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Phytochemical Investigation and Analgesic Activity of Stem Bark Extract of *Sapindus trifoliatus* Linn

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The study is designed to isolate and charecterize the phytoconstituents, and screen for the analgesic activity of stem bark extracts of *Sapindus trifoliatus* Linn.
Methodology: The cleaned, dried and powdered stem barks of *Sapindus trifoliatus* were subjected to extraction by maceration process. The concentrated ethanolic extract of stem bark on was further subjected to preliminary phytochemical studies. The fractionated extracts were then packed into column chromatography for the isolation of phytoconstituents and they were characterized by IR, ^{1}HNMR, ^{13}CNMR and mass spectroscopy.Acute toxicity was performed to establish the lethal dose of the extract and *In vivo* analgesic activity was performed by tail flick and acetic acid induced writhing methods in experimental animals.
Results: Preliminary phytochemical studies showed the presence of steroids, terpenoids, flavonoids, saponins and carbohydrates. Isolation of extracts led to give compounds like saponin glycoside, a steroid and triterpenoids. The extract was found to be safe up to 2000 mg kg bodyweight. Analgesic activity was found significant at level *P* = 05 when compared with control by tail flick and acetic acid induced writhing models in experimental animals.

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Conclusion: From ethanolic extract isolated a saponin glycoside,from petroleum ether stigmasterol and triterpenoids ursolic acid. The presence of saponin glycoside, triterpenoids, steroids might be responsible for the analgesic activity of the stem bark extract of *Sapindus trifoliatus* Linn.

Keywords: Sapindus trifoliatus; sapindaceae; stigmasterol; ursolic acid; tail flick method; acetic acid writhing model; analgesic.

1. INTRODUCTION

Pain is an unpleasant physical sensation linked with damages in the tissue/s [1]. Pain is normally originated by noxious stimuli and spread over neural networks to the CNS where, it is interpreted as such. It acts as a method to shield the body from likely damage [2]. To different obnoxious stimuli like injury to the tissue or any infections, inflammation is a protective response which is a result of both innate and adaptive immune systems [3,4].

Inflammation and pain still remain as one of most challenging health issues affecting around 80% of worlds adult population even though there is access to adequate medications [5]. In majority of communities across the world it is considered as a major clinical and socio-economical problem [6]. Untreated and chronic pain is widespread condition which results in both psychological and physical damage [7]. Non sorted inflammation results in major inflammatory ailments which includes pelvic inflammatory disease, hypersensitivities, hay fever, inflammatory bowel diseases, asthma, reperfusion injury, rheumatoid arthritis, glomerulonephritis, autoimmune diseases and it might also lead to deprivation from social activities. If these conditions are not properly addressed they may lead to major complications that might result in death [8].

Presently used drugs against inflammation and pain act as center piece for treating these problems. But, even these are accompanied by various adverse effects and toxicities like ulceration, GIT irritation, variations in kidney function, changes in Blood Pressure, liver damage and inhibition of platelets resulting in bleeding. Use of NSAIDs especially COX 2 inhibitors result in elevated risk of CVS abnormalities [8,9]. Even, opioid related analgesics are also accompanied with large number of adverse effects and toxicities which includes constipation, hearing loss, pruritis, nausea and vomiting, drowsiness, disturbance in hormonal homeostasis, addiction and respiratory problems. In the light of this, there is a requirement to boost medicinal plant research because they are claimed to be potent anti-inflammatory agents to manage pain [10].

Sapindus is a genus of about five to twelve species of shrubs and small trees in the lychee family, Sapindaceae, native to warm temperate to tropical regions of the world. The genus includes both deciduous and evergreen species. Members of the genus are commonly known as soapberries or soapnuts because the fruit pulp is used to make soap [11].

Sapindus trifoliatus (ST) Linn. family *Sapindaceae* is medium-sized deciduous tree found in south India. High content of saponins and sugars have been reported in the pericarp. The saponin moiety is characterized as hederagenin group of glycosides. The pericarp is reported for various medicinal properties. It is reported to possess emetic, tonic, astringent and used in the treatment of asthma [12], colic due to indigestion, diarrhoea and paralysis of limbs, a thick watery solution of the pulpy mesocarp is introduced into the nose of the patients for the relief of hemicrania and for restoring consciousness during epileptic and hysteric fits. In the folklore literature, it is also reported that there was more or less relief in almost every case of hemicrania in which the solution was tried, but the cases of hysteria and epilepsy benefited by it were very less. ST has been reported for its antispermatogenic, antiandrogenic activities and antirheumatic activities.

Hence, objective of the present study was to investigate the phytochemical constituents ethanolic extract of ST *in vivo* animal models to study its effects on [13].

Current study was performed for phytochemical investigation and to examine the analgesic activity of *Sapindus trifoliatus* stem bark extract

2. MATERIALS AND METHODS

2.1 Preparation of Ethanolic Extract

The stem bark of *ST* were obtained from Mangaluru, Karnataka. The *ST* plant was

authenticated by botanist Dr. Noeline J. Pinto, Head of botany Department, St. Agnes College, Mangaluru. The stem barks were washed, dried and subjected for size reduction into a coarse powder using mechanical grinder. Obtained powder was then passed through sieve no. 40 and extracted using ethanol by process of maceration. Resulting extract was dried using flash evaporator under controlled temperature and reduced pressure.

2.2 Preliminary Phytochemical Screening

To know the existence of secondary metabolites related to analgesic activity of *S. trifoliatus Linn.* Standard phytochemical examination was performed [14,15].

2.3 Isolation of Compounds from Ethanolic Extract

The powdered stem bark (500 g) of *ST* was subjected to continuous soxhlet extraction and maceration process by using ethanol. Resulting extract was concentrated on water bath (yield 35 g). The ethanolic residue (30 g) was suspended in water (5 mL). This was then loaded onto a silica gel column (150 g) prepared in water. The column was eluated with 75% aqueous methanol and 100% methanol. These eluates were combined and concentrated to yield a light brown colored semi solid mass (15 g). This residue (15 g) again suspended in 5 mL of methanol, subjected to repeated column chromatography using silica gel (150 g). It was eluated with increasing polarity of solvents. Every time 10 mL of eluates were collected and continuously monitored by TLC (visualization; vanillin: H_2SO_4 reagent). The eluates of ethyl acetate: ethanol: water (25:5:2) showed single spot on TLC and then chloroform: methanol: water (40:10:1) showed a single spot in TLC. They separately concentrated and recrystallised from methanol and designated as compound I (45 mg).

2.3.1 Hydrolysis of compound I

Added 5mL of conc. 2 N HCl to solution of compound (5 mg) in MeOH (5 mL). Refluxed the resultant mixture for 2 h at 100 ^{0}C and under reduced pressure evaporates it to dryness. 5mL of distilled water was added and extracted using ether. Extract was washed with water and solvent was evaporated. Then it was dried over an. sodium sulfate to get powder of light yellow color.

2.3.2 Identification of the sugars

Rotary evaporator was used to evaporate aqueous hydrolysis which was left after extraction using ether and resulting residue was dissolved using 2 drops of pyridine. It was co-chromatographed with standards like xylose, fucose, rhamnose, fructose, glucose, galactose and arabinose. Spots developed by sugars were visualized using spray reagent aniline hydrogen phthalate and heating at 105 °C. *n*-butanol: EtOH:H_2O (4:2:1) was used as developing solvent. Arabinose, glucose and xylose were identified.

2.4 Isolation of Compounds from Petroleum Ether Extract

The petroleum ether extract (20 g) was dissolved in $CHCl_3$ (20 mL) and adsorbed onto silica gel (20 g). Solvent was evaporated, after evaporation it was loaded onto silica gel column (70-300 Mesh, 150 g) prepared in petroleum ether (60-80°C). Pet ether 100% was eluted first followed by graded mixture of petroleum ether: $CHCl_3$ (95:5, 90:10, 80:20, 70: 30), $CHCl_3$ 100%, graded mixture of $CHCl_3$: MeOH (95:5, 90:10, 80:20) and finally with methanol. The TLC was used to monitor the elution (Silica gel G; visualized using spray reagent aniline hydrogen phthalate and heating at 105 °C). Every time 10 mL were collected in a test tube and same volume of eluates (TLC monitored) were combined and was concentrated to 5 mL and placed in a desiccator.

Elution which was carried out using pet ether (60-80°C): $CHCl_3$ graded mixture (95:05) gave single spoton TLC (petroleum ether: $CHCl_3$; 95:05) and was named as compound II (50 mg). Elution carried out using pet ether: $CHCl_3$ graded mixture (80:20) gave single spot on TLC (petroleum ether: $CHCl_3$; 80:20) and was named as compound III (55 mg).

2.4.1 Acetylation of compound II

Compound II (5 mg) was taken up in dry pyridine (200 µL) and freshly distilled AC_2O (1 mL) was added to it. The mixture was kept at room temperature overnight, then added to the crushed ice, stirred and kept for 2 hours, filtered and dried.

2.5 Acute Toxicity Studies

Female adult albino rats were used to study acute toxicity in accordance with “Up and Down”

[16] method and OECD guidelines 425 [17]. Guidelines of IAEC of KSHEMA, Deralakatte, Mangaluru (KSHEMA/AEC/28/2010) was followed during experimentation.

2.6 Screening of Analgesic Activity

2.6.1 Tail flick method [18]

Study was performed using albino rats whose average body weight was 200 mg. Rats were separated into 5 groups of 6 animals. All the rats were individually numbered and weighed. First group was taken as control. Rats in second group were treated with standard drug Pentazocine. Ethanolic extract of *ST* stem bark (suspended in 0.5% sodium CMC) was given to third, fourth and fifth group 100 mg/ kg, 200 mg/ kg and 400mg /kg body weight (per oral). Every rat was kept in restrainer, prior to treatment basal reaction time was measured by keeping distal one-third portion of tail. Tail was kept on wire of nichrome without touching it. After this step extracts were administered orally, reaction time was measured for every 30 min. intervals for 2 hours after administering the drug. To prevent tissue damage 10 seconds of cut off time was employed. After every 30 minutes reaction time was recorded for 120 minutes and after each time interval an average value of reaction time was calculated and was compared with pretest value by analysis of significance.

2.6.2 Writhing by acetic acid [18]

Study was performed using albino mice with body weights between 20-25 gms. Mice were separated into 5 groups of 6 animals. All the mice were individually numbered and weighed. 0.1ml acetic acid(0.6% *v/v) by* intraperitoneally was given to all animals. First group was taken as control. Standard aspirin was given to animals in second group. 30 min prior to administration of acetic acid ethanolic extract of *Sapindus trifoliatus* stem bark (suspended in 0.5% Na CMC) was given to third, fourth and fifth group 100, 200 and 400mg/kg body weight (per oral.). By injecting acetic acid into peritoneal cavity of mice pain is produced. Stretching of abdomen with simultaneous stretching of at least one hind limb indicated writhing effect. This was observed for 30 min and change in number of writhing in test group compared with standard treated and control treated groups.

The percentage inhibition was calculated by using the formula.

$$\text{Percentage inhibition} = [1 - R_t \div R_c] \, 100$$

Where:

R_t = Mean number of writhing in treated group'
R_c = Mean number of writhing in control group.

2.7 Statistical Analysis

The results were expressed as mean ± SEM. The total variation present in the data was analysed by one way analysis of variance (ANOVA) followed by Post hoc test (Dunnett's test).

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening

Preliminary phytochemical screening, the stem bark of ST showed and confirmed the presence of steroids, flavonoids, glycosides, triterpenoids and saponins as Secondary Metabolites

3.1.1 Analysis of compound I

Physical state: Yellow colored crystals,Rf Value: 0.72 (Solvent system; $CHCl_3$: MeOH, 80:20)
Melting point: 274-276 °C

Spectral characteristics of compound I:

IR (KBr cm^{-1}):	3428.81 (OH) 2931.27 (C-H stretch in CH_2 of alkanes),1737.55 (C=O) 1645.95 (C=C stretch C 12 (13) double bond,1464.67 C-H deformation of methyl and methylene (CH_3/CH_2), 1372.1 gemdimethyl grouping 1350-1264.11 (C-O stretch of primary alcohol), 1043.30 (C-O stretch).
^{1}HNMR (DMSO-d6):	δ 12.05 (CO-OH protons),δ 5.15 CH=C (C_{12} vinyl group), δ 0.5-2 aliphatic protons(CH_3,CH_2 protons),δ 3.6 O-CH_2 protons of primary alcoholic group. δ 3.1 broad O-H protons, δ 3.38 OH protons of the sugar
13CNMR (DMSO-d6):	Chemical shifts of the aglycone moieties δ38.2 (C-1), 31.9 (C-2), 76.3 (C-3), 39.9 (C-4), 45.47 (C-5), 29.0 (C-6), 32.8 (C-7), 33.3 (C-8), 47.13 (C-9), 35.9 (C-10), 25.5 (C-11), 121.58 (C-12), 143.84 (C-13), 45.7 (C-14), 30.37(C-15),

29.2 (C-16), 45.4 (C-17), 46.2 (C-18), 41.4 (C-19), 29.0 (C-20), 32.1 (C-21), 31.9(C-22), 62.4 (C-23), 27.2 (C-24), 25.5 (C-25), 29 (C-26), 27.2 (C-27), 178.54 (C-28), 32.8 (C-29), 24.1 (C-30), δ9.6 (C-1'), 76.3 (C-2'), 72.9(C-3'), 69.9 (C-4'), 68.2(C-5'), 103.1(C-1''), 79.4(C-2''), 73.4(C-3''), 70.9 (C-4''), 68.4 (C-5''), 63.1 (C-6''), 105.8 (C-1'''), 81.2 (C-2'''), 73.8 (C-3'''), 72.4 (C-4'''), 69.4 (C-5'''), 64.9(C-6''').

Compound I gave pink color for Liebermann-Burchard and responded for Molisch's tests indicating the presence of glycones and test for saponins The ^{1}H NMR spectrum of compound I showed six methyl groups. The ^{13}C spectral data exhibited the presence of 30 carbon signals, consisting of six methyl carbon at δ 25.5 (C-24), 25.3 (C-25), 29 (C-26), 27.9 (C-27), 24.2 (C-30), 32.1 (C-29) and presence of an acid signal at δ178.54 (C-28). Inspection of ^{13}C NMR spectral data of compound I showed that the presence of three sugar moieties (anomeric carbons at δ 99.6, 103.1, 105.08 along with 30 carbon signals for a glycones. The chemical shift of C-3 (δ76.3) suggested that in compound I the sugar moieties were linked at C-3. From the above IR, ^{1}H NMR, ^{13}C NMR data the proposed structure of compound I as given below (Fig. 1).

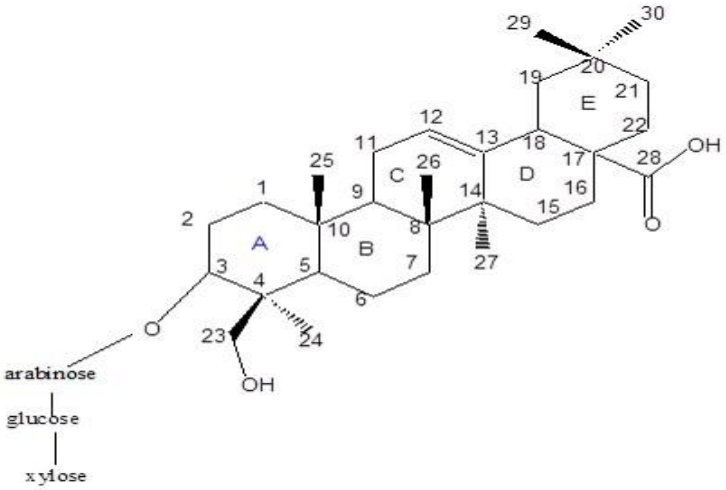

Fig. 1.

3.2 Analysis of Compound II

Physical state: Pearl white crystals,R_f value: 0.8 (solvent system-Pet ether: $CHCl_3$; 95:05)
Melting point: 168ºC.It gave a red color in Salkowski's test and a green color in Libermann-Burchard's test.

Spectral characteristics of compound II

IR (KBr cm^{-1}): **IR (KBr cm^{-1}):** 3429.0 cm^{-1} (br, OH str), 2919.0 cm^{-1} (C-H str. in CH_3), 2850.0 cm^{-1} (C-H str. in CH_2),1656.0 cm^{-1} (C=C str.), 1108.0 cm^{-1} (C-H deformation in gem dimethyl), 1006.0 cm^{-1} (C-O str. Of secondary alcohol), 615 cm^{-1} (rocking vibration of CH_2).

^{1}HNMR(DMSO-d6): **^{1}HNMR ($CDCl_3$)** δ 0.76 (s, 3H, H-19), δ 0.79 (s, 3H, H-18), δ 0.94 (s, 3H, H-21), δ 0.83 (t, 3H, H-24"), δ 0.87 (d, 3H, H-26), δ 1.07 (d, 3H, H-27), δ 2.17 (d, 1H,OH), δ 4.69 (m, 1H, H-3), δ 5.35 (s, 1H, vinylic proton), δ 5.15 (d, 2H, allylic proton),δ 1.13 to δ 1.25 (m, 16H, 8 x CH_2),δ 1.38 to δ 1.68 (m , 9H, methine protons).

^{13}CNMR(DMSO-d6) δ 141.62(C-5), 137.65 (C-22), 128.29 (C-23), 120.17 (C-6), 70.25 (C-3) 56.53 (C-17),50.87 (C-20), 55.87 (C-13), 40.33(C-4),39.38 (C-12), 37.17(C-15), 31.79(C-1), 36.32 (C-8), 31.57(C-10), 31. 26 (C-7),28.12 (C-25), 24.71

(C-14), 23.94 (C-27), 21.04 (C-29), 20.60(C-19), 18.9(C-21), 19.10(C-18).

Mass Spectra (EI-MS): Molecular formula: $C_{29}H_{48}O$, Molecular weight: 412 GC-MS (m/z), 412 (M^+,$C_{29}H_{48}O$), the other peaks appeared at 394, 351, 300, 271, 255,213, 159, 145, 133,105, 83, 55 (100%).

Isolated compound II gave a characteristic colour reaction of Sterols. It gave red colour in Salkowski's test and a green u in Libermann-Burchard's test. It formed an acetate m.p. 143-145 °C. The peak at 1656.0 cm^{-1} in IR spectra indicated C=C stretching while peaks at 1108.0 cm^{-1}, 1006.0 cm^{-1} and 615 cm^{-1} indicated the C-H deformation in gem dimethyl, C-O stretching of secondary alcohol and rocking vibration of CH_2 respectively. IR Peak at 3429.0 cm^{-1} indicated the presence of OH group while the ^1H NMR signal at δ 2.17 indicated OH at C-3 position. The ^1H NMR signal at δ 5.35 gave singlet which indicated one vinylic proton at C-6 position while the ^1H NMR signal at δ 5.15 gave doublet indicated two allylic protons at C-7 position. The most down field signals at The most down field signals at δ 141 was accommodated for sp2 (olefinic) carbon at C-5 and the next downfield signal at δ 137 ppm and 128 ppm to C-22 and C-23. The angular methyl group and the side chain methyl carbons gave signals in the region δ 14.8- δ 18.4 ppm The EI-MS spectra showed the molecular ion peak m/z 412. The Mass fragmentation was typical to that of Stigmasterol. Comparison of spectral data with the known compound (steroids) supported its characterization as Stigmasterol [19] (Fig. 2).

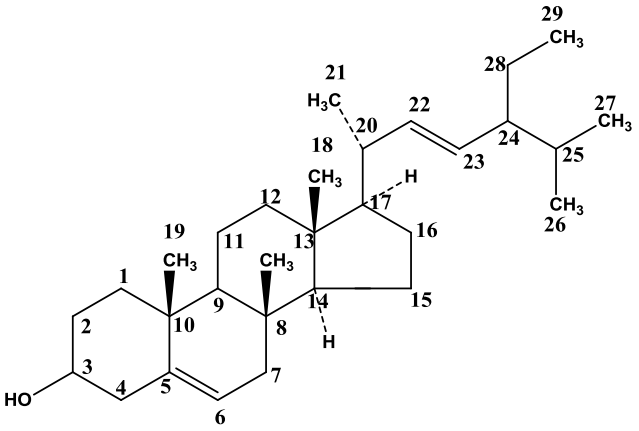

Fig. 2.

3.3 Analysis of Compound III

Physical state: Pale yellow powder, R_f value: 0.38 (solvent system- Petroleum ether: $CHCl_3$; 80:20), Melting point 272 ºC, It gave a green color in Libermann-Burchard's test.

Spectral characteristics of compound III

IR (KBr cm^{-1}): 3375.32 cm^{-1} (br, OH str), 2939.24 cm^{-1} (C-H str. in CH_3), 2372.90 cm^{-1} (C-H str. in CH_2), 1644.70 cm^{-1} (C=C str.), 1457.25 cm^{-1} (C-H deformation in CH_3), 1378.20 cm^{-1} (C-H deformation in gem dimethyl), 1035.43 cm^{-1} (C-O str. Of secondary alcohol), 650.18 cm^{-1} (Rocking vibration of CH_2)

^1H NMR ($CDCl_3$): δ 1.03 (s, 3H, H-23), δ 0.91 (s, 3H, H-24), δ 0.76 (d, 3H, H-25), δ 0.94 (s, 3H, H-26), δ 0.97 (s, 3H, H-27), δ 0.87 (s, 3H, H-29), δ 0.83 (s, 3H, H-28), δ 2.39 (d, 1H, H-18), δ 1.07 to δ 1.78 (m, 16H, 8×CH_2), δ 1.89 to δ 2.17 (m, 4H, 4×CH), δ 4.69 (d, 2H, allylic protons), δ 2.37 (m, 1H, OH), δ 3.21 (m, 1H, H-3), δ 5.13 (s, 1H, vinylic proton), 271,259, 231, 205,177, 137 and 95.

Mass spectra (LC-MS): Molecular formula: $C_{30}H_{48}O_3$, Molecular weight: 456LC-MS (m/z): 456 (M^+,$C_{30}H_{48}O_3$), the other peaks appeared at 440, 425, 409(100%), 391, 353, 325, 313.

Compound III gave characteristic color reaction for triterpenoids. It gave a green color in Libermann-Burchard's test. The peak at 1644.70 cm^{1},1378.20 cm^{-1} and 1035.43 cm^{-1} in IR spectra indicated C=C stretching, C-H deformation in gem dimethyl and C-O stretching of secondary alcohol respectively. Peak at 650.18 cm^{-1} in IR spectra indicated rocking vibration of CH_2 while peak at 3375.32 cm^{-1} indicated the presence of OH - group. The ^{1}H NMR signal at δ 2.37 indicated OH- at C-3 position. The ^{1}H NMR signal at δ 5.13 gave singlet which indicated one vinylic proton at C-12 position while the ^{1}H NMR signal at δ 4.69 gave a doublet which indicated two allylic protons at C-11 position. The LC-MS spectra showed the molecular ion peak at m/z 456. The mass fragmentation was typical to that of Ursolic acid. Comparison of spectral data with the known compound (triterpenoids) supported its characterization as Ursolic acid [20] (Fig. 3).

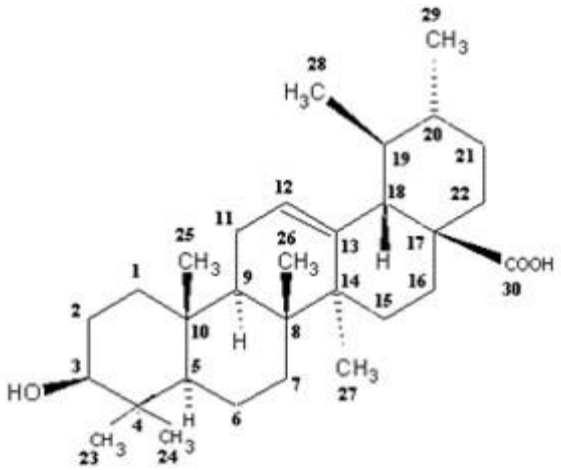

Fig. 3.

3.4 Acute Toxicity Studies

There were no signs of mortality after administration of ethanolic extract of ST in rats.So,the ethanolic extract of stem bark of *ST* was found to be safe up to 2000 mg/kg body weight.

3.5 Analgesic activity

Table 1. Effect of ethanolic stem bark extract of *Sapindus trifoliatus* on tail flick method

Treatment	Dose (mg/kg)	Reaction time in minutes				
		0	30	60	90	120
Control	5 ml/kg	2.3±0.1	2.3±0.038	2.42±0.109	2.28±0.19	2.19±0.23
Pentazocine	10	2.57±0.02	4.56±0.62	7.3±0.27	8.24±0.27	7.07±0.10
	100	2.51±0.02**	3.39±0.33**	4.46±0.05**	4.84±0.14**	4.07±0.02**
EEST	200	2.40±0.01**	3.29±0.01**	4.10±0.01**	4.95±0.005**	4.71±0.016**
	400	2.53±0.01**	4.71±0.01**	5.24±0.05**	5.97±0.01**	4.95±0.01**

*Note: EEST= Ethanolic extract of ST;** The mean difference is significant at the P= 0.05 level, when compared to the control group*

Table 1 shows the effect of drug and the test extracts treatment on tail flick method. Reaction time of 400 mg kg dose of ethanolic extract of *ST* stem bark which is equivalent to that of 10 mg kg body weight of Pentazocine. On comparison with control in all dose levels reaction time was found to be significant (P= 0.05). Sensitivity to strong analgesics and limited tissue damage were reasons behind selecting this model to evaluate extracts central analgesic potential. Significant elevation in pain threshold (P= 0.05) was observed in all the extract test doses (100, 200 and 400 mg/ kg) by increasing the reaction time starting from 30 min of observation onwards as compared to the control group. At 120 min maximum analgesic effect was observed for extracts with their values being 4.07, 4.71, 4.95 respectively as compared with standard

Table 2. Effect of ethanolic stem bark extract of *ST* on writhing by acetic acid model

Treatment	Dose (mg /kg)	Writhing	% inhibition
Control	0.5% CMC	46.00 ± 1.78	-
Standard Aspirin	30	14.00 ± 0.89*	70%
EEST	100	37.67 ± 2.25**	19%
	200	32.00 ± 0.89**	31%
	400	21.33 ± 1.36**	54%

*Note: EEST= Ethanolic extract of ST; **The mean difference is significant at the P=0.05 level, when compared to the control group*

Pentazocine. (10 mg kg^{-1}), it had analgesic value of 7.07 at 120 min. Analgesic activities was shown by extract doses during all the times of observation in dose dependent manner. To attain maximal effect extract doses took longer time for all doses it was found to be 120 min. It may be because of probable time lag for the drug to enter in to the central compartment and distribute into the target site or formation of active metabolites that are endowed with analgesic activity with better half-life. High concentration of active metabolites might be the reason for relatively better action of 400 mg/kg at all observation time. Activating the periaqueductal gray matter (PAG) to release endogenous peptides (i.e., endorphin or enkephalin) may be the possible mechanism of extracts central analgesic effects. These peptides descend the spinal cord and work as pain impulse transmission inhibitors at synapse on dorsal horn or through peripheral mechanisms involved in the inhibition of Prostaglandins, leukotriene, and other endogenous substances that are important in central pain transmission [21].

Table 2 shows the effect of drug treatment on acetic acid writhing method. The ethanolic stem bark extract of *ST* at dose (100, 200 and 400 mg/ kg) inhibited the writhing and also showed significant dose proportionate inhibited writhing. The inhibitory effect of the 400 mg/kg body weight of ethanolic extract was found to be equivalent to that of 30 mg/kg of Aspirin. Significant (P= 0.05) peripheral analgesic activities was seen in all the doses of stem extract (100, 200 and 400 mg/ kg) by decreasing writhing numbers with the values of 19%, 31%, 54% as compared to control. These results proved that there was dose dependent increase from lower dose (100 mg /kg) to the higher dose (400 mg/ kg) in peripheral analgesic activity of extract. Dose dependent increase in analgesic activity might be due to higher concentration of active constituents with maximum dose. The possible mechanism by which the extract produced peripheral analgesia in this model might be associated with inhibiting the synthesis and release of various endogenous inflammatory mediators and suppression of sensitivity of peripheral nociceptors in the peritoneal free nerve endings for chemical-induced pain. These proposed mechanisms are in line with the principles that stated, any agent that decreases the number of writhing will demonstrate analgesia by inhibiting the synthesis and release of PGs, and by inhibiting the peripheral pain transmission [22].

4. CONCLUSION

The preliminary phytochemical analysis of the ethanolic extract of the stem bark showed the presence of flavonoids, triterpenoids, steroids, resins, tannins and reducing sugars. Further, thorough analysis of the stem bark extract by the separation of the chemical constituents and characterization by various methods like IR, ^{1}H NMR, ^{13}CNMR and mass spectroscopy revealed the presence of a saponin glycoside(compound I),a sterol stigmasterol(compound III) and a triterpenoids ursolic acid (compound III). Further the analgesic activity was analyzed using *in vivo* methods like tail flick method and acetic acid induced writhing method in experimental animals. So, it can be said that the analgesic effects exhibited by the extract may be because of the presence of these above mentioned and currently identified phytoconstituents, this suggestion is in line with the reports that stated, phytoconstituents such as alkaloids, flavonoids, steroids, and tannin isolated from plants have been reported to have a potent analgesic activity.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

"All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-

23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee" (KSHEMA/AEC/28/2010)

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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