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"Evaluation of Anti-Inflammatory and Antioxidant Activities of Stem Bark of *Toddalia asiatica* (L.) Lam. Using Different Experimental Models"

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Abstract: This study was designed to investigate the anti-inflammatory and free radical scavenging potential of 50%ethanolic extract of stem bark of *Toddalia asiatica* (L) Lam. The anti-inflammatory activity of 100, 200, 400 mg/kg of 50% ethanolic extract of stem bark of *Toddalia asiatica* was assessed by Carrageenan-induced rat paw edema method and Cotton pellet granuloma method. All the three doses (100, 200, 400 mg/kg) of the extract showed reduction in paw volume from first hour to third hour significantly. The extract at a dose of 400 mg/kg p.o. of the extract showed the maximum value 44.37% of anti-inflammatory activity. The 100, 200, 400 mg/kg p.o. of the extract significantly ($p<0.01$) reduced the granuloma dry weight when compared to the control. *Toddalia asiatica* showed good radical scavenging activity at various concentrations (200-1000µg/ml) against DPPH with IC_{50} value of 240.07µg/ml, OH radical with IC_{50} value of 432.17µg/ml, NO radical with IC_{50} value of 324.81µg/ml and the same showed moderate scavenging activity against Chelation of Fe^{2+} ions with IC_{50} value of 483.21µg/ml. Our study indicates that 50%ethanolic extract of stem bark of *Toddalia asiatica* (L) Lam possessed effective anti inflammatory and antioxidant activity.

Key words: *Toddalia asiatica* · Free radical scavenging activity · Cotton pellet granuloma method

INTRODUCTION

Inflammation is the complex patho physiological process. Inflammatory diseases including different types of rheumatic disorders are very common through out the world [1]. Inflammatory diseases are accompanied by the chronic release of cytokines and Reactive Oxygen Species (ROS). The reactive oxygen and nitrogen species may further increase the tissue injury [2]. There is much evidence has shown that the production of reactive species occurs at the site of inflammation and contributes to the tissue damage [3]. Although several modern drugs are used to treat these types of disorders but their prolonged use may cause several adverse effects like gastric intestinal irritation [4], consequently there is a need to develop new anti-inflammatory agents [5]. Herbal medicines are being accepted and used increasingly by general populations in both eastern and western countries because of the ethnic acceptability and compatibility having less side effects [6]. Several plants are being used in the traditional medicine for treating the inflammatory disorders.

Toddalia asiatica (L.) Lam. (Family: Rutaceae) can reach the height of 10m in the forests as it uses other trees too as the support. The attractive shiny trifoliate leaves are light to dark green and are extremely aromatic, smelling of lemon when crushed. *Toddalia asiatica* widely used as a folk medicine in India [7]. Pharmacological studies undergone in the plant were comparatively low and the studies undergone showed that the plant constitutes the tumor selective cytotoxicity [8], antibacterial and antifungal activity [7], anti malarial activity [9], anti viral activity against influenza type A virus [10]. Moreover to date no anti-inflammatory and anti oxidant investigations have been reported in this plant. Therefore, the present study was undertaken to investigate the free radical scavenging potential and anti-inflammatory activity of 50% ethanolic extract of stem bark of *Toddalia asiatica* (L.) Lam.

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MATERIALS AND METHODS

Chemicals: All chemicals used in the study were of analytical grade and were purchased from reliable firms (S.D fine chemicals, Mumbai and Sigma chemicals, St Louis, USA).

Animals: Male Wistar rats (125-150g) were used for the study. Animals were procured from the Perundurai medical college, Perundurai, Tamilnadu and were maintained in the standard environmental conditions and have free access to feed (Amrut feeds, India) and water *ad libitum*. The Institutional Animal Ethical Committee approved the protocol of this study.

Plant Materials: The fresh stem bark of *Toddalia asiatica* was collected from the Kolli hills of Namakkal District, Tamilnadu, India in the month of December and authenticated by Dr. G.V.S. Murthy, Botanical Survey of India (Southern circle) Coimbatore. Voucher specimen number of the plant is TA-1718 and stored.

Preparation of the Plant Extract: The stem bark was shade dried and pulverized to make the coarse powder, extracted with 50% ethanol in soxhlet extractor for 72 hours. Then the extract were concentrated under reduced pressure at 50°C using Rota vapour (Buchi U.S) and dried in a vacuum dryer. Thus the obtained extract was used directly for the assessments of anti-inflammatory and antioxidant activities.

Determination of LD_{50}: Dried crude extract was freshly suspended in 2% (w/v0 carboxy methyl cellulose (CMC) prepared in distilled water. LD50 of the extract was determined following the Organizations for Economic Co-operation & Development guidelines (acute toxicity class method: OECD guideline No. 423) were followed for the testing of extract. A Limit test was performed to categorize the toxicity class of the compound and then Main test was performed to estimate the exact LD50. The animals (female wistar albino rats) were fasted overnight with free access to water, weighed before dosing and test substance administered. All the animals survived at limit test on 2000mg/kg and subsequently on 4000mg/kg dose. The 50%ethanolic extract of stem bark of *Toddalia* asiatica is practically a non toxic compound as it is non toxic upto 4000mg/kg dose. A dose of 100, 200 and 400 mg/kg was selected for pharmacological action [31].

Carrageenan-Induced Rat Paw Edema: Inflammation in rats was produced by carrageenan according to the method already described [11]. The rats were divided in to five groups, each group consist of six animals. Tween 80 (1% v/v in saline) was used as a vehicle for suspending the extract as well as standard anti-inflammatory drug Aspirin. The group I served as a control and received only vehicle, the group V received standard drug Aspirin (20mg/kg p.o.) [30]. Group II, III and IV were treated with 100mg/kg, 200mg/kg and 400mg/kg of 50% ethanolic extract of stem bark of *Toddalia asiatica* respectively. All the doses of extract were given orally. After 30 minutes of sample treatment, acute inflammation was produced by sub-planter injection of 0.1ml of 1% carrageenan in normal saline in the right hind paw of rat. Mean increase in paw volume were measured using plethysmometer (UGO Basile, Italy) at 0hr, 1hr, 2hr and 3hr after carrageenan injection to each group. 0 hr readings are considered as initial paw volume of the animals. Percentage inhibition of the paw volume was calculated at each hour by using the formula:

$$\% \text{ of Inhibition} = 1 - (Vt/Vc) \times 100$$

Where, Vt is the paw volume of test group and Vc is the paw volume of control group.

Cotton Pellet Granuloma: The method was described by [12]. In this method, some giant cells and connective tissue can be observed besides the fluid infiltration after several days of subcutaneous implantation of pellets of cotton in rats [13]. The rats were divided in to five groups, each group consist of six animals. Sterile cotton pellets (10±0.7 mg) were implanted subcutaneously in axilla under ether anesthesia. The group I served as a control and received only vehicle, the group V received standard drug Diclofenac sodium (13.5 mg/kg p.o.) [20]. Group II, III and IV were treated with 100mg/kg p.o., 200mg/kg p.o. and 400mg/kg p.o. of 50% ethanolic extract of stem bark of *Toddalia asiatica* respectively for seven consecutive days from the day of cotton pellet implantation. On the eighth day, animals were sacrificed and granulation tissue with cotton pellet was removed and dried to constant weight at 60°C and dry weight was taken. The mean weight of the pellets of the control group as well as of the test group was calculated and compared to calculate percentage inhibition.

Free Radical Scavenging Activity Using 1,1-diphenyl-2-picrylhydrazyl Radical (DPPH): The ability of the 50% ethanolic extract of stem bark of *Toddalia asiatica* to scavenge the free radicals were estimated by in vitro method using a stable nitrogen centered radical viz. DPPH [14]. Briefly 0.05 ml of extract dissolved in methanol was added to a methanolic solution of DPPH (100 µM, 2.95 ml) at different concentration (200-1000µg/ml) and the absorbance was recorded at 517 nm.

Nitric Oxide Scavenging Activity: Nitric oxide (NO) radical scavenging activity was measured by using a spectrophotometer [15] Sodium nitroprusside (5mM, 1.5ml) in phosphate buffered saline was mixed with different concentrations of 50% ethanolic extract of stem bark of *Toddalia asiatica* (200-1000µg/ml) dissolved in methanol and incubated at 25°C for 30 min. A control without test compound but with equivalent amount of methanol was taken. Thirty minutes after incubation, 1.5ml of the incubation solution were removed and diluted with 1.5ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% Naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with Naphthyl ethylene diamine was measured at 546nm.

Hydroxyl Radical Scavenging Activity: The ability of the test extract to scavenge the hydroxyl (OH) radical was determined using ascorbic acid iron-EDTA (Ethylene diamine tetra acetic acid) model, OH generating system. The standard reaction mixture consisted of 100mM phosphate buffer, pH 7.4, 167µM iron-EDTA complex, 0.1 mM EDTA, 2mM ascorbic acid and 33mM DMSO (Dimethyl sulphoxide) in a final volume of 3ml. Iron catalyzed oxidation of ascorbic acid at 37°C was used to generate formaldehyde from DMSO. Appropriate controls, reaction mixtures without ascorbic acid, were maintained. Test extract was added to obtain final concentrations ranging from 50-400 µg/ml separately. D-mannitol (50mm) was used as standard. The reaction was stopped by the addition of 1ml of ice cooled Trichloro acetic acid (17.5% w/v). The decrease in formaldehyde formation due to scavenging or decreased formation of OH was assayed spectro photometrically [16].

Chelation of Ferrous (Fe^{2+}) Ions: Concentration of free ferrous ions (fe^{2+}) was estimated using chelating agent 2, 2'bipyridyl [17]. Briefly, the reaction mixture (1ml) contained 50µM sodium chloride (pH 7) and different concentrations of test extract and were incubated for 30 min, at the end of which 2ml of 2, 2'bipyridyl (1mM) was added. Absorbance of Ferrous bipyridyl complex was measured at 525 nm against the blank devoid of ferrous sulphate.

Statistical Analysis: All the data are expressed as mean ± S.E.M. Statistical significance was determined by one way ANOVA (Analysis of Variance) followed by Dunnet multiple comparison test by using the Graph pad Instant version 3.01. All data on all antioxidant activity tests are the average of triplicate analyses. Linear regression analysis was used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

The carrageenan induced paw edema test is the simplest and most widely used acute inflammatory model for studying anti-inflammatory model for studying anti inflammatory agents [18]. Mechanism of induction of carrageenan edema has been extensively investigated [19]. Carrageenan induced inflammation is a biphasic process [20]. The initial phase which lost for one hour is attributed to the release of histamine and serotonin [19], but the platelet activating factor and arachidonic acid metabolites are also play important roles [21]. The second phase (3rd hour) is related to release of bradykinin and prostaglandins [22, 23]. The second phase is sensitive to most chemically effective anti-inflammatory drugs [19, 24]. All the three doses (100, 200, 400mg/kg) of 50% ethanolic extract of stem bark of *Toddalia asiatica* showed reduction in paw volume from first hour to third hour (Figure 1) significantly. Among the values of Percentage of anti-inflammatory activity, 400mg/kg p.o. of the extract showed the maximum value 44.37% (Figure 2) at third hour. Next to that, 200mg/kg showed 38.13% of anti-inflammatory activity (Figure 2).

The 50% ethanolic extract of stem bark of *Toddalia asiatica* was further evaluated for its anti-inflammatory activity by cotton pellet induced granuloma formation to understand its potential in the chronic inflammatory phase. Further the method has been widely employed to evaluate the transudative, exudative and proliferative components of chronic inflammation [20]. The 100, 200, 400mg/kg p.o. of the 50% ethanolic extract of stem bark of *Toddalia asiatica* significantly ($p<0.01$) reduced the granuloma dry weight when compared to the control (Table 1). The inhibition percentage too calculated, 400mg/kg of the extract showed the maximum inhibition value of 44.53% (Table 1). Thus the extract exhibits the anti-inflammatory activity.

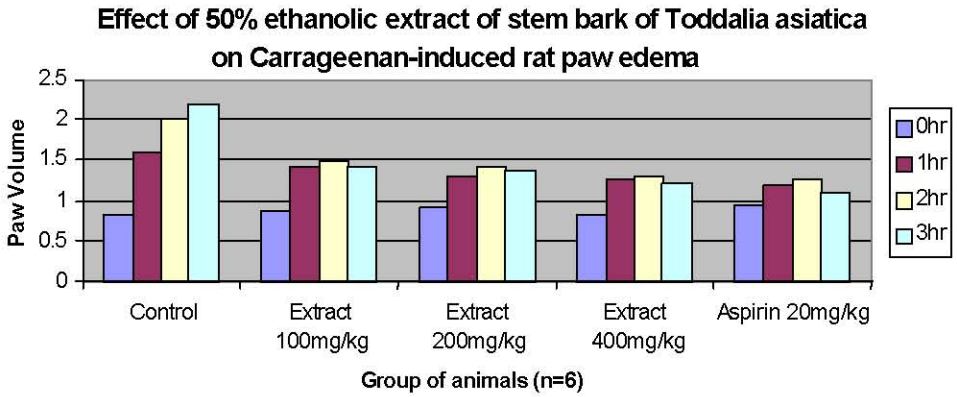

Fig. 1:

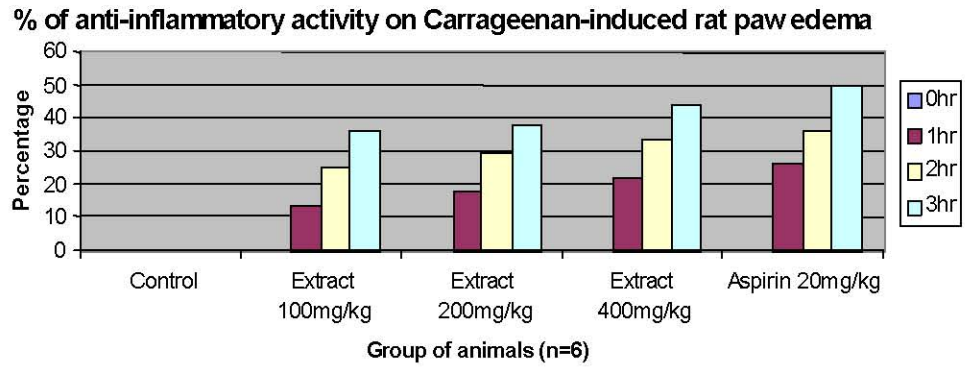

Fig. 2:

Table 1: Effect of 50% ethanolic extract of stem bark of *Toddalia asiatica* on Cotton pellet induced granuloma in rats

Group (n=6)	Dose (mg/kg p.o.)	Granuloma dryweight (mg)	Inhibition %
Group A Control	-	31.22±1.37	-
Group B Extract	100	22.83±0.34**	26.88%
Group C Extract	200	20.17±1.31**	35.40%
Group D Extract	400	17.32±0.92**	44.53%
GroupE (Diclofenac sodium)	13.5	15.36±0.81**	50.81%

Each value is expressed as mean ± S.E.M. **p<0.01 Statistical significance was determined by one way ANOVA (Analysis of Variance) followed by Dunnet multiple comparison test by using the Graph pad Instant version 3.01.

Table 2: Free radical scavenging potential of 50% ethanolic extract of *Toddalia asiatica* stem bark

Methods	IC_{50} values	% inhibition of standard Ascorbic acid (100µM)
DPPH	240.07	88.07
OH Radical	432.17	78.72
NO Radical	324.81	92.10
Chelation of Fe^{2+} ions	483.21	93.21

Inflammation is a complex process, ROS plays a vital role in the pathogenesis of inflammatory diseases. ROS generated in the body by two ways endogenously and exogenously both are associated with the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and aging process [25].

The Free radical scavenging activity using DPPH is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 517 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is

generated accompanied by loss of color [14]. Such reactivity has been widely used to test the ability of the plant extracts to act as free radical scavengers. The extract scavenged the DPPH radical in a dose dependent manner with IC_{50} value of 240.07µg/ml (Table 2).

Hydroxyl radical (OH) has been shown to promote lipid peroxidation. Protein oxidation and DNA strand breakage. In fact, much of the hydrogen peroxides that promotes alteration in the DNA and other organic substrates has been suggested to result from the site specific, metal-catalyzed production of OH via the fenton reaction [26]. Several studies showed that the plant phenolic compounds are efficient scavengers of free radicals and blockers of lipid peroxidation. They are able to chelate transition metals, such as iron and copper ions and there by to prevent the redox cycle of these metals. [27, 28]. The 50% ethanolic extract of stem bark of *Toddalia asiatica* also inhibited the Hydroxyl radical potently in the dose dependent manner with IC_{50} value of 432.17µg/ml (Table 2).

The free radical scavenging activity using nitric oxide is based up on sodium nitroprusside serves as a chief source of free radicals. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with napthylene diamine is used as a marker for NO scavenging activity [20]. Nitric oxide (NO) is recognized as a mediator and regulator of inflammatory responses [29]. NO can also interact with molecular oxygen and superoxide anion to produce reactive nitrogen species that can modify various cellular functions. The 50% ethanolic extract of stem bark of *Toddalia asiatica* also potently inhibited NO in dose dependent manner (Table 2) with the IC_{50} being 324.81µg/ml.

Chelation of Fe^{2+} ions test is based on the measurement of change in fe^{2+}/fe^{3+} ratio. The below results represented that chromogen formation is inhibited in a dose dependent fashion. The test extract showed the moderate inhibition of the ferryl-bipyridyl complex with the IC_{50} being 483.21µg/ml (Table 2). Thus the extract exhibits the potent anti-oxidant effect.

CONCLUSION

In conclusion, the study has shown that, 50% ethanolic extract of stem bark of *Toddalia asiatica* (L.) Lams possess effective anti-inflammatory and anti-oxidant activities. Thus it can be said that, free radical potential of the extract may be one of the factor pillered the anti inflammatory activity of the same. Anyhow, further studies needed to bring out the active principles and exact mechanism of the extract.

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