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# *In vivo* anti-inflammatory activity of *Tabernaemontana divaricata* leaf extract on male albino mice

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**[ABSTRACT] AIM:** To study the *in vivo* anti-inflammatory activity of *Tabernaemontana divaricata* leaf extract on male albino mice. **METHODS:** Aqueous decoction and methanol leaf extracts were tested for their ability to reduce croton oil-induced edema in the mouse ear after topical application. The methanol leaf extract dose-dependently inhibited the croton oil-induced ear edema in mice ($ID_{50}$ <500 μg·cm$^{-2}$). A bioassay-guided liquid–liquid fractionation of this methanol extract gave four active fractions: water insoluble (F1), hexane (F2), ethyl acetate (F3) and water (F4). **RESULTS:** The hexane fraction showed a very high activity (42.1% inhibition at 0.7 μg·cm$^{-2}$) as compared to the control. The other fractions were less active (F1: 56.1% at 506.2 μg·cm$^{-2}$; F3: 57.3% at 289.3 μg·cm$^{-2}$; and F4: 31.9% for 203.8 μg·cm$^{-2}$) while indomethacin gave 48.8% of inhibition at 90 μg·cm$^{-2}$. The activity of F1 and F3 may be at least in part explained by the presence of anti-inflammatory flavonoids, while the activity was not correlated to the tannin contents. No compounds were detected in the most active F2 fraction. **CONCLUSIONS:** The results give a rational support to the traditional use of *T. divaricata* in tropical India as anti-inflammatory agent.

**[KEY WORDS]** *Tabernaemontana divaricata*; Anti-inflammatory; Mouse ear edema; Quercitrin; Plant extract; Traditional medicine

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## 1 Introduction

Inflammation involves a complex web of intercellular cytokine signals [1], and is implicated in the pathogenesis of many diseases, including cancer, diabetes, cardiovascular, neurodegenerative, and other life-threatening and debilitating diseases [2]. Macrophages play a central role in the inflammatory response, and serve as an essential interface between innate and adaptive immunity [3]. In the process of inflammatory response, macrophages release nitric oxide (NO), a reactive molecule originated from the guanidine nitrogen of L-arginine, catalyzed by nitric oxide synthase enzymes (NOS) and other cytokines, e.g. interleukin-6 (IL-6) [4]. Excessive NO production leads to the development of many inflammatory-related diseases [5]. Prostaglandin E2 ($PGE_2$), generated by specific COX-2 function, is another anti-inflammatory parameter [6]. Moreover, MDA and SOD, due to their contributions to the alleviation of inflammatory responses [7], were both detected for their levels to assess the radical scavenge abilities of samples. Macrophages also play an important role in inflammatory diseases relating to overproduction of pro-inflammatory cytokines including interleukin-10 (IL-10), interleukin-6 (IL-6) and tumor necrosis factor [8]. Elevated productions of these mediators have been detected in many tissues after exposure to immune stimulants including LPS [9]. Thus, these inflammatory mediators, MDA, NO, SOD, $PGE_2$, IL-6, IL-10, and TNF-α, are important targets in the treatment of inflammatory diseases.

*Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult. (Apocynaceae) is a widely distributed plant in Asia. It is used in the traditional medicine systems of many Asian countries for the treatment of bacterial, fungal, parasitic and inflammatory disorders. *T. divaricata* is one of the plants that is used in Ayurveda, Chinese and Thai traditional systems of medicine. *T. divaricata* is traditionally used by people in many parts of the world to treat various disorders like abdominal tumors, arthralgia, asthma, diarrhea, epilepsy, eye infections, fever, fractures, headache, inflammation, leprosy, mania, edema, paralysis, piles, rabies, rheumatic pain, skin diseases, ulceration. It is also used as antihelmintic, antihypertensive, aphrodisiac, diuretic, emmenagogue, hair

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growth promoter, purgative, remedy against poisons and tonic for brain and liver Growing evidences suggests that this plant has medicinal benefits and its extracts could possibly be used as pharmacological intervention in various diseases. Phytochemical studies on various parts reveal that this plant contains at least 66 indole alkaloids, non-alkaloidal constituents like enzymes, flavonoids, hydrocarbons, phenolic acids, phenyl propanoids, steroids and terpenoids. Alkaloids, flavonoids and terpenoids are the main secondary metabolites that exhibit many physiological and pharmacological properties on living cells [12]. *T. divaricata* flowers contain 3, 4, 14, 19-tetrahydro-olivacine, 11-methoxy-*N*-methyl dihydropericyclivine, 19-*epi*-voacangine, apparicine, isovoacangine, isovoacristine, tabernaemontanine, tabersonine, voaphylline, *N*-1-methyl-voaphylline, and vobasine. There are still many *T. divaricata* alkaloids and & their derivatives whose pharmacological activities are yet to be studied.

## 2 Experimental

### 2.1 *Plant material*

The fresh green leaves of *T. divaricata* were collected in Rajendra nagar, Indore and identified by Prof. S. R. Upadyaya. A voucher specimen (COPIPS/097/T/ 2012) has deposited at the Institute.

### 2.2 *Extraction*

Leaves were sun-dried and pulverized. Three extracts were prepared from the powdered leaves: decoction 20 min ($H_2O$-D), maceration 24 h in methanol (MeOH-M) and Soxhlet extraction for 4 h with methanol (MeOH-S). The obtained extracts were dried under vacuo using a rotary evaporator or lyophilised ($H_2O$-D).

### 2.3 *Fractionation*

The MeOH-S extract was dissolved in boiling water and kept for 24 h at room temperature. Filtration gave a residue (F1) and a water-soluble fraction. This aqueous soluble part was then successively extracted (50 mL, four times) with hexane and ethyl acetate. Afforded organic fractions were dried over anhydrous sodium sulfate, filtered and concentrated. The residual aqueous extract was lyophilized. This gave four fractions: water insoluble (F1), hexane (F2), ethyl acetate (F3) and residual aqueous fractions (F4).

### 2.4 *Animals*

Male albino Swiss mice (28−33 g) of were used. Animal quarters were maintained at 22 °C and 60% humidity with 12 h light/12 h dark cycle.

### 2.5 *Chemicals*

Croton oil, indomethacin, pyrogallol and ellagic acid; ketamine hydrochloride; hyperoside and quercitrin; hide powder CRS.

### 2.6 *Anti-inflammatory activity (AIA)*

Cutaneous inflammation was induced to the inner surface of the right ear (surface: about 1 $cm^2$) of anesthetized mice (145 $mg \cdot kg^{-1}$ ketamine hydrochloride, intraperitoneally) by application of 15 μg of acetone or acetone–water (1 : 1) solutions containing 80 μg of croton oil as irritant (control animals). For treated animals, appropriate amounts of indomethacin (reference drug) or of the tested extracts were dissolved in the croton oil-containing solution and applied as for controls. After 6 h, mice were sacrificed and a punch (5 mm of diameter) was excised from both ears. Inflammation was measured as edema formation and quantified by the weight difference between the treated and the untreated (opposite) ear samples [10]. The anti-inflammatory activity was expressed as a percentage of the edema reduction in treated mice compared to the control mice. The baseline edema level in non-inflammed mice was zero. At least two experimental groups of five animals were tested for each dose level. The experimental design was approved by the IAEC (Institutional Animal Ethical Committee).

### 2.7 *Dosage of tannins and flavanoids*

Dosage of tannins was realized as per the European Pharmacopoeia [11] and expressed in pyrogallol. Total flavonoids were quantified following the method described for hawthorn (*Craetegus* spp.) leaves and flowers and expressed as hyperoside [12].

### 2.8 *TLC analysis*

TLC on silica gel 60 (Merck plates, 0.25 mm) was performed eluting with EtOAc–HCOOH–$CH_3COOH$–$H_2O$ (100 : 11 : 13 : 26, *V*/*V*) (A), toluene–EtOAc–HCOOH (8 : 2 : 1, *V*/*V*) (B), or 30 mL of B with 5 mL of MeOH (C) as mobile phases, at room temperature. Developed TLCs were examined under UV254 nm and UV366 nm light prior to spraying. Flavonoids were detected using aminoethanol diphenylborate–PEG 400 mixture and visualised at 365 nm [13]. Dragendorff reagent [14] was used for alkaloid detection. Anisaldehyde/$H_2SO_4$ reagent was used to reveal the presence of terpenoids. For polyphenolic compounds, iron (III) chloride reagent R3 [15] was used. For each sample, 10 μL of 5 $mg \cdot mL^{-1}$ solutions in the appropriate solvent were spotted and allowed to migrate over 10 cm. Ellagic acid ($R_f$ 0.30 in system C), hyperoside ($R_f$ 0.13 in system C) and quercitrin ($R_f$ 0.22 in system C) were used as references at a concentration of 1 $mg \cdot mL^{-1}$.

### 2.9 *Statistical analysis*

The pharmacological data were analyzed by Student's *t*-test and significance was assumed for *P*-values lower than 0.05.

## 3 Results

### 3.1 *Extraction yield of plant material*

Extraction yields (*W*/*W*) were 11.46% for aqueous decoction ($H_2O$-D), 13.08% for methanol macerate (MeOH-M), and 24.34% for the Soxhlet extract (MeOH-S) in term of dry weight. Among the fractions from MeOH-S, fraction F1 (water insoluble, 50.62%) had the highest yield followed by the

ethyl acetate fraction (F3, 28.93%) and residual aqueous (F4, 20.38%). The F2 (hexane fraction) yield (0.07%) was the least.

3.2 *Anti-inflammatory test*

The $H_2O$-D, MeOH-M and MeOH-S samples were tested at 2 000 μg·cm$^{-2}$. At this concentration, the MeOH-S extract showed the highest activity, while the water decoction demonstrated only a mild effect (Table 1). These initial experimental results were confirmed by a second set of tests on the MeOH-S extract, giving a higher yield than the macerate, which exhibited dose-dependent activity (Table 2).

The activity of a quantity of each fraction corresponding to that present in MeOH-S (1 000 μg) was analyzed. At these doses, all fractions induced a significant reduction of the edematous response (Table 3). From these results, it can be seen that the hexane fraction (F2) was the most active, giving 42% edema inhibition at 0.7 μg·cm$^{-2}$ (0.07% of 1 000 μg·cm$^{-2}$).

3.3 *TLC analysis*

TLC analysis revealed the presence of tannins, flavonoids, terpenoids, and alkaloids in the crude methanolic extracts [16]. These extracts have the same chromatographic profiles. Tannins were present in F1 and F3, while all extracts except F2 contained flavonoids. In the methanol extracts, the most abundant flavonoids were hyperoside and quercitrin. These molecules were also found in F1 and F3 fractions. All alkaloids were concentrated in F3 fraction and, apparently, only F1 fraction contained terpenoids.

3.4 *Dosage of tannins and flavanoids*

MeOH-M extract contained more tannins (12.0%) than MeOH-S (8.9%) and $H_2O$-D extracts (10.0%), while the concentrations of flavonoids in MeOH-M and MeOH-S were in the same range (2.9 and 3.2%, respectively). The decoction contained less flavonoids (2.0%) (Table 4). The most active fraction (F2) contained neither flavonoids nor tannins. All tannins were concentrated in the F1 and F3 fractions. F3 contained far more flavonoids than F1 and F4.

**Table 1 Anti-inflammatory activities of $H_2O$-D and MeOH crude extracts (mean ± SE)**

| Substance | Dose/(μg·cm$^{-2}$) | *n* | Edema/mg | Inhibition/% |
|---|---|---|---|---|
| Control | – | 10 | 8.2 ± 0.11 | – |
| $H_2O$-D | 2 000 | 11 | 7.3 ±0.29 | 11.1 |
| MeOH-M | 2 000 | 9 | 3.4 ± 0.27* | 56.6* |
| MeOH-S | 2 000 | 8 | 0.7 ± 0.05* | 91.6* |
| Indomethacin | 90 | 9 | 3.7 ± 0.08 | 48.8* |

*$P$ < 0.05

**Table 2 Anti-inflammatory activities of MeOH-S extract at different doses (mean ± SE)**

| Substance | Dose/(μg·cm$^{-2}$) | *n* | Edema/mg | Inhibition/% |
|---|---|---|---|---|
| Control | – | 10 | 7.2 ± 0.3 | – |
| MeOH-S | 2 000 | 8 | 0.6 ± 0.2 | 91.6 |
| MeOH-S | 1 000 | 9 | 0.8 ± 0.2* | 88.4* |
| MeOH-S | 500 | 9 | 2.4 ± 0.7* | 66.6* |
| Indomethacin | 90 | 9 | 3.7 ± 0.6* | 48.8* |

*$P$ < 0.05

**Table 3 Anti-inflammatory activities of MeOH-S fractions (mean ± SE)**

| Substance | Dose/(μg·cm$^{-2}$) | *n* | Edema/mg | Inhibition/% |
|---|---|---|---|---|
| Control | – | 10 | 7.2 ± 0.11 | – |
| Insoluble (F1) | 506.2 | 8 | 3.0 ± 0.7* | 56.1* |
| Hexane (F2) | 0.7 | 7 | 4.0 ± 1.3* | 42.1* |
| EtOAc (F3) | 289.3 | 7 | 2.9 ± 0.9* | 57.3* |
| $H_2O$ (F4) | 203.8 | 12 | 4.7 ± 0.6* | 31.9* |
| Indomethacin | 90 | 9 | 3.7 ± 0.6* | 48.8* |

*$P$ < 0.05

## 4 Discussion

The MeOH-S extract produced dose-dependent reduction of the croton oil-induced ear edema with an $IC_{50}$ <500 μg·cm$^{-2}$. All fractions of the MeOH-S extract had some activities and contribute to the activity of the total extract. However, the hexane fraction (F2) showed the highest activity (0.7 μg·cm$^{-2}$ gave 42.1% of inhibition). This lipophilic fraction (F2) can be regarded as a potential source of highly active anti-inflammatory compounds with a higher potency

than the pure non-steroidal anti-inflammatory drug (NSAID) indomethacin. This is quite unusual for a crude extract since their active compounds are often diluted by other bulk substances. However, synergistic or additive effects will be investigated. In fraction F3, which also has an interesting activity, the presence of ellagic acid, hyperoside and quercitrin was demonstrated. These three compounds were also present in the F1 fraction but in lower quantity. This is the first time that the presence of quercitrin in *Tabernaemontana divaricata* is reported. Lee *et al*. 1993 [17] showed that hyperoside had a significant activity on the mice ear edema test after oral administration. Guided bioassay of the inhibitory activity on TPA-induced ear edema in mice on *Erythrospermum monticolum* Thouars. (Achariaceae) identified quercitrin among the two active compounds [18]. Quercitrin was also identified in an anti-inflammatory fraction of Solanum melongena leaves [19]; furthermore Sanchez de Medina et al (1996) shows the inhibitory effect of quercitrin on inflammation [20]. Previous reports about *T. divaricata* showed that ellagic acid was responsible for anti-plasmodial activity *in vitro* [21]. Ellagic acid was also successfully tested for its protective activity against colitis inflammation induced by dextran sulfate sodium [22] at a concentration of 10 mg/kg given twice daily for 6 days, per os in microsphere capsules. The studies of Giovannelli *et al*. (2000) on complex polyphenols and tannins from wine concluded that they might both have a protective and a therapeutic potential in oxidative damage related pathologies [23]. In 1996, after topical test for heterogeneous tannins' ability to inhibit the biomarkers of tumor promotion in mouse skin *in vivo*, Perchellet *et al*. (1996) concluded that some foliage tannins have potent antioxidant and anti-inflammatory activities [24]. However, in these experiments, it was observed that the most active crude extract (MeOH-S) had the lowest tannins content (Table 4), and that the aqueous decoction containing about 10% tannins had non-significant activity at a dose of 2 000 μg·cm$^{-2}$. Furthermore, the yields of tannins in fractions F1 (12.00%) and F3 (8.9%) can not explain the difference between the observed anti-inflammatory activity, since F1 contained more tannins and was proportionally less active than F3. On the other hand, the concentrations of flavonoids in F1, F3 and F4 were correlated to the anti-inflammatory activity. Nevertheless, the most active fraction (F2) was highly lipophilic. Purification and characterization of the compounds from this fraction are in process. The ethanolic extract of the leaves of *T. divaricata* also possess free radical scavenging and anti-fertility activity [25-26].

**Table 4 Presence of tannins (expressed as pyrogallol) and flavonoids (expressed as hyperoside) in crude extracts**

| Extracts | Tannins/% | Flavonoids /% |
|---|---|---|
| $H_2O$-D | 10.0 | 2.0 |
| MeOH-M | 12.0 | 2.9 |
| MeOH-S | 8.9 | 3.2 |

## 5 Conclusion

The MeOH extracts of *T. divaricata* leaves showed strong dose-dependent anti-inflammatory activity after topical application. This activity may be explained at least partially by unidentified lipophilic compounds and by flavonoids (e.g. hyperoside and quercitrin), but the degree of activity was not related to tannin contents. This work is the first report on the presence of quercitrin in *T. divaricata*. Nevertheless, other compounds probably also contribute to the anti-inflammatory activity. Further studies will be undertaken to elucidate the mechanism of action by which the extracts exert their anti-inflammatory activity and eventually isolate active compound(s). However, our results give a rational support to the traditional use of *T. divaricata* in tropical India as anti-inflammatory agent.

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