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**Research Article**

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# Evaluation of anti-inflammatory and antinociceptive activity and isolation of two new alkaloids from leaves extract of *Tabernaemontana sananho*

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**ABSTRACT**

*Tabernaemontana is genius of flowering plant in the family of apocynacea, plant is rich in indole alkaloids and parts of the have been used to relieve pain in some parts of Asian countries. In present study from the methanol extract of Tabernaemontana sananho leaves two new alkaloids compounds were isolated and structure was elucidated by spectral studies as TS-01, a subtype of ervatamine and TS-02 belonging to monoterpene indole alkaloid. Methanolic extract of Tabernaemontana sananho leaves was evaluated for anti inflammatory and antinociceptive activity at dose of 150 mg/kg and 300 mg/kg respectively, and a significant activity dose dependent (p< 0.001) activity was exhibited, confirming the folk- fore use of Tabernaemontana sananho in treating inflammation .*

**Key words:** *Tabernaemontana sananho* leaves, indole alkaloids, anti inflammatory

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## INTRODUCTION

*Tabernaemontana* is genus of flowering plants in the family of apocynaceae, these plants grows as small trees and are rich in indole alkaloids like conolidine ,voacangine, ibogaine . Some genus finds their use for treating dementia [1], as additive in some psychedelic drink [2], conolidine has been reported as new class of pain reliever [3]. *Tabernaemontana sananho (TS)* commonly known as Lobo sanango, grows as a evergreen dense shrub and is used medicinally by several tribes for different aliments. *TS* shrub is traditionally used by Iberoamerican, the leaves are used in the treatment of rheumatic pains [4]. The bark juice is applied for toothache; pulp is used as gargle for sore throat and colds [5]. The latex mixed with water is used to heal eye wounds. It is also considered sudorific, tonic, used for colds, obesity, rheumatism and syphilis [6]. In the present research work isolation of alkaloid is carried out by column chromatography and structure are established by various spectral data's and anti inflammatory and antinociceptive activity for methanolic extract of TS leaves are reported.

## EXPERIMENTAL SECTION

### 2.1 General

Carrageenan and indomethacin (Sigma Aldrich,) dicolfenac sodium, formalin and solvents for extraction were obtained from Merck, prepared TLC plates ( Merck) were used for monitoring the isolates from the various fractions ; NMR spectra were recorded on Bruker DPX 400 Switzerland (400 MHz for $^{1}$H NMR and 100 MHz for $^{13}$C NMR), respectively and ES-MS was were recorded on a TOF Spec 2E Micromass (UK), a MS route JMS-600H, Jeol, Japan, IR recorded on FTIR Perkin Elmer .

**2.2 Plant material**

*Tabenaemontana sananho* leaves (TSL) were collected from Gangakhed, Parbhani district, Maharashtra in the month of July, 2009 The leaves were identified and authenticated from the Department of Botany, Ganabharati, Bangalore University, Karnataka.

**2.3 Animals**

The protocol for the study was approved by the Ethical Committee of Krupanidhi College of Pharmacy, Bangalore. Male and female Albino rats (200–250 g) were used in these experiments. The animals were obtained from the animal house, Krupanidhi College of Pharmacy. All animals were housed in standard cages at room temperature (20 ± 2°C), provided with pellet food and water *ad libitum*. Prior to administration of the drugs, the rats were fasted for 12 h with free access to water.

**2.4. Formalin induced paw licking Test**

Albino rats 200-250 g of either sex were divided into four groups (n = 6). Group I was treated as control ( normal saline) 1ml/kg, group II was administered with Indomethacin 10 mg/kg p.o., group III was given a low dose 150 mg/kg and group IV was treated with a high dose of 300 mg/kg p.o methanol leaf extract suspension of TS. Diluted formalin (1 %) 50 µl was injected subcutaneously into the dorsal surface of the right hind paw of rats; nociceptive behavior of animals was observed immediately after formalin injection. Nociceptive behavior was quantified as the numbers of flinches of the injected paw during one minute period for every 5 min, up to 60 minute after injection [7]. The initial acute phase (0–5 min) was followed by a relatively short quiescent period (10 - 20 min), which was then followed by a prolonged tonic (inflammatory phase) response (25–60 min).

**2.5. Carrageenan induced paw edema**

Either sex of albino rats weighing 220-250 g were selected and were divided into five groups each consisting of six animals. Group I, received normal saline (1ml p.o.), group II were treated for toxic control, group III were treated with dicolfenac sodium (10 mg/kg p.o.) and group IV and V were given 150 mg/kg and 300 mg/kg p.o of methanol leaf extract of TS respectively. After 30 min of administration of the test dose, the animals were injected 0.1 ml of 1% (w/v) carrageenan in the plantar region of the left paw of control. The non-inflamed right paw served as reference for comparison. The difference between the right and left paw volume of the treated animals were observed at interval of 15, 30, 60, and 120 min after carrageenan challenge [8]. The measurement of foot volume was accomplished by a displacement technique using a plethysmometer immediately before and 3 h after the injection. The percent difference in the right and left paw volumes of each treated animal was calculated and expressed as percent edema inhibition.

**2.6 Statistical analysis**

Results are expressed as mean ± SEM (standard error of the mean). The results were tabulated and data were statistically analyzed using one way ANOVA followed by Dunnet's test, $p < 0.05$ was considered to be significant.

**2.7 Extraction and Isolation**

Dried leaf powder was subjected for cold maceration with petroleum ether (40-60 $^0$C). After the complete extraction, the marc was dried and macerated with methanol. The methanol extract was collected and concentrated under vacuum. Dried methanol extract (150 g) was treated with 10 % HCl, the acid layer was neutralized with ammonia solution and was extracted with chloroform. The free alkaloids were isolated from dried chloroform fraction, (38g), over silica gel column chromatography using dichloromethane and chloroform in increasing polarity. Various fractions collected were monitored by TLC visualized under UV 365 nm; total ten different fractions A-J were collected. From fraction Fr I, sub fractions were collected from chloroform and methanol in gradient elutions ; Fr I $_{(6\text{-}9\ )}$ yielded **TS 1** and from sub-fraction Fr J $_{(1\text{-}4)}$ **TS 2** was isolated ( fig1). Compounds were purified from chloroform: methanol (1: 9) and were subjected for spectral analysis.

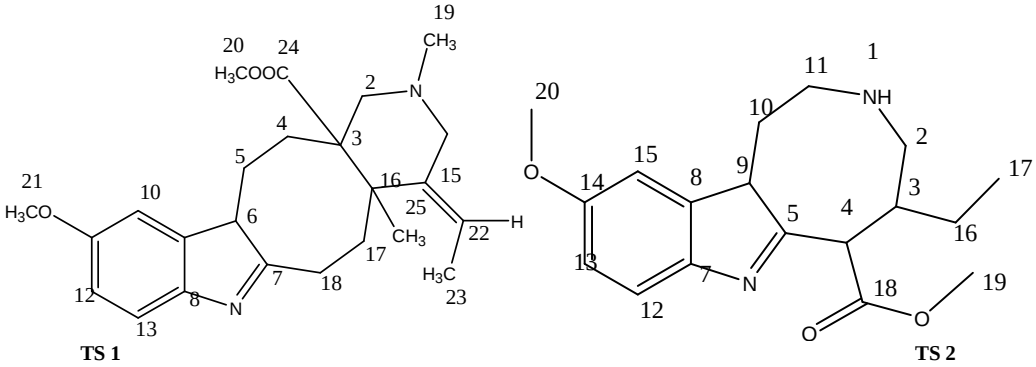


**Fig1- Alkaoids TS1 and TS2 isoalted from TS leaves methanol extract**

**Compound TS 1**- White powder, EI-MS m/z: 382 $M^+$ calcd $C_{23}H_{30}N_2O_3$ ; 47 (97%) 85 (87%); 239 (58%); 313 (75%); 331 (30%) . IR (KBr) $cm^{-1}$ at 3430 (N-H), 2925 (C-H), 2853 (C-H.), 2617 (COOR), 1734 (C=O), 1625 (C=O); $^1H$ and $^{13}C$ NMR see table 1

**Compound TS 2**- White powder, m.p.120°C (uncorrected), EI-MS m/z: 316 (15%) $M^+$ calcd $C_{18}H_{24}N_2O_3$; 43(100%); 57(80%); 83(70%); 126(40%); 109(35%); 149(20%). IR(KBr) $cm^{-1}$ at 3430 (N-H), 2925 (C-H), 1734 (C=O),1625 ( C=O),1246 (C-O ). $^1$ H and $^{13}$ NMR see table 1.

**Table 1 - $^{13}C$ and $^1H$ NMR data of compound TS1 and TS 2 in $CDCl_3$**

| Position | TS1 | | TS2 | |
|---|---|---|---|---|
| | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ |
| 1 | | | | 1.94,s,1H |
| 2 | 67 | 3.9, m,1H | 50.4 | 2.52,t, 2H |
| 3 | 47 | | 29 | 2.45, m,1H |
| 4 | 28 | 2.07,m , 1H | 34 | 2.47, d, 1H |
| 5 | 56 | 2.8, m , 1H | 168 | |
| 6 | 160 | 2.2,m,1H | | |
| 7 | | | 139 | |
| 8 | 147 | | 136 | |
| 9 | 131 | | 31 | 2.20, m, |
| 10 | 113 | 6.86,d,1H,J=2.4Hz | 32 | 1.26,m, 2H |
| 11 | 152 | | 50 | 1.86,m,2H |
| 12 | 112 | 6.65,dd, 1H,j= 1.6, 5.6 | 123 | 6.91, d, 1H |
| 13 | 124 | 7.05,d,1H,j=5.6 | 112 | 6.72,d,1H |
| 14 | 61 | 1.25,s,2H | 107 | |
| 15 | 149 | | 109 | 7.4,d,1H |
| 16 | 34 | | 24 | 1.54,q,2H |
| 17 | 29 | 2.03, m ,2H | 12 | 0.78,t, 3H |
| 18 | 31 | 2.09, m, 2H | 174 | |
| 19 | 43 | 2.28, s, 3H | 113 | 3.59,s,3H |
| 20 | 173 | 3.66, s, 3H | 26 | 3.80,s,3H |
| 21 | 53 | 3.8,s 3H | | |
| 22 | 120 | 5.35, s, 1H | | |
| 23 | 13 | 1.54,s, 3H | | |
| 24 | 22.5 | | | |
| 25 | 14 | 0.87, s, 3H | | |

## RESULTS AND DISCUSSION

The anti nociceptive activity of TSL methanol extract exhibited dose dependant significant inhibition ($p<0.01$***) in phase I and II of acute and chronic inflammation, while significant inhibition was seen in Phase Q (table 2). In Carrageenan induced paw edema**,** methanol extract of TSL showed significant inhibition of inflammation (table 3 ) shows dose dependant significant anti inflammatory activity of TSL extract with respect to the toxic control.

Compound **TS 1** was obtained as white powder, EI-MS showed a molecular ion peak at m/z 382 from which the molecular formula was calculated $C_{23}H_{30}N_2O_3$, and an absorption peak at 3443 $cm^{-1}$ indicated the presence of indole N-H and a peak at 1738 $cms^{-1}$ for carbonyl group of an ester [9]. The $^1$HNMR spectra in $CDCl_3$ showed the presence

of a singlet at δ 3.86 for the presence of aromatic methoxy group which was supported from carbon spectra which exhibited a peak at δ 152; the multiplet peaks seen at δ 6.8 and doublet at δ 7.05 confirmed the presence of aryl ring [9], this was supported from the peaks of $^{13}$CNMR at δ 131 and 124 respectively. A singlet peak at δ 5.24 indicated the vinyl proton and its presence was established from the absorption peak at 1404 cms$^{-1}$ and from peaks in $^{13}$CNMR seen at δ 149 and δ 120 establishing the presence of olefin carbons; further singlet at δ 1.54 indicated the olefin methyl proton which was supported from $^{13}$C peak at δ 12. A broad singlet at δ 2.28 in $^{1}$H NMR indicated the presence of N-$CH_3$ [9-11] which was confirmed from $^{13}$C singlet seen at δ 43 ; $^{1}$HNMR spectra exhibited mutilplet for the three methylene group protons at δ 1.25 and further the presence of two methylene present in the environment of electro negativity was confirmed from $^{1}$HNMR peak at δ 2.03 and in $^{13}$CNMR peaks at δ 67 and 61 respectively [12-14]; peaks seen at δ 34 indicated the presence of quaternary carbon the up field value showing the presence of an adjacent sp$^3$ carbon the presence of latter was confirmed from singlet peaks seen in $^{1}$HNMR at δ 0.87. The down field shift of quaternary carbon seen at δ 47 in $^{13}$C indicated the presence of an adjacent carboxymethyl group, the presence of which was revealed by IR and confirmed from singlet peak observed at δ 3.6 and from the peak at δ 173 in NMR spectra's. A multiplet peak at δ 2.8 indicated methine proton this was confirmed from the peak seen at δ 56 indicating a cycloheptane ring fusion with indole nucleus [15]. From the spectral analysis and with cited literature it was confirmed the obtained compound was an alkaloid of Ervatamine subtype [16,17] substituted with aryl methoxy, N-methyl, olefenic methyl and carboxymethyl ester.

Compound **TS 2** – EI-MS exhibited the molecular ion peak at m/z 316 and molecular formula was calculated as $C_{18}H_{24}N_2O_3$. The IR spectra showed an absorption peak at 3430 cm$^{-1}$ indicated indole N-H, 1734.21cm$^{-1}$ for the carbonyl group of an ester [18-19], the presence of which was confirmed from the peak seen at δ 174 in $^{13}$C NMR . A singlet peak at δ 3.59 indicated the presence of carboxymethyl protons [20-22]; multiplet peaks seen at δ 6.9 and a doublet at 7.4 further confirmed the presence of indole ring; $^{1}$H NMR showed a triplet at δ 0.78 indicated methyl group and quartet at δ 1.86 confirmed an adjacent methylene group, confirmed from the absorption peak at 2925 cm$^{-1}$ in the IR spectra. The $^{1}$HNMR exhibited peak at δ 3.8 for the presence of an aryl methoxy group [23-25], and same was supported from the peak seen at δ 159 in $^{13}$C NMR; a broad singlet was seen at δ 1.94 confirmed the presence of secondary amine group; $^{1}$H NMR exhibited multiplet peaks at δ 2.52 for aliphatic methylene; the presence of methine proton was observed from multiplet at 2.27. A downfield value of δ 2.4 multiplet indicated two methine protons and their presence was further confirmed from the peaks seen at δ 34 and δ 31 in $^{13}$CNMR respectively [26,27]. From the spectral analysis and molecular mass it would be analysed that a methoxyindole nucleus was fused to octazepine nucleus having carboxymethyl ester and an ethyl side chain. Very significant inhibition of phase I and II of antinociceptive and anti inflammatory activity of TS extract with respect to the toxic control was exhibited by TSL methanol extract The formalin test is different from most models of pain, as it can assess the way animals respond to moderate, continuous pain generated in injured tissue [28,29] . It is a very useful method not only for assessing antinociceptives, but also for elucidating the mechanism of pain and analgesia, whether the site of action is central and/or peripheral [30].The formalin test consists of two distinct phases, possibly reflecting different types of pain . The early phase starts immediately after an injection of formalin and lasts for 3-5 min corresponding to acute neurogenic pain. The second phase (lasting from 15 to 30 min) corresponding to inflammatory pain due to direct chemical stimulation of nociceptors. This phase can be inhibited by centrally acting antinociceptives [31-33]. The late phase starts approximately lasts for 20-40 min, seems to be due to the combination of an inflammatory response in the peripheral tissue [33]. Centrally acting drugs such as opioids inhibit both phases equally, but peripherally acting drugs such as aspirin, indomethacin , and dexamethasone only inhibit the late phase [34] .This phase can be inhibited by NSAIDs and steroids, as well as centrally acting drugs [35]. Inflammation consists of body's response to injury and is characterized by a series of events that includes inflammatory reaction, a sensory response is perceived as pain, and a repair process. The results of our present study indicate the TSL methanol extract can relieve pain mediated centrally as well as peripherally as it inhibited both phases of nociceptive, development of edema in the paw of the rat, a biphasic event is also inhibited; the initial phase, observed around 1hr, can be attributed to the release of PG like substances and inhibition of proliferate phase of the inflammation of the microphages, neutrophils, fibroblasts and collagen formation which are basic source for the granuloma formation; therefore decrease in the granuloma formation indicates the suppression of the proliferate phase[32]. The extract of TSL showed strong inhibition on the paw edema in the early phase and late phase of the inflammation, implying that extract exhibit anti-inflammatory effect by acting on both phase of the inflammation.

**Table 2: Anti inflammatory activity of methanol leaves extract of *T. sananho* in formalin induced rat model.**

| Treatment | Dose | Different phases of anti inflammatory activity | | |
|---|---|---|---|---|
| | | Phase-I | Phase-Q | Phase-II |
| Control (normal saline) | 01 ml/kg | 13 ± 0.5744 | 03 ± 0.408 | 15 ±0.5774 |
| Indomethacin | 10 mg/kg | 04± 0.5774*** | 0.5±0.2880*** | 5±0.5774*** |
| Methanol extract | 150 mg/kg | 7.5 ± 0.8660** | 1.2±0.2880** | 8.5±0.2887*** |
| Methanol extract | 300 mg/kg | 06± 1.1500*** | 01 ± 0.4787** | 6.5±0.2887** |

*All values are mean ±SEM, n =6, *p>0.05, ** p<0.01, *** p<0.001*

**Table 3- Effect of Methanol Leaves Extract of *T Sananho* in carrageenan induced paw edema in rats.**

| Treatments | Paw volume in ml due to carrageenan at different time intervals (mean ±SEM) | | | | |
|---|---|---|---|---|---|
| | 0 (min.) | 15 (min.) | 30 (min.) | 60 (min.) | 120 (min.) |
| Normal Control | 0.019± 0.001 | 0.018± 0.001 | 0.019± 0.002 | 0.014± 0.002 | 0.012± 0.005 |
| Toxic Control | 0.01± 0.001* | 0.034± 0.003** | 0.34± 0.002* | 0.029± .001** | 0.034± 0.003** |
| Standard (diclofenac sodium) | 0.02± 0.002aa | 0.026± 0.002 | 0.025± 0.002 | 0.018± 0.001 a | 0.002± 0.002 |
| Methanol extact TS (150 mg/kg) | 0.014± 0.001 | 0.025± 0.002 | 0.018± 0.004 a | 0.015± 0.003 aa | 0.013± 0.001 aa |
| Methanol extact TS (300mg/kg) | 0.015± 0.001 | 0.019± 0.002 a | 0.014± 0.003 aa | 0.013± 0.002 aa | 0.011± 0.003 aa |

*All values are mean ±SEM, n=6, * p>0.05 ** p<0.01, *** p<0.001 when compared with normal control; a p>0.05, aa p<0.01, aaa p<0.001 when compared with toxic control.*

## CONCULSION

In conculsion this work reports isoaltion of two new alkaloides, **TS 1** belonging Ervatamine subtype and **TS 2** methoxy indole from methanolic leaves extract of *T. Sananho* and extract exhibited very good analgesic effect.

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