ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 3, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

EVALUATION OF AQUEOUS FRUIT EXTRACT OF *TAMARINDUS INDICA* (L) FOR INHIBITION OF TUMOR NECROSIS FACTOR-α AND CYCLO OXYGENASE ENZYMES IN EXPERIMENTAL ANIMAL

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Received: 11 October 2017, Revised and Accepted: 23 November 2017

ABSTRACT

Objective: The main objective of this study was to assess tumor necrosis factor-α (TNF)-α and cyclooxygenase enzymes (COX) inhibition potency of *Tamarindus indica* Linn. in comparison to standard drug (indomethacin).

Methods: Three plants are selected for the studies, namely: *Aloe vera* (L.), *Terminalia chebula* Reitz., *and T. indica* Linn. Estimation of TNF-α in serum (at 1:10 dilution in PBS) was performed using the immunoenzymatic (ELISA) technique. COX inhibitor screening assay kits were used for estimation of COX.

Result: All three plant extracts showed a potent significant inhibition of the COX enzyme as compared to the positive control and standard drug when the animal was administered with 400 mg/kg. These studies indicate that the *T. indica* plant extract showed significant COX inhibition even at low dose. All the extracts were effective anti-inflammatory in nature, however, *T. indica* extracts at a dose of 400 mg/kg were found to be most potent. It was found to be comparable with that of Indomethacin 10 mg/kg body weight.

Conclusion: The anti-inflammatory activity expressed by all the three plants *A. vera* (L.), *T. chebula* Reitz., *and T. indica* L. Among all three plants *T. indica* (L) was found to be more active against both TNF-α and COX, and it was comparable to standard drug Indomethacin. Need further studies to elucidate the exact secondary metabolite by which these plants express this activity.

Keywords: Inflammation, *Tamarindus indica* (L), Tumor necrosis factor-α, Cyclooxygenase enzymes.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i3.23027

INTRODUCTION

Inflammation is a defensive process that protects the body against harmful stimuli and hastens the recovery process [1]. The stimulus may be in the form of a wound, a pathogenic bacterium or virus or an endogenous trigger. This can be recognized by redness, pain, swelling, immobility, and heat [2]. The process of inflammation is to activate the immune system to the site of injury by increasing the blood supply and altering the permeability of the blood vessels which accounts for some the above-mentioned characteristics such as the swelling and heat [3]. The inflammatory response is complex as it involves a group of reactions that include enzyme activation, innate immune cell activation and migration, release of reactive oxygen species and tissue repair that act to remove the cause and repair the damage of the injury [4]. The migrated immune cells are called macrophage and T lymphocyte cells that become activated to release pro-inflammatory cytokines. The cytokines are released to magnify inflammatory process at the site of injury to resolve the source of injury [5]. Inflammation is part of the innate immune response which means that is it not specific for any particular cause of injury. The non-specific nature of the response means that the reactions are similar despite the cause of the injury which can be detrimental if the injury is persistent. Therefore, the inflammatory reactions must be actively regulated to prevent unnecessary impairment to tissues [6]. The improper regulation of the process for the resolution of injury can lead to a perpetual state of inflammation which can become chronic.

Inflammation is mediated by chemokines that include tumor necrosis factor-α (TNF)-α, nuclear factor kappa beta (NFKβ), interferon (IFN)-γ, nitric oxide (NO), and interleukins. The mediators are from different sources and influence different reactions within the inflammatory process. Most of the inflammatory mediators are newly synthesized in the injured tissues or by the floated immune cells during an inflammatory event. Pathological organisms such as bacteria activate NFKβ through receptors found on macrophages through several signaling pathways. The activated macrophages can release TNF-α which is responsible for the up-regulation of the production of other inflammatory mediators that include prostaglandins and NO. The membrane-bound glycoproteins, cyclooxygenase (COX) enzymes are found in the endoplasmic reticulum [7]. The cell membrane-bound arachidonic acid is converted into prostaglandin H_2 by COX enzymes. Arachidonic acid is a precursor molecule for all the eicosanoid molecules that include prostaglandins and thromboxanes. There are two main types of COX enzymes; namely, COX1 and COX2 that differ mostly in their array of expression.

The current approaches for the treatment of the inflammatory condition are mainly interrupting the synthesis or action of critical mediators that drive the host's response to injury. Steroidal, Narcotic and nonsteroidal anti-inflammatory drugs (NSAIDs) are the current treatment approach for inflammatory disorders. However, the available drugs have reduced efficacy against inflammatory conditions due to adverse effects and relatively high potency. For example, steroidal drugs are in use as anti-inflammatories due to their specific mechanisms of action that are considered to be responsible for their adverse effects as well. Steroidal anti-inflammatories inhibit basal physiological function such as leukotriene inhibition. The unwanted effects reported as hypertension due to the similarity of the steroidal drugs to the steroid hormones. The nonsteroidal drugs have relatively fewer and less adverse effects than the steroidal that include gastrointestinal bleeding and improper clotting of blood [8].

Therefore, there is a need for treatment options that have minimum side effect and maximum efficacy and must be comparable to a standard drug. To fulfill this demand currently, researchers are looking for alternate source of drug rather than synthetics one. The best option is an herbal crude drug that is frequently used in rural part of India as a folk medicine. Present research work layout in such a manner to explore the molecular basis of the particular activities that can inhibit expression of TNF-α and COX.

MATERIALS AND METHODS

Plant collection

On the basis literature review three plants are selected for the studies:

1. *Aloe vera* (L.)
2. *Terminalia chebula* Reitz.
3. *Tamarindus indica* L.

After collection of all three plants it was identified and authenticated by a pharmacologist and letter a voucher specimen (No. IU/PHAR/HRB/7/11) was deposited at the Department of Pharmacognosy Integral University, Lucknow, Uttar Pradesh, India. Aqueous extractions of all these three plants were done according to standard procedure.

A. vera Linn

For aqueous extraction of *A. vera* leaves were collected, washed in cold water; spines around the leaves were removed using a blade after which the leaves were sliced by knife. 200 g of the sliced material were mixed with of distilled water and blended in an electric blender for 3 min to obtain 400% (w/v) extract. The blended material was squeezed through a muslin cloth. The filtrate was freeze-dried at −50°C under vacuum using a lyophiliser and kept at −20°C until use. Various dose levels of the *A. vera* were made by reconstituting the extract at concentration of 1% (w/v) [9].

T. chebula Retiz

The fruits of *T. chebula* were used for the experiment. Preparation of extract of *T. chebula* shade dried fruits of *T. chebula* was grounded into a fine powder in an electric grinder. About 890 g of grinded powdered material were extracted with 80% ethanol at a temperature of 60°C for about 48 h. The mixture was filtrated. While volume is reduced, it was then poured in watch glass of large surface area to make it more condensed and allow the rest solvent to evaporate. While the condensed filtrate turned into a gummy concentrate, it was obvious that we found the crude ethanolic extract. The ethanolic extract was further evaporated to dryness to obtain the dried ethanolic extract. The percentage yield of extract was 12.1% w/w with respect to the original air-dried powder was obtained [10]. the extract was finally stored in airtight container at 2°C–8°C for further use during the experiment [11]. The dose was adjusted as per dosing schedule following standard procedure.

T. indica L

The extraction was performed using maceration technique [12]. The coarse powder of *T. indica* seeds (100 g) was subjected to maceration for 72 h at room temperature using 500 ml methanol. The extract was filtered, and the solvent was evaporated under vacuum to obtain a powdered residue. After that during experiment dose was adjusted as per dosing schedule following standard procedure.

Preparation of solutions (extract and standard)

To evaluate the activities of different plant extract, two different concentration of extract were used, 200 and 400 mg/kg body weight (b.w.) for each plant extract. For the preparation of standard solution indomethacin 10 mg/kg b.w. adjusted by dissolving in distilled water.

Qualitative analysis of phytochemical constituents

Preliminary chemical tests were carried out for the all extracts of plants to identify different phytochemical constituents present in the plants as per the protocol described [13].

Assay of COX inhibition

Ultrapure water, COX inhibitor screening assay kits and lipoxygenase (LOX) inhibitor screening assay kits were obtained from Cayman Chemicals, USA. All reagents and solvents used were of analytical grade from Sigma-Aldrich, India, and from HiMedia Laboratories, India. 96 well plates were read on the iMark Microplate absorbance reader and washed with the model 1575 immunowash microplate washer from BioRad, India. Spectrophotometric assays were conducted on ultraviolet 1800 spectrophotometer from Shimadzu, Japan. The COX inhibitor screening directly measures prostaglandin F2 alpha by stannous chloride reduction of COX-derived prostaglandin H2 produced in the COX reaction [14]. The reaction system consists of reaction buffer, heme, enzyme and plant extract pre-incubated at 37°C for 20 min with background and enzyme controls. The reaction was started with the incorporation of arachidonic acid and incubated for 2 min at 37°C temperature. The reaction was stopped with add of saturated stannous chloride solution and 5 min at room temperature (25°C). The prostaglandins are quantified by EIA. An aliquot of these reactions was added to the precoated plates in triplicates together with AChE tracer and antiserum and incubated for 18 h at room temperature on an orbital shaker. The plate was finally developed with Ellman's Reagent and kept on an orbital shaker in the dark at room temperature for 1 h. The absorbance was read at 420 nm. The data were plotted as standard bound/maximum bound versus log concentration using a 4-parameter logistic curve fit. The concentration of each sample was determined from a standard curve with appropriate dilutions and used to calculate the percent inhibition as per the formula is given below:

$$\text{Percent inhibition}(\%)=\frac{(\text{Activity of control}-\text{Activity of test})}{\text{Activity of control}}\times 100$$

The percent inhibition was plotted against the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). The dosing scheme for all the plant extracts, control and standard followed same as for the other experiments [15].

Estimation of TNF-α in serum

Estimation of TNF-α in serum (at 1:10 dilution in PBS) was performed using the immunoenzymatic (ELISA) technique, taking advantage of high sensitivity Quantikine HS ELISA human TNF-α (R and D systems, USA) kits, manifesting mean minimum detectability of 0.106° pg/ml. The absorbance value noted at λ *max* 490° nm, obtained using reader 250 (bioMerieux, France). The final result of the studied cytokine concentration involved a product of the readout on the standard curve and the applied dilution (×10) [15,16].

Acute toxicity study

The oral acute toxicity studies of all three plants extract where examined as per Organization for Economic Cooperation and Development (OECD) guideline 423 on BALB/c mice (20–30 g) [17], where the limit test dose of 4000 mg/kg was used. All the animals were kept at overnight fasting before every experiment with free excess to water. The animals were divided into four groups, each comprising five animals. The 1st group served as a negative control, while 2nd, 3rd, and 4th was considered as tested groups received orally plant extract (dissolved in normal saline) extract at a dose of 300 mg/kg, 2000 mg/kg, and 4000 mg/kg. Before dose administration, the b.w. of each animal was determined, and the dose was calculated according to the b.w. The animals were observed for any toxic effect for first 4 h after the treatment period. Further animals were investigated for a period of 3 days for any toxic effect. Behavioural changes and other parameters such as body weight (b.w), urination, food intake, water intake, respiration, convulsion, tremor, temperature, constipation changes in eye and skin colors.

Sub-acute toxicity study

The oral sub-acute toxicity study was carried as per OECD guideline 407 (OECD guidelines for the testing of chemicals). The adult healthy BALB/c mice (20–30 g) of each sex were divided into three groups of

five animals each and were kept under standard conditions. Group I was treated as control and the other two groups which were considered as tested groups received the plant extract at a dose of 600 and 1000 mg/kg b.w., respectively, for 28 consecutive days [18].

Animals and experimental design

Animal handling during the experimental procedure was done as per university guidelines and animal ethics. The rats were allowed free access to standard commercial rat pellets (Hindustan Lever Li, India). Clean water was provided *ad libitum* throughout the experimental periods.

In all the experiments, animals were categorized as following:
Group I: Control group received only 1 ml saline water (0.91% w/v).
Group II: 200 mg/kg b.w as per oral for each plant extract.
Group III: 400 mg/kg b.w as per oral for each plant extract.
Group IV: 10 mg/kg b.w as per oral as a standard drug (Indomethacin)

Statistical analysis

Data are summarized as mean ± standard deviation (SD). All studies compare between standard and at two different dose level for all the three plants were done using one-way ANOVA and $p<0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Qualitative analysis of phytochemical constituents

A (+) score was recorded if the reagent produced only a slight positive reaction; a (++) score for a definitive positive reaction and a (+++) score was recorded if heavy reactions were obtained. Statistical analyses: In all the studies, the values of three independent experiments were expressed as mean ± SD for n determinations where n=3 unless otherwise stated. The result is shown in a tabular form for all plant extract in Table 1.

Screening of plant extracts for effect on COX activity

All plant extracts were used as per protocol to study the effect on the activity of COX, and the results are as shown in Table 2.

All three plant extracts showed a potent significant inhibition of the COX enzyme as compared to the positive control and standard drug when the animal was administered with 400 mg/kg. These studies indicate that the *T. indica* plant extract showed significant COX inhibition even at low dose. The anti-inflammatory activities of this plant extract may be due to potential inhibition COX even at low dose. The results presented in this section have conclusively established the presence of bioactive molecules in all these three plant extracts capable of dual inhibition of COX enzymes involved in the biosynthesis of pro-inflammatory leukotrienes and prostaglandins. The identification of secondary metabolites present in plant extracts could provide lead-like compounds involved in the anti-inflammatory activity reported in these plant extracts. The phytochemical analysis of these all three plant extracts indicates the presence of terpenoids, saponins, and alkaloids. The presences of flavonoids are also found in all the three plant extracts. The extracts exhibit weak positive results for cardiac glycosides, tannins, and reducing sugars. The medicinal plants used for the present study are routinely used in Ayurvedic and Unani formulations and folkloric medicines in a different part of India. The findings of the present study reveal the presence of potential bioactive molecules capable of dual inhibition of COX enzymes. The findings of the present study open up new avenues for identification of bioactive molecules which could be used for development of safer anti-inflammatory molecules compared to current anti-inflammatory NSAIDs. The exact nature of the activity of the plant extract and the bioactive compounds involved are being investigated.

Serum TNF–α estimation

All the three plants extract showed the inhibition of TNF-α, and it is dose-dependent but in case *T. indica* showed highest % inhibition. The result showed in a tabular form at a different dose for different plant extract in Table 3.

This study revealed an anti-inflammatory effect of all three plant extracts. All the extracts were effective anti-inflammatory in nature, however, *T. indica* extracts at a dose of 400 mg/kg were found to be most potent. It was found to be comparable with that of Indomethacin 10 mg/kg b.w. It has been reported that various mediators are released by carrageenan in the rat paw. The initial phase is attributed to the release of histamine and 5-hydroxytryptamine. A second phase is mediated by kinins, and finally, in a third phase, the mediator is suspected to be prostaglandin.

The chemical analysis of all plant extracts revealed the presence of flavonoids. A variety of *in vitro* and *in vivo* experiments have shown that selected compounds of this large group of naturally occurring products possessed various important pharmacological activities including anticancer, anti-inflammatory, and anti-allergic activities [19].

Studies have shown that the anti-inflammatory activity of flavonoids is mediated by the inhibition of arachidonic acid metabolizing enzymes, COX, and LOX as well as by antioxidative properties. It has been previously demonstrated that several flavonoids such as flavone chrysin, 3-hydroxyflavone, and galangin, some chalcones (having a3–4 hydroxyl substitution) were COX inhibitors. In TNF-α estimation using ELISA, among all extract showed a significant decrease in TNF-α production in a dose-dependent matter. This further proves

Table 1: Phytochemical investigation of plant extract

Name of plant	Alkaloid	Flavonoid	Cardiac glycoside	Saponins	Terpenoids	Tannin	Reducing sugar
A. vulgaris	+	++	++	-		+	++
T. chebula	+	++	+	+	+	+++	-
T. indica	++	+++	+	+	-	++	+

(−) - Not presence (+) - Low presence, (++) - Moderate presence, (+++) - High presence. *A. vulgaris: Aloe vulgaris, T. chebula: Terminalia chebula, T. indica: Tamarindus indica*

Table 2: Effect of plants extracts on COX activity

Sl. No.	Plant extracts	% inhibition of COX at 200 mg/kg dose	% inhibition of COX at 400 mg/kg dose
1	Control	0%	
2	*A. vulgaris* Lam.	20.98±1.68	29.13±1.03
3	*T. chebula* Retiz.	32.67±1.87	43.17±1.99
4	*T. indica*	53.94±2.50	62.68±1.01
5	Indomethacin standard at standard dose 10 mg/kg b.w.	75.15±0.965	

n=3, ±: Standard deviation. *A. vulgaris: Aloe vulgaris, T. chebula: Terminalia chebula, T. indica: Tamarindus indica*, COX: Cyclooxygenase, b.w: Body weight

Table 3: Estimation of TNF-α of different plant at two different dose

Sl. No.	Treatment group	% inhibition of TNF-α (pg/ml) at 200 mg/kg b.w.	% inhibition of TNF-α (pg/ml) at 400 mg/kg body weight
1	Vehicle/control	0%	
2	*A. vulgaris*	23.10±0.981	31.98±1.03
3	*T. chebula*	25.18±1.93	35.09±1.00
4	*T. indica*	40.29±1.30	59.01±1.09
5	Indomethacin standard at standard dose 10 mg/kg body weight	70.34±0.59	

n=3, ±: Standard deviation, TNF-α: Tumor necrosis factor-α, b.w.: Body weight, *A. vulgaris: Aloe vulgaris, T. chebula: Terminalia chebula, T. indica: Tamarindus indica*

Table 4: General appearance and behavioral observations of acute toxicity study for control and treated groups

Observation	Control group	300 mg/kg	2000 mg/kg	4000 mg/kg
Digestion	NO	NO	NO	NO
b.w.	Normal	Not change	Not change	Not change
Temperature	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal
Urination	Normal	No effect	No effect	No effect
Rate of respiration	Normal	No effect	Normal	No effect
Change in skin	No effect	Normal	No effect	No effect
Drowsiness	Not present	Not present	Not present	Not present
Sedation	No effect	No effect	Observed	Observed
Eye color	No effect	No effect	No effect	No effect
Diarrhea	Not present	Not present	Not present	Not present
General physique	Normal	Normal	Lethargy	Lethargy
Coma	Not present	Not present	Not present	Not present
Death	Alive	Alive	Alive	Alive

b.w.: Body weight, NO: Nitric oxide

that *T. indica* regulates the inflammation by a significant decrease of pro-inflammatory cytokines, i.e., TNF-α which mediates many crucial events for the initiation of acute, sub-acute, and chronic inflammation. Furthermore, it is having an anti-proliferative activity and the potential to directly react with free radicals. The present research work validates the traditional claim of this plants on modern scientific line.

Acute toxicity study

The acute toxic effect of all three plant extracts was determined as per the OECD guideline 423, where the limit test dose of 4000 mg/kg was used. No treatment-related toxic symptom or mortality was observed after oral administration of the tested plant extract at a dose of 300, 2000, and 4000 mg/kg. The general behavioral of the extract treated animals, and control group was observed first for a short period (4 h) followed by long period (72 h), did not display any drug-related changes in behavior, breathing, skin effects, water consumption, and impairment in food intake and temperature. Therefore, the extract seems to be safe at a dose level of 4000 mg/kg, and the LD50 was considered be >4000 mg/kg. However, there was sign of sedation, lethargy, and drowsiness after the administration of plant extract at a dose of 0.6 g/kg and 1.0 g/kg, compared to control group. The parameters observed for acute toxicity study after the administration of these plant extract compared with normal group are presented in Table 4.

Sub-acute toxicity study

The sub-acute toxic study of all the three plant extract was determined as per OECD guideline 407. All the tested group animals treated with plant extract at a dose of 600 and 1000 mg/kg dairy survived throughout the 28 days. No clinical toxicity signs were observed in all the three plant treated group compared to the control group. In conclusion, the pharmacological activities of all the three plants of its use in folk medicine as antipyretic activity, analgesic activity and anti-inflammatory activity but further studies are needed to elucidate the exact mechanism by which secondary metabolite of these plants express these activities.

There were no mortality or morbidity observed in animals through the 14 days period following single oral administration at all selected dose levels for all the plant extract. No observed adverse effect limit was >5000 mg/kg when administered orally.

CONCLUSION

The anti-inflammatory activity expressed by all the three plants. However, *T. indica* L. is more active against TNF–α and COX, and it was comparable to Indomethacin. Need further studies to elucidate the exact secondary metabolite by which these plants express this activity.

ACKNOWLEDGMENT

Authors are thankful to the Intuitional Ethic Committee for providing animals for the studies.

AUTHORS CONTRIBUTION

declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content ofthis article will be borne by the authors. Mohammad Daud Ali collected the data , analyzed the data, all the laboratory workperformed , wrote the introduction, discussion and the material and method part. Prof. Dr. Atul Gupta, Dr. Mohd. AamirMirza and Sabir Afzal proof-read the whole manuscript. Prof. Dr. Arif Naseer helps in designed the study.

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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