabolic enzymes and small molecules of living systems. The interaction of ROS and cellular components result in cellular damage and tissue injury prompting Inflammation. These reactive oxygen species have been found to play an important role in the initiation and progression of various diseases such as cancer, gout, atherosclerosis, cardiovascular diseases, aging, and respiratory diseases [4, 5]. ROS also initiate a wide range of toxic oxidative reactions in body like initiation of lipid peroxidation, inhibition of metabolic enzymes, inhibition of membrane sodium potassium ATPase activity and oxidative modifications of proteins. All these toxic effects lead to development of inflammation [6], a fundamental protective response or a local response of living mammalian tissue injury. Inflammation is protective and defense mechanism of the body. During inflammatory conditions various pathological changes are take place. The production of active inflammatory mediators was triggered by microbial products or by host proteins; such as proteins of the complement, kinins, and coagulation systems, which was activated by microbes and damaged tissues. It is a body defense phenomenon in order to eliminate or limit the spread of injurious agent. Various components of inflammatory reaction can lead to various symptoms such as tissue injury, edema formation, and leukocyte infiltration [7]. The possible mechanism behind this action of antioxidants was explored; when substance with antioxidative property is likely to be one, that it gets readily oxidized by itself [8]. Inflammatory response occurs in three distinct phases. The first phase is caused by an increased in vascular permeability resulting in exudation of fluids from the blood into the interstitial space, the second phase involves the infiltrations of leukocytes from the blood into the tissue and in third phase granuloma formation and tissue repair. Mediators of inflammation originate either from plasma (e.g. complement proteins [kinins]) or from cells (e.g. histamine, prostaglandins, cytokines). Generally the mediators of inflammation are histamine, prostaglandins (PGs), leukotrienes (LTB_4), nitric oxide (NO), platelet-activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines, and growth factors. Large amount of experimental studies and detailed knowledge that

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arises of mediators of inflammation has been carried out [9, 10]. This review will address the commonly used animal models for the evaluation of anti-inflammatory activity in laboratory practice. It is also giving the principle and procedure behind using each animal model. This review hopefully fills the expectation to provide the *in vivo* models in area of inflammation. For anti-inflammatory activity, use of different inflammatory mediators induces paw edema sub acute inflammation using carrageenan air pouch model and chronic inflammation by cotton pellet induced granuloma formation in rats. Inhibition of eicosanoid generation from rat neutrophils and pro-inflammatory cytokine (tumor necrosis factor-α, TNF-α) release from macrophages are used as markers for *in vitro* tests for inflammation [11, 12]. This review, explains the major *in-vitro* and *in-vivo* methods for determination of anti-inflammatory assays for natural and synthetic drugs.

ANTI-INFLAMMATORY ACTIVITY ASSAYS

Conjugated Diene Assay

For Conjugated diene assay firstly there is need to prepare RBC cell membrane

PREPARATION OF RBC MEMBRANE

The blood sample, collected with addition of EDTA as an anticoagulant, was centrifuged and the plasma was aspirated. The blood cells were washed three times using saline. To cells in ice-cold distilled water was added and left overnight at 0 ^{o}C, the hemolysate was separated by centrifugation at 10000 RPM for 20 min at 4 ^{o}C. Pellets was washed twice with distilled water, followed by centrifugation for 10 min and then suspended to 50 ml of Tris-HCl buffer (0.1 M, pH 7.4) and the resultant solution was used as a membrane solution.

ASSAY

The membrane solution was mixed with chloroform: methanol (2:1 v/v). The mixture was centrifuged at 100 x g for 15 min to separate the two phases. The chloroform layer was removed by using separating funnel and dried at 45 ^{o}C in a water bath. The lipid residue was dissolved in cyclohexane. The hydro peroxides generated were detected at 233 nm spectrophotometrically against cyclohexane, as a blank. Acetylsalicylic acid (1mM) was used as a standard drug.

The % activity was calculated by using the formula: % Activity = (1-T/C) x 100

Where T = absorbance of test sample and C = absorbance of control sample [13-15].

HET-CAM ASSAY

Hen's egg Chorioallantoic membrane was used for this assay.

Phase 1

Pellet preparation: Sodium dodecyl sulfate (SDS) was dissolved with or without test drug or fractions or controls (hydrocortisone, phenylbutazone) in 2.5 % hot agarose solution. These gelling solutions were used for the assay.

Phase 2

Execution: The fertile hen's eggs were incubated for 65-70 hours at 37 ^{o}C and a relative humidity of 80%. The eggs were placed in a horizontal position and rotated several times. They were opened on the snub end for the aspiration of 10 ml of albumin from the hole on the pointed end. At two-third of the height from the pointed end, the eggs were traced with a scalpel and after that the shells were removed with forceps. The aperture was covered with Whatman papers and the eggs were incubated at 37 ^{o}C at a relative humidity of 80 % for 75 hours. One pellet per egg was kept on the formed chlorioallanthoic membrane (CAM), which was about 2 cm in diameter. The eggs were incubated for a day and then evaluated. For every test, 10-15 eggs were utilized. For evaluation of these effects, as positive irritation controls, CAMs were treated with SDS only. As positive controls, hydrocortisone and phenylbutazone were tested at a concentration of 72.5 mg/pellet respectively, and in the presence of SDS (50 µg/pellet). As a negative control, the drug was assayed at a concentration of 500 mg/ml, without SDS. As a blank CAM were treated with agarose solution only. The inhibition or otherwise of the membrane irritation was observed. A positive effect, corresponding to anti-inflammatory activity exists if the irritation of the membrane induced by SDS decreased and the blood vessel net appear normal. The number of experiments with a positive effect was given in percentage, indicating the level of anti-inflammatory effect [16].

ASSAY OF ANTI-PROTEOLYTIC ACTIVITY

In this method, solutions of individual drugs were incubated with trypsin for 20 min followed, by addition of bovine serum albumin (BSA), in 50 ml phosphate buffer, pH 7.6. This solution was incubated for 20 min at 37 ^{O}C, after incubation, the reaction was terminated by using 3 ml, 5% trichloroacetic acid. The resulted precipitates were estimated by using standard Lowry *et al.* (1951) method, Salicylic acid (1 mM) used as standard [17, 18].

β- GLUCURONIDASE INHIBITION ASSAY

For this assay, the P-nitrophenyl- β-D-glucopyranosiduronic acid was incubated with individual drug with known volume in acetate buffer for 5 min followed by addition of β-glucuronidase solution. The mixture was further incubated for 30 min followed by addition of sodium hydroxide (NaOH) for termination of the reaction. The amount of reaction product formed was observed and absorbance was recorded by spectrophotometrically at 410 nm. The salicylic acid used as reference drug for comparative study [19].

WST-ANTI-INFLAMMATORY ASSAY

This *in-vitro* assay is based on the reduction of highly water-soluble tetrazolium salt WST-1 in the presence of activated neutrophils. Anti-inflammatory activity was determined in a total volume of modified Hank's Solution (MHS) with pH 7.4 containing neutrophils, WST-1 [1, 2-(4-iodophenyl)-3- (4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt and various concentrations of the test samples. The control contained was only buffer, neutrophils and WST-1. All samples were equilibrated at

37 ^{o}C and the reaction initiated by adding Zymosan-activated serum [ZAS] (Sigma Chemicals, St. Louis, USA) for 30 min. Indomethacine was used as reference. Absorbance was measured at 450 nm using Spectra MAX 340-plus microplate reader. Aspirin and indomethacine were used as positive controls because both are widely accepted as non-steroidal, anti-inflammatory drugs (NSAIDs) used for the treatment of several inflammatory disease. IC_{50} values were calculated by comparing with the DMSO as black and expressed as % inhibition of superoxide produced.

ISOLATION OF HUMAN NEUTROPHILS

Heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank and neutrophils were isolated. Whole blood was layered on to histopaque in 1:1 ratio, centrifuged and Buffy layer was collected and washed with modified HBSS buffer pH 7.3 (*Composition:* Na_2Cl_2 135 mM, HEPES 20 mM, KCl 4 mM, Na_2HPO_4 1 mM, $CaCl_2$ 2 mM, 1 mM $MgCl_2$, And 10 mM glucose). The cells were then re-suspended in the same buffer.

MHS -ANTI-INFLAMMATORY ASSAY

The anti-inflammatory assay was determined as, the total volume of 250 ml Modified Hank's solution (MHS) with pH 7.4 containing neutrophils/ml, WST-1 [1,2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (Dojindo Laboratories, Kumamoto, Japan) and various concentration of the drug samples. The control contained only buffer, neutrophils and WST-1. All samples were equilibrated at 37 ^{o}C and the reaction initiated by adding Zymosan activated serum ZAS (Sigma Chemicals, St. Louis, USA) for 30 min. Indomethacine was used as reference. Absorbance was measured at 450 nm using Spectra MAX 340-plus microplate reader (molecular devices, Sunnyvale, California, USA). IC_{50} values were calculated by comparing with DMSO, as blank and expressed as percent inhibition of superoxide produced [20, 21].

HRBC-MS ASSAY

Wister male albino rats weighing 150-200 g were used for the anti-inflammatory studies. The animals were housed under standard conditions of temperature (23 ^{o}C± 1 ^{o}C), 12 hours light/dark cycle and fed with water ad libitium. Before performing the experiment the ethical clearance was obtained from institutional animal ethics committee.

The human red blood cell membrane stabilization method was used for this study. The blood was collected from healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (Procured from, Sigma-Aldrich, Mumbai) and centrifuged at 3,000 rpm. The packed cells were washed with saline and suspension was made. Various concentrations of drugs were prepared using distilled water and to each concentration of phosphate buffer, hyposaline and HRBC suspension were added. It is incubated at 37 ^{o}C for 30 min and centrifuged at 3,000 rpm for 20 min. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac was used as reference standard and a control was prepared omitting the drugs [22].

TPA-INDUCED ASSAY

This assay was performed using acetone solution of the drugs was applied on the left ear of a mouse (male, 6 weeks old). After 30 min, an acetone solution of an inflammation inducer, 12-0-tetradecanoylphorbol 13-acetate (TPA) was applied to both the left and right ears of the same mouse. The ear disk (0.6-cm diameter) was punched and weighed 6.5 h after TPA applications; Anti-inflammatory activity was estimated by the percent inhibition of the ear edema, which was calculated using the following formula [23, 24].

% Inhibition = {[(weight of the ear to which TPA was applied) – (weight of the ear to which TPA and drug were applied)] / [(weight of the ear to which TPA was applied) – (weight of the ear to which only vehicle was applied)]} x 100.

TNF-α ASSAY

Human leukemia monocytes THP-1 cells were cultured in 75 cm^2 plastic flasks bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cell culture was maintained at 37 ^{o}C in a humidified 55% CO_2 atmosphere. According to this method, cells at the exponential growth phase were trypsinized and the cell suspension was prepared in complete medium at 5 x 10^5 cells/ml. Cell suspension was pre-activated with phorbol ester (PMA) in a 24-well plate for 48 h to undergo transformation in to macrophages before pre-incubation with various concentrations of drug. Cells with dexamethasone and vehicle (DMSO) were used as a reference drug and control, respectively. After 30 min pre-treatment with test compound, cells further incubated with LPS for 24 h. Cell free supernatants were collected and TNF-α level was determined by EIA reader [25].

CARRAGEENAN-INDUCED PAW EDEMA IN RATS

This model is based on the principle of release of various inflammatory mediators by carragenan. Edema formation due to carrageenan in the rat paw is biphasic event. The initial phase is attributed to the release of histamine and serotonin. The second phase of edema is due to the release of prostaglandins, protease and lysosome. Subcutaneous injection of carrageenan in to the rat paw produces inflammation resulting from plasma extravasation, increased tissue water and plasma protein exudation along with neutrophil extravasation, all due to the metabolism of arachidonic acid. The first phase begins immediately after injection of carrageenan and diminishes in two hours. The second phase begins at the end of first phase and remains through third hour up to five hours. In this method the animals are divided into three groups (n=6) starved overnight with water ad libitum prior to the day of experiment. The control group receives vehicle orally, while other group receives test drug and standard drug respectively. Left paw is marked with ink at the level of lateral malleolus; basal paw volume is measured plethysmographically by volume displacement method using plethysmometer (UGO Basile 7140) by immersing the paw till the level of lateral malleolus. The animals are given drug treatment. One hour after dosing, the rats are challenged by a subcutaneous injection of 0.1ml of 1% solution of carrageenan into the sub-plantar side of the left hind paw. The paw volume is

measured again at 1, 2, 3, 4 & 5 hours after challenge. The increase in paw volume is calculated as percentage compared with the basal volume. The difference of average values between treated animals and control group is calculated for each time interval and evaluated statistically. The percent Inhibition is calculated using the formula as follows [26, 27].

% edema inhibition = [1- (Vt / Vc)] X 100

Vt and Vc are edema volume in the drug treated and control groups respectively.

HISTAMINE INDUCED PAW EDEMA IN RATS

Histamine induced paw edema is said to occur in earlier stage in mounting of vascular of the vascular reaction in the chemically induced inflammation. In this, swelling occurs primarily due to action of histamine. Generally histamine is released following the mast cell degranulation by number of inflammatory mediators including substances interleukin-1 (IL-1). This is likely to evoke the release of neuropeptide as well as release of prostaglandins and monohydroxy eicosatetranoic-acid from endothelial cell leading to hyperalgesia and other pro-inflammatory effects. This assay is similar to that of Carrageenan-induced paw edema, only instead of carrageenan the rats are challenged by a subcutaneous injection of 0.1 ml of 1% solution of histamine into the sub-plantar side of the left hind paw. The paw volume is measured. The percent inhibition of the inflammation is calculated using the formula and compared with control group [28, 29].

% Inhibition = Vc - Vt / x 100

Vt and Vc edema volume in the drug treated and control groups respectively.

ACETIC ACID-INDUCED VASCULAR PERMEABILITY

The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by acetic acid by releasing inflammatory mediators. 10 Mediators of inflammation, such as histamine, prostaglandins and leukotrienes are released following stimulation of mast cells. This leads to a dilation of arterioles and venues and to an increased vascular permeability. As a consequence, fluid and plasma protein are extra vaseted and edemas are formed. Animals are divided into three groups (n=6). The control group received vehicle orally, while other groups received test drug and standard drug respectively followed by the injection of acetic acid intraperitoneally. Immediately after administration, Evan's blue is injected intravenously through the tail vain. Thirty minutes after Evan's blue injection, the animals are hold by a flap of abdominal wall and the viscera irrigated with distilled water over a Petri dish. The exudate is then filtered and makes the volume up to 10 ml. The dyes leaking out into the peritoneal cavity measured spectophotometrically using visible spectra at 510 nm and compared with the control group [30, 31].

PHORBOL MYRISTATE ACETATE-INDUCED EAR EDEMA IN MICE

Phorbol myristate acetate (PMA) is a protein kinase C (PKC) promoter, which induces the formation of free radicals *in vivo*. It has been also demonstrated that pre-treatment of mouse skin by antagonists of PKC suppresses inflammation and ROS (reactive oxygen species). This species involved in the synthesis of mediators and regulate the production of TNF-α in turn stimulate PLA2 activity, which releases arachidonic acid from phospholipids and stimulate the activity of COX and LOX (Lipoxygenase) these enzyme involved in release different inflammatory mediators. Ear of PMA, in acetone, is applied to the both ear of each mouse. The left ear (control) receives the vehicle. Test drug is administered 1 h before PMA application. Two control groups are used, one group with application of PMA on the right ear and second positive control group with standard drug. Six hours after PMA application, the mice was killed by cervical dislocation and a 6 mm diameter disc from each ear is removed with a metal punch and weigh. Ear edema is calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment), and is express as a reduction in weight with respect to the control group [32,33].

MYELOPEROXIDASE (MPO) ASSAY

MPO is an enzyme present in neutrophil, monocytes and macrophages. It is well know that the level of MPO activity is directly proportional to the neutrophil concentration on the inflamed tissue. Inhibition of MPO activity by the drugs prevent the generation of oxidants such as hypochlorous acid. Tissue samples of each ear, from the PMA model, are assessed biochemically with neutrophil marker enzyme MPO. All the ear tissue is homogenized in 50 mM K_2PO4 (pH 6) containing hexadecyl trimethylammonium bromide (HTAB) using a Polytron (Ultra-turax T-25) homogenizer. After freezing and thawing the samples were centrifuged at 2500 rpm for 30 min at 4 ^{o}C and the resulting supernatant is assayed spectrophotometrically at 460 nm for MPO determination. MPO activity data is presented as units per mg of tissue. One unit of MPO activity is defined as that degrading 1 μmol of peroxide per minute at 25 ^{o}C [34, 35].

OXAZOLONE-INDUCED EAR EDEMA IN MICE

The oxazolone-induced ear edema model in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a herbal and synthetic drugs following topical administration. Animals used in this models are divided into six groups (n=6). Before each use a fresh solution of oxazolone (4- ethoxymethylene-2-phenyl-2-oxazolin-5-one) is prepared using acetone. The mice are sensitized by application of the shaved abdominal skin or the inside of both ears under halothane anesthesia. The mice are challenged 8 days later again under anesthesia by applying oxazolone solution the inside of the right ear (control) or oxazolone solution, in which the test drugs or the standard drug is solved. Groups of 10 to 15 animals are treated with the irritant alone or with the solution of the test drugs. The left ear remains untreated. The maximum inflammation occurs 24 h later. At this time the animals are sacrificed under anesthesia and a disc of 8 mm diameter is punched from both sides. The discs are immediately weighed on a balance. The weight difference is an indicator of the inflammatory edema [36, 37].

CARRAGEENAN INDUCED GRANULOMA POUCH MODEL

Carrageenan induced granuloma pouch model is an excellent sub acute inflammatory model in which fluid extravasations, leukocyte migration and various biochemical exudates involved in inflammatory response can be detected readily. The air pouch has the advantage of supplying a suitable space for the induction of inflammatory responses. The injection of irritants such as carrageenan into subcutaneous air pouch on the dorsal surface of rats initiates an inflammatory process. The rats used in this method are divided into five groups (n=6), fasted overnight and allowed free access to water. The animals are administered with vehicle, standard drug and test drug. One hour after dosing, the back of the animal is shaved and disinfected. With a very thin needle subcutaneous dorsal granuloma pouch is made in ether anaesthetized rats by injecting 6 ml of air, followed by injection of carrageenan in normal saline into it to avoid any leakage of air and the treatment continued for seven consecutive days as follows [38, 39]. On day 8, the pouch is opened under anesthesia and the amount of exudates was collected with a syringe. The average volume of exudates, total WBC count and weights of granuloma is determined.

FORMALIN-INDUCED PAW EDEMA

This model based upon the ability of test drug to inhibit the edema produced in the hind paw of the mice after injection of formalin. The non conceptive effect of formalin is biphasic, an early neurogenic component followed by a later tissue-mediated response. In the first phase there is release of histamine, 5-HT and kinin, while the second phase is related to the release of prostaglandins. The mice are divided into three groups (n=6). In mice of all groups, inflammation is produced by supplanted injection of freshly prepared 2% formalin in the right hind paw of mice. The paw thickness is measured by plethysmometrically 1 h before and after formalin injection. The drug treatment is continued for 6 consecutive days. The increase in paw thickness and percentage inhibition are calculated and compared with control group [40, 41].

COTTON PELLET-INDUCED GRANULOMA IN RATS

This model is based on the foreign body granuloma which is provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histological giant cells and undifferentiated connective tissue can be observed beside the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan. The rats divided into six groups (n=6), fasted overnight and allowed free access to water. The animals are administered with vehicle, standard drug and test drugs, after the first dosing the animals are anesthetized with anesthetic ether and the sterile cotton pellet is inserted one in each axilla and groin of rats by making small subcutaneous incision. The incisions are sutured by sterile catgut. The animals are sacrificed by excess anesthesia on the 8^{th} day and cotton pellets are removed surgically. Pellets are separated from extraneous tissue and dried at 60°C until weight become constant. The net dry weight, i.e. after subtracting the initial weight of the cotton pellet will be determined. The average weight of the pellet of the control group as well as of the test groups is calculated. The percent change of the granuloma weight relatively with vehicle control is determined and statistically evaluated. The percentage inhibition increase in the weight of the cotton pellet is calculated [42, 43].

% Inhibition = Wc - Wd / Wc X 100

Where, Wd = difference in pellet weight of the drug treated group. Wc = difference in pellet weight of the control group.

CONCLUSION

The above mentioned models, have given broad spectrum for the evaluation of the anti-inflammatory activitivites. In different models, the inflammation has produced by different inducers by releasing inflammatory mediators. Each is having different mechanism of action for producing inflammation either by increased in vascular permeability, the infiltrations of leukocytes from the blood into the tissue, granuloma formation, and tissue repair. Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. The main aim of this review is to describe and give optimized evaluation for a high throughout assays, which can be used to monitor oxidation reduction progress of herbal and synthetic drugs. These assays could be valuable tools for identifying better anti inflammatory herbal and synthetic drugs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

BSA = Bovine serum albumin

CDCl3 = Deuteriochloroform

CI = Chemical Ionization

EC = Enzyme commission

ED = Effective dose

EDTA = Ethylene diamine tetra acetic acid

GMP = Guanisine monophophate

HAP = Hydroxyapetite

HMP = 2-hydroxy-6-methylpurine

NK = Natural Killer

IC_{50} = Concentration of the inhibitor that is required for 50% inhibition *in vitro*

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