



ANTI-INFLAMMATORY ACTIVITIES OF THE AQUEOUS EXTRACT OF THE STEM OF *Tinospora crispa* (FAMILY MENISPERMACEAE)

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ABSTRACT - *Tinospora crispa* of the family Menispermaceae is a popular folk medicine in the Cordillera for the treatment of common pains such as arthritis, rheumatism and sprains. The aim of this study was to evaluate the anti-inflammatory properties of the aqueous extract of the dried stem of the plant in order to provide scientific grounding to its use. Acute inflammation was induced in rat models by introducing carageenan in hind paws causing edema. The extract, at all concentrations (50mg/kg, 100mg/kg and 150 mg/kg), significantly inhibited swelling of the paw. In vitro anti-inflammatory assays were also conducted. *Tinospora crispa* was found to cause stabilization of cellular membranes at 5%w/v and 7.5% w/v concentrations and inhibition of protein denaturation. These activities are therefore suggested as the possible mechanisms for the anti-inflammatory action of *Tinospora crispa*.

KEY WORDS: *Tinospora crispa*, makabuhay, anti-inflammatory activity, membrane stabilization, protein stabilization

INTRODUCTION

Plants are one of nature's richest sources of medicine. Throughout history, great discoveries on compounds useful for treatment and prevention of various diseases were found and isolated from plants. These include digoxin from *Digitalis* spp., quinine from *Cinchona* spp., atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum* (Tyler, 1988). Even with the development of new treatment methods, a great number of the Philippine population especially in the rural settlements, still resort to herbal preparations due to their availability and due to the high cost of modern medicine. Herbs are sources of crude drugs that are used to treat pathologic conditions, often chronic in nature, or to achieve or maintain a state of improved health. Various cultures have characteristic use of plants to treat various ailments. This traditional knowledge on plant use has been orally passed on through several

generations, thus their existence and practice until now. Such collection of folk views on medical practice compiled over the centuries by trial and error, and presumably using the patient as the experimental animal throughout, must surely contain some material worthy of further investigation. This, therefore, calls for the conduct of scientific studies on such plants to verify the claims of community folks on their medicinal effects.

Tinospora crispa (Willd.) Miers ex Hook. F. & Thoms (syn *T. cordifolia*) is a climber found in South East Asia and in tropical India. It naturally occurs in primary rainforests or mixed deciduous forests up to 1,000 m above sea level (Sinha et al 2004). In the Philippines, it is popularly known as makabuhay. This plant is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. Its stem is rather succulent and corky with long filiform fleshy aerial roots from the branches. The bark is creamy white to grey spotted with large lenticels.

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The leaves are cordate, thin, ovate, 6-12 centimeters long and 7-12 centimeters wide. The flowers are small and yellow or greenish yellow. Racemes are solitary or in pairs arising from the axis of the leaves, pale green, and short-pedicelled. The fruit is 7-8 millimeters long (Merrill 1912; Singh et al. 2003, Sengupta, Sharma and Chakraborty 2011).

South East Asian folk medicine considers *Tinospora crispa* as a ‘universal’ medicine since it had long been used in the alleviation of various health conditions. Traditionally, it had been prepared as an aqueous extract for the treatment of flatulence, indigestion and diarrhea. Moreover, the plant is claimed to be effective for the treatment of various inflammatory disorders like rheumatism and arthritis when prepared as a poultice with coconut oil (Quisumbing, 1978). Such method had been a popular traditional treatment among Filipino rural folks. *Tinospora crispa* is also a common component of many conventional Indian herbal preparations. It is used as a tonic, antispasmodic, anti-inflammatory, antiarthritic, antiallergenic, and antidiabetic (Singh et al., 2003).

Tinospora crispa contains a number of chemical constituents which could account for the plant’s medicinal properties. These include alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoids, phenolics, aliphatic compounds and polysaccharides. Leaves are rich in protein (11.2%) and are fairly rich in calcium and phosphorus (Srivastava, 2011). In addition, two triterpenes have been isolated from the stems of *Tinospora crispa*, namely, cycloeucalenol and cycloucalenone (Konghathip et al. 2002). Recent studies have been conducted to verify the efficacy of *Tinospora crispa* as treatment for various diseases. Using experimental animals, the extract of the plant was found to cause increased insulin secretion thereby lowering glucose levels in the diabetic subjects (Noor and Ashcroft, 1998). In addition, evidences had been provided for the plant’s usefulness in the treatment of malaria and cancer (Jagetia and Rao, 2006).

Studies on the anti-inflammatory action of the plant, on the other hand, are limited. Inflammation is a normal protective response of tissues against injury. Its complex metabolism involves increase in vascular permeability, increase of protein denaturation and membrane alterations; and is often associated with pain (Umopathy et al. 2010). If not addressed however, it may lead to more serious conditions such as osteoarthritis and rheumatism.

It is believed that currently available drugs such as opioids and non –steroidal anti-inflammatory drugs (NSAIDS) are not useful in all cases of inflammatory disorders, because of their side effects and extent of potency (Benyamin et al. 2008). Hence, this inadequacy is constantly being bridged by on-going researches in pharmacognosy and pharmacology. Selected plants used in folkloric medicine are constantly being screened for their anti-inflammatory properties and may later prove to be safer and more effective alternatives to synthetic drugs in the market.

Philippine ethnopharmacology has always taken *Tinospora crispa* in high esteem. Its common name, “makabuhay” which literally means “to give life,” lends to its multiple medicinal uses especially against the common maladies. Elaborating on *Tinospora crispa*’s medical properties by subjecting it to various biological assays to ascertain its analgesic and anti-inflammatory properties will definitely provide a scientific grounding to our consideration of *Tinospora crispa* as a real panacea. This study was then conducted to evaluate the anti-inflammatory effect of the aqueous extract of its stem using in vivo and in vitro assays.

MATERIALS AND METHODS

Plant material

The stems of *Tinospora crispa* were collected from Cabalayanan, Bauang, La Union. These were washed with water then dried in an oven at a temperature of not greater than 70°C for two days. The stems were then powdered using

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mortar and pestle and stored in airtight containers until extraction.

Plant extraction

Sufficient quantity of the dried stem powder to fill the thimble for Soxhlet extraction was weighed in a digital balance. Using distilled water as solvent, the stems were subjected to Soxhlet extraction. The solvent was completely removed by concentrating under hot water bath and the resulting solid mass was weighed (yield 11.7%). This was then stored in vials and refrigerated at 4°C prior to use. Suspensions of the extract were prepared freshly for the animal studies using sterile water as solvent.

Animals

Forty five albino rats (*Rattus norvegicus*) of either sex weighing 180-200g were used for the Carageenan-induced paw edema assay. The animals were housed and acclimatized two weeks before the experiment in the Natural Science Research Unit, Saint Louis University at an ambient temperature of 25°C with dark and light cycle (14/10h). They were placed in individual cages and were given free access to dry pellet diet and water.

Carageenan-induced paw edema

Acute inflammation was induced using the method described by Lawal et al. (2010) with some modifications. The albino rats were first grouped into five using random selection. One hour before treatment of the test animals, the diameter of their right hind paw were measured using a vernier caliper. Administration of the five treatments followed via oral route. The treatments were the following: *Tinospora crispa* extracts at 50mg/kg, 100mg/kg and 150mg/kg, ibuprofen 10mg/kg (positive control) and sterile water (negative control). After 30 minutes, 0.1ml of a 1% carageenan solution was injected into the subplantar region of the right hind paws of the test animals. The first hour after injection was used as the basis of reference for the observations for the next succeeding hours. These were computed as percentage of the measurement in the first hour.

Beginning at this point and up to the fifth hour, the paw diameter was measured using the caliper. All measurements were the average of two readings. Percentage of edema was calculated using the following formula:

$$\% \text{ edema} = \frac{\text{size of paw (at a specific hr)} - \text{initial size}^*}{\text{Size of paw at 1 hr} - \text{initial size}^*} \times 100$$

*initial size is the measured size of the non-edematous paw

In vitro Membrane Stabilization test

The protocol described by Gambhire, Juvekar, & Sakat (2009) was used in this study with some modifications. Briefly, human blood from healthy individuals was obtained from the Clinical Laboratory of the College of Natural Sciences, Saint Louis University. Blood samples were washed three times with isotonic buffered solution (154mM NaCl) in 10mM sodium phosphate pH 7.4 for 10min at 3000g. Reaction mixtures consisted of the stock red blood cell suspension (0.5ml), 5 ml of hypotonic solution (50mM NaCl) in 10 mM sodium phosphate buffer pH 7.4 and the test samples: 2.5%w/v, 5% and 7.5% *Tinospora crispa* extract or ibuprofen (0.5%w/v). The mixtures were allowed to stand for 10 min at 25°C then were centrifuged at 3000 g for 10 min. The supernatant was then collected and absorbance was read at 540nm. Percentage inhibition of hemolysis was calculated by the following formula:

$$\% \text{ Inhibition of haemolysis} = 100 \times [A1 - A2/A1].$$

Where: A1 = Absorption of hypotonic-buffered saline solution alone

A2 = Absorption of test sample in hypotonic solution

In vitro Protein stabilization test

The method described by Cantoria (1993) involves the use of albumin as the sample protein. Albumin solution (1%) was prepared in normal saline solution. Varying concentrations of the extract (2.5%, 5.0% and 7.5% w/v) were

added directly into the solution. The reaction mixtures were incubated at room temperature for 15 min. Denaturation was then induced by placing the reaction mixture at 70°C water bath for 10 minutes. After cooling, absorbance at 660 nm was measured. Percentage inhibition of denaturation was calculated from control where no extract and drug was added.

$$\% \text{ inhibition} = \left[\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right] \times 100$$

Statistical analysis

Data were subjected to a One-Way ANOVA at $p < 0.05$, using SPSS program. Means were compared by Fisher's least-significant differences (LSD) to determine significantly different groups. Different letter(s) indicate the values are significantly different ($p < 0.05$).

RESULTS AND DISCUSSION

Carageenan-induced paw edema

The experiment on the reduction of induced rat paw edema evidently confirmed the anti-inflammatory activity of *Tinospora crispa* extracts in all the concentrations used. Figure 1

shows that the extracts are capable of reducing edema size in carageenan treated rat paws. From the third hour to the fifth, the different concentrations of the extracts were significantly different from the negative control (sterile water) and were not significantly different from the positive control (ibuprofen) as determined by One-Way ANOVA ($p < 0.05$).

The carageenan-induced edema in the experimental rats is a common procedure employed in screening compounds as potential remedy for acute inflammation. This type of inflammation is biphasic; the early phase (1-2h) is mediated by histamine and serotonin and characterized by increased synthesis of prostaglandin while the second phase is characterized by sustained prostaglandin release (Agbaje and Fageyinbo, 2012). The results above show that *Tinospora crispa* extract at various concentrations was able to reduce edema after the first hour up until fifth hour similar to the standard, ibuprofen. This means that the extract acts on both early and late phases of acute inflammation. Moreover, this indicates that even at lower doses (500mg/kg) the extract targets the inflammatory mediators (histamine and serotonin) and controls the release of prostaglandin in order to stop the progress of inflammation.

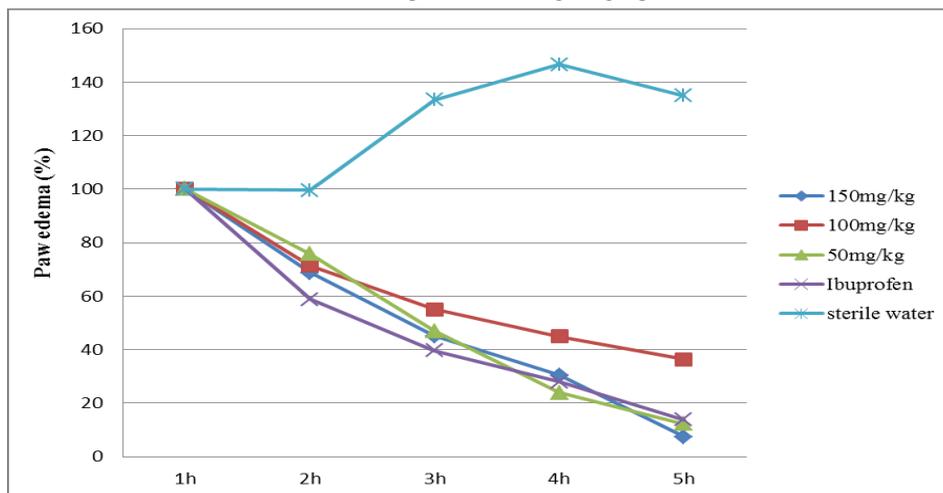


Fig. 1. Effect of *T. crispa* extracts on paw edema (%) after induction of inflammation using carageenan. Each value represents the mean of nine test animals (Data were analyzed using One-Way ANOVA; $p < 0.05$)

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In vitro Membrane Stabilization test

Screening for anti-inflammatory activity using in vitro methods were also conducted in this study. In the first assay, *Tinospora crispa* extract was tested if it is able to prevent hypotonicity-induced lysis of red blood cells. Figure 2 shows that the percentage inhibition of lysis was very similar among the 5% and 7.5% concentration of the extract and the positive control, ibuprofen. However, the 2.5% concentration of the extract had a significantly lower inhibitory property. This may indicate that the extract's efficacy as an anti-inflammatory agent is dose-dependent. Hence, a higher concentration of at least 5% w/v of the extract is required in order to observe comparable effects with standard anti-inflammatory drugs.

Membranes of cell organelles are the targets of oxidation and injury during the inflammatory process (Gambhire et al., 2009). Neutrophils, a class of white blood cells are commonly activated when tissues are inflamed. Moreover, Govindappa et al (2011) explains that such cells store in their lysosomes various proteins

such as phospholipases, proteases and bactericidal enzymes which cause further tissue inflammation and damage upon extracellular release and the activation of other mediators that exacerbate the inflammation.

Anti-inflammatory agents control the biochemical processes involved during the inflammatory response by stabilizing the membranes of the lysosomes (Govindappa et al., 2011). Such compounds are known for their ability to interfere with the release of phospholipases; thus preventing damage to surrounding tissues. (Gambhire et al., 2009; Padmanabhan & Jangle, 2012). The RBC membrane is analogous to the lysosomal membrane in the assay performed in this study. Exposure of RBCs to a hypotonic solution results to lysis of cells. With the addition of *Tinospora crispa* extracts, however, hemolysis was significantly controlled. This result then provides evidence of membrane stabilization as a possible mechanism for the anti-inflammatory effect of the extract.

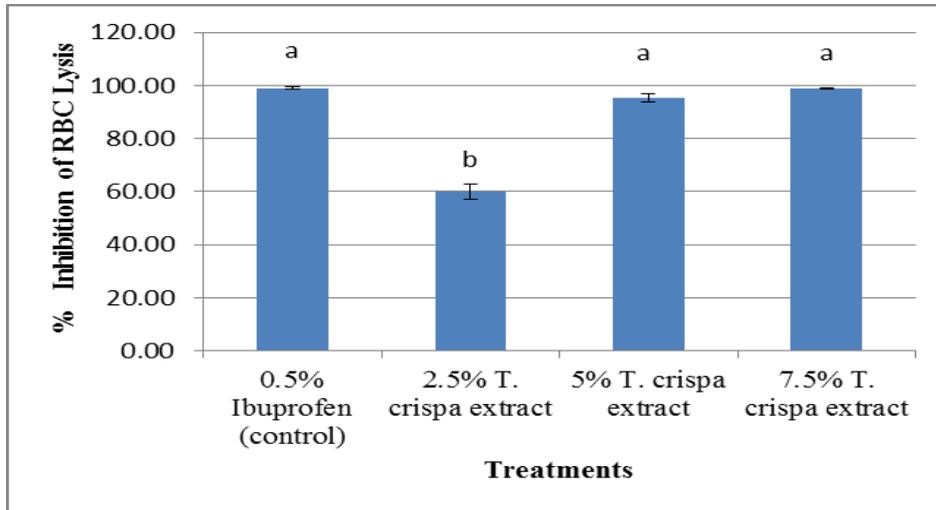


Fig. 2. Hemolysis inhibition (%) of the *T. crispa* aqueous extracts. Each value is expressed as mean \pm standard deviation (n = 9, LSD P > 0.05).

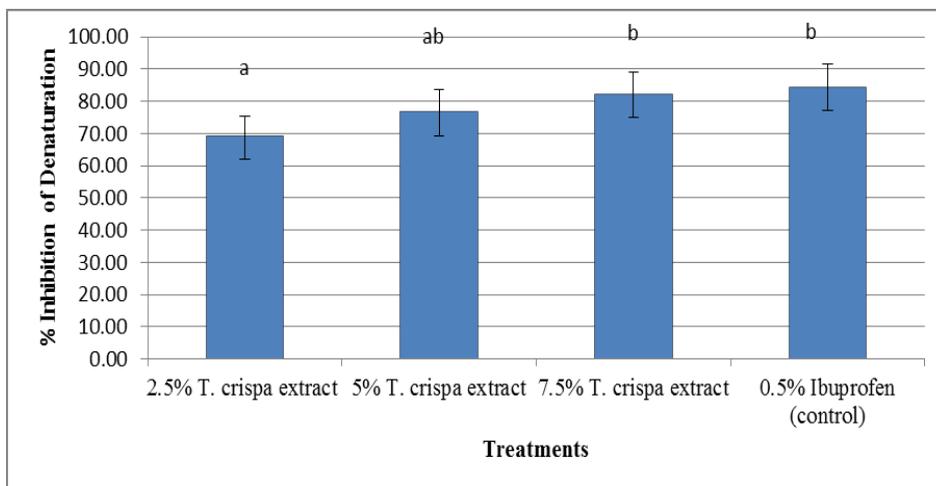


Fig. 3. Inhibition of protein denaturation (%) by the *T. crispa* aqueous extracts. Each value is expressed as mean \pm standard deviation ($n = 9$, LSD $P > 0.05$).

Inhibition of Protein Denaturation

Samples were also tested for their capacity to inhibit protein denaturation. Figure 3 shows that all of the treatments were able to inhibit heat-induced denaturation of the albumin solution. Among the different concentrations, 7.5% *Tinospora crispa* extract exhibited the highest inhibitory property and was followed by 5% and 2.5%, respectively. The standard anti-inflammatory drug, ibuprofen, exhibited the highest inhibition and this was comparable with the effect of the 7.5% extract.

Similar results were presented in other plants including *Coffea arabica* (Chandra et al. 2012) and *Murraya koenigii* (Gambhire 2009). Many synthetic anti-inflammatory drugs combine with proteins to stabilize them and protect them from denaturation by heat, chemicals and agitation (Cantor 1993). During the degenerative and necrotic stages of inflammation, there is a characteristic increase in denatured proteins (Seidler and Yeagans 2002). Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compounds, thereby losing their

biological functions. In this study, *Tinospora crispa* extract showed a comparable effect with ibuprofen in inhibiting heat-induced denaturation of albumin. Hence, the plant's anti-inflammatory effect may also be attributed to its protective properties on cellular proteins.

The three assays performed above reveal consistent results on *Tinospora crispa*'s anti-inflammatory properties. These therefore show that the aqueous extract is effective in ameliorating inflammation and thus supports the folkloric use of the stem in the treatment of various inflammation disorders. As the plant is readily available in the rural areas, it is a good alternative to costly synthetic drugs.

SUMMARY AND CONCLUSION

Aqueous extracts of *Tinospora crispa* stems were used in vivo and in vitro tests to determine its anti-inflammatory activities. Different doses (50 mg/kg, 100 mg/kg and 150 mg/kg) of the aqueous extract were administered to albino rats. Results of the rat paw edema assay revealed that the different concentrations of the extract have significantly reduced paw edema in rats and that the reduction was comparable to

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effects of ibuprofen. This was linked to the capacity of the extract to inhibit the actions of inflammatory mediators and prostaglandin. Protein and membrane stabilization assays using the aqueous extracts were also done to further determine the probable mechanisms of the extract in eliciting its anti-inflammatory property. In the membrane stabilization assay, the comparison of the inhibitory capacities of the extract and the positive control against hypotonicity-induced hemolysis revealed similar results at 5% and 7.5% concentrations. This provided evidence on the possible anti-inflammatory mechanism of action of the extract, that is, the extract is able to stabilize lysosomal membranes of neutrophils. Meanwhile, the protein stabilization test investigated the capacity of the extract in protecting proteins against denaturation. In this assay, the 7.5% concentration of the extract had comparable effects with ibuprofen in preventing protein (albumin) denaturation. This shows another possible mechanism of the extract as an anti-inflammatory agent. *Tinospora crispa* combines with plasma proteins to stabilize them protecting these molecules from proteolytic enzymes which are secreted extensively in the later stages of inflammation. The results of this study thus, justify the folkloric application of the plant for treating inflammation disorders such as sprains, arthritis and rheumatism.

*Statement of Authorship

Regina Lourdes B. Hipol conceptualized the research, conducted the assays and drafted the manuscript.

Roland M. Hipol carried out the in vivo assay, performed the statistical analysis and edited the manuscript.

Maria Faye Nnette M. Cariaga conceptualized the research and edited the final manuscript.

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