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Comparative Hepatoprotective Potential of *Tinospora cordifolia, Tinospora sinensis* and *Neem-guduchi*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SJ and OK designed the study and wrote the protocol. Authors BN, RK, PB and DK conducted the experimental works. Authors BN and DK wrote the first draft of the manuscript, performed the statistical analysis and managed the literature searches and analyses of data. Authors OK, AH and SJ managed overall revision and submission. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Aim of this study was to evaluate the comparative efficacy of *Tinospora cordifolia* (Willd.) Miers ex Hook. F., *Tinospora sinensis* (Lour.) Merrill and *T. cordifolia* growing on *Neem* (*Azadirachta indica* A. Juss.) called *Neem-guduchi.*
Study Design: Selected species have been widely used in the traditional medicine systems in various dosage forms to treat liver disorders. They are of common occurrence and are being used as substitutes to each other. There is no comparative hepatoprotection study yet published, therefore, present study has carried out.
Place and Duration of Study: Interactive Research School for Health Affairs, Pune and

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Amrutvahini College of Pharmacy, Sangamner, between November 2011 and August 2012.
Methodology: *Guduchi-Satwa*, a well-known dosage form was prepared according to the traditional procedure. Hepatoprotective potential was assessed using paracetamol-induced hepatotoxicity model in rats and evaluated by using biochemical parameters viz. alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (BIL).
Results: Both *T. cordifolia* and *T. sinensis Satwa* significantly reduced the paracetamol induced elevated levels of serum ALT, AST, ALP and BIL at dose of 200 mg/kg, i.p. as compared to *Neem-guduchi.*
Conclusions: *Satwa* preparation form of *T. sinensis* offers exploitable level of hepatoprotection potential.

Keywords: Tinospora cordifolia; Tinospora sinensis; Neem-Guduchi; comparative hepatoprotection.

1. INTRODUCTION

Liver diseases are a worldwide health problem. In India use of medicinal plants and their formulations are common for the treatment of liver diseases [1]. Liver injuries can be caused by consumption of modern drugs, toxic chemicals, alcohol consumption and viral infections [2]. Most of the liver damage instances are associated with redox imbalance and oxidative stress [3] Due to paucity of a reliable hepatoprotective drugs in modern medicine, herbal drugs are being recommended for the treatment of liver diseases [4]. However, no scientific evidence is available to support these claims and for their mechanism of action.

Guduchi is one of the most commonly practiced herbs being prescribed for various disorders for its curative as well as preventive role. In Indian sub-continent, four different species of *Tinospora* are found, viz. *T. cordifolia* (Willd.) Miers ex Hook. F. & Thoms, *T. sinensis* (Lour.) Merr, *T. crispa* (L.) Miers ex Hook. F. & Thoms and *T. glabra* (Burm f.) Merrill. The plant is locally known as *Amrita, Amritavalli, Chinnobhava, Chakralakshanika, Guduchi, Gulvel, Gurch, Kaduvel, Kundalini, Madhuparni, Sudarsana Tantrika, Vatsadani* etc. Out of these four species, *T. cordifolia* and *T. sinensis* are described as medicinal species [5,6].

Most practitioners believe that *Guduchi* as described in *Ayurveda* is *T. cordifolia*, although, the description matches very well with both, moreover, better with *T. sinensis*. They are a large, glabrous, perennial, deciduous, climbing shrub of family Menispermaceae [5,7,8] and widely used in folk and *Ayurvedic* systems of medicine [9,10].

1.1 *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms

T. cordifolia is distributed throughout the tropical and subtropical Indian subcontinent and China. In India, it is fairly common inhabitant of deciduous and dry forests, growing over hedges and small trees. It is one of the major constituent of several *Ayurvedic* preparation used preferably for general debility, dyspepsia, fever and urinary diseases [11,12]. Apart from other studies, hepato-protective potential validated with respect to *T. cordifolia* by scientific research includes a clinical study for normalization of altered liver functions [13]; antihepatotoxic activity in CCL_4 induced liver damage, normalizing liver function in goats [14]; significant increment in the functional capacities of rat peritoneal macrophages [15]. As

preventive antitubercular drug [16,17] and bile salts induced hepatic damage [6]; for jaundice [18] and activity against hepatitis B and E [19]. The chemical constituent reported in *T. cordiofolia* belongs to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides [6].

1.2 *T. sinensis* (Lour.) Merrill (syn. *Tinospora malabarica*)

T. sinensis is native of south and Southeast Asia, Nepal, Srilanka and Bengal. In India it occurs in Assam, Bihar, Orissa, Maharashtra, Andhra Pradesh, Karnataka, Kelala and Tamilnadu [20]. The mature stem of *T. sinensis* has been used to treat fever, jaundice and burning sensation [21]. In china, the fresh leaves and stem are used in the treatment of chronic rheumatism [22], for treatment in piles and ulcerated wounds [23]. The scientific validation studies on *T. sinensis* reported to possess anti-inflammatory [23] and anti-diabetic [24] activities but there is no report on its hepatoprotective potential.

In *Ayurvedic* practice, both *T. cordifolia* and *T. sinensis* are used as "*Guduchi*" often mixed together in various proportions. As *T. cordifolia* is easily available therefore it is used in major proportion localy. Interestingly however, it was observed that the description of *Guduchi* as described in *Ayurvedic* literature matches accurately with *T. sinensis* rather than with *T. cordifolia.* In *Ayurvedic* literature, it is also mentioned that *Guduchi* that grows on *Neem* tree has a better potential and preferentially used in treatment of certain diseases, presumably due to close vicinity to *Neem* [5,25].

Considering these contexts, the present study was designed to evaluate comparative hepatoprotective potential of *T. cordifolia, T. sinensis* and *Neem-guduchi.* We have prepared *Ayurvedic* formulation known as "*Guduchi Satwa*" following procedure described in *Ayurveda* and compared their biological activity using Paracetamol intoxication induced hepatotoxicity model in rats. It is of utmost interest to identify *Guduchi* that is described in *Ayurvedic* literature as well as validate the claim about *Neem-guduchi* having better biological activity.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Stems of *T. cordifolia, T. sinensis* and *Neem-guduchi* were collected during November 2011 from Pune, India. The plants were identified and voucher specimen has been deposited at the herbarium of Medicinal Plants Conservation Center, Pune *Tinospora_cordifolia* (Willd.) Miers ex Hook. F. & Thoms (MPCC 3464), *Tinospora sinensis* (Lour.) Merr. (MPCC 3525) and *Neem-guduchi* (*T. cordifolia* (Willd.) Miers ex Hook. F. & Thoms) (MPCC 3526).

2.2 Preparation of *Guduchi Satwa*

Fresh stems of selected three variants of *Tinospora* sp. were used for the preparation of *Guduchi Satwa*. The preparation was defined in *Ayurvedic* literature as sediment extract, which is predominantly starchy in nature. In brief, freshly collected stem parts were washed with water and cut into small pieces. They were hand-macerated in water and left overnight to sediment. Next morning, the water was decanted, sediment that remained was completely air dried for couple of days and made into find powder, which was collected as *Guduchi Satwa* [26]. This *Satwa* was re-suspended in water at the time of oral administration.

2.3 Experimental Animals

The study was carried out on male Wistar rats (150–250 g). Animals were maintained under standard husbandry conditions (temperature 25±2ºC, 12-h light: 12-h dark cycle) and fed with standard pellet diet (Amrut, Sangali, M.S., India) and water *ad-libitum.* All animal experiments were handled according to the international guidelines for the care and use of laboratory animals of National Research Council (1996). This study was carried out in accordance with CPCSEA guidelines (Committee for the purpose of control and supervision of experimental animals). The study was approved by institutional animal ethical committee (1153/ac/07/CPCSEA) of Amrutvahini College of Pharmacy, Sangamner.

2.4 Paracetamol-induced Hepatic Damage

Comparative hepatoprotective potential of *T. cordifolia*, *T. sinensis* and *Neem- guduchi* was studied against paracetamol-induced hepatotoxicity, according to method described by Sadashivan et al. [27]. Animals were randomly divided into eight groups (n=6) and received feed and water normally throughout the study. Paracetamol (Crocin, Remidix Pharma Pvt. Ltd., India) was suspended in 2 ml of water and administered p.o., at a dose of 2.5 g/kg to induce hepatic toxicity in all groups except healthy control on day 4, 30 min after drug administration. Group I, was the healthy control group maintained without paracetamol and without any formulation. Group II, was the paracetamol control group and did not receive any drug. In group III and IV animals received *Satwa* of *T. cordifolia* (suspended in water) at a dose 200 and 400 mg/kg p.o. respectively, for 4 days. Similarly, Group V and VI received *Satwa* of *T. sinensis* (suspended in water) at doses 200 and 400 mg/kg p.o. respectively for 4 days. Group VII and VIII received *Satwa* of *Neem-guduchi* (suspended in water) at doses 200 and 400 mg/kg p.o. respectively for 4 days. The animals were sacrificed 48 h after paracetamol administration by mild ether anesthesia. Blood from all animals were collected by retro-orbital puncture, allowed to clot and serum was separated at 3500 rpm for 15 min and used for biochemical studies.

2.5 Blood Biochemical Markers Assay

Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin were estimated using standard kits (Merck Specialties Pvt. Ltd. India) according to instruction of the manufacturer with an autoanalyzer (Nihon Kohden, Japan).

2.6 Histopathological Studies

For histopathological analysis, liver specimens fixed in 10% formalin were embedded in paraffin, sliced 5-µm thick, stained with hematoxylin and eosin (H and E). The liver sections then assessed for pathological changes [28].

2.7 Statistical Analysis

The statistical analysis was one-way ANOVA followed by Dunnette comparison test using graphpad prism 5.00 for Windows, GraphPad Software, San Diego California USA. All values are expressed as Mean ± S.E.M.

3. RESULTS

In the present study, comparative hepatoprotective potential of *T. cordifolia*, *T. sinensis* and *Neem-guduchi Satwa* were evaluated by assessing activities of serum enzymes AST, ALT, ALP and total bilirubin. The animals of paracetamol treated group showed significant elevated levels of AST, ALT, ALP and bilirubin, as compared with healthy control group (Table 1). The results of comparative hepatoprotective potential of *T. cordifolia*, *T. sinensis* and *Neem-guduchi Satwa* on paracetamol treated rats are also summarized in Table 1. *T. cordifolia Satwa* pretreated groups exhibited significantly decreased, paracetamol intoxication elevated activities of serum enzymes AST, ALT and total bilirubin at dose 200 mg/kg, p.o. *T. cordifolia Satwa* at dose 200 mg/kg, p.o. shows 92.2%, 83.2% and 76.9% recovery of AST, ALT and total bilirubin respectively. However surprisingly, activities of serum enzymes ALT, ALP along with total bilirubin were found to be further elevated at dose 400 mg/kg, p.o. Similarly, group pretreated with *T. sinensis Satwa* at dose, 200 mg/kg, p.o. showed significant decrease in levels of AST, ALT, ALP and total bilirubin, increased by paracetamol intoxication at dose 200 mg/kg, p.o. It shows 104%, 84%, 110% and 84.6% recovery of AST, ALT, ALP and total bilirubin accordingly (Table 1). But, group treated with *T. sinensis Satwa* at dose 400 mg/kg, p.o. showed non-significantly decreased activities of ALT, ALP and total bilirubin, when compared with paracetamol control group. Interestingly, the groups of animals treated with *Neem-guduchi Satwa* at doses, 200 mg/kg and 400 mg/kg, p.o., exhibited non-significant decreases in paracetamol intoxication elevated levels of AST, ALT, ALP and total bilirubin (Table 1) which has not supported the traditional claims [5,25].

The results of microscopic examination of liver sections of animals from healthy control group showed normal liver architecture (Fig. 1a). The liver sections of paracetamol intoxicated group rats exhibited infiltration of macrophages and ballooning degeneration in liver parenchymal cells. Lesions of necrosis, pyknosis and nuclear degeneration were evident (Fig. 1b). Liver sections of rats treated with *T. sinensis* showed near-normal liver architecture (Fig. 1c). Treatment of *T. cordifolia* was found to be effective in restoring paracetamol induced hepatic damage when compared with healthy control as it restored near-normal cellular architecture (Fig. 1d). Contrary to expectations, treatment of *Neem-guduchi* showed limited recovery form disturbed cellular architecture in which lesions of nuclear degeneration could be seen (Fig. 1e).

Table 1. Comparative hepatoprotective effect of aqueous stem extract of *T. cordifolia*, *T. sinensis* and *Neem-guduchi* on serum ast, alt, alp and total bilirubin against paracetamol intoxication

Sr. No.	Groups	AST (IU/ml)	ALT (IU/ml)	ALP (IU/ml)	Total bilirubin (mg/dl)
I.	Healthy control	156.0±12.3[c]	81.3±6.18[c]	448.0±26.9[b]	0.27±0.016[b]
II.	Paracetamol control	440.0±23.1	302.0±22.0	859.0±107	0.40±0.006
III.	*T. cordifolia* (200mg/kg p.o.)	178.0±13.5[c] (92)	118.3±9.1[b] (83)	511.0±54.7	0.30±0.007[a] (77)
IV.	*T. cordifolia* (400 mg/kg p.o)	254.0±52.5[b] (65)	207.0±26.2	871.0±41.5	0.37±0.007
V.	*T. sinensis* (200 mg/kg p.o)	143.0±3.1[c] (104)	125.0±24.3[b] (80)	404.0±52.3[b] (110)	0.29±0.006[a] (85)
VI.	*T. sinensis* (400mg/kgp.o)	230.0±36.9[c] (74)	174.0±28	756.0±103	0.33±0.017
VII.	*Neem-guduchi* (200 mg/kg p.o)	328.0±46.8	193.0±52.2	637.0±81.7	0.35±0.034
VIII.	*Neem-guduchi* (400mg/kg p.o)	306.0 ±19.9	207.0±26.2	637.0±81.7	0.37±0.028

Values are mean ± S.E.M., n=6 animals per group.

Values in the parenthesis indicate percent protection in individual biochemical parameters from their elevated values.

The percentage of the protection is calculated as 100 × (values of paracetamol control − values of sample)/(values of paracetamol control – values of control).

[a], P < 0.05, [b], P < 0.01, [c] , P < 0.001, All groups compared with paracetamol control

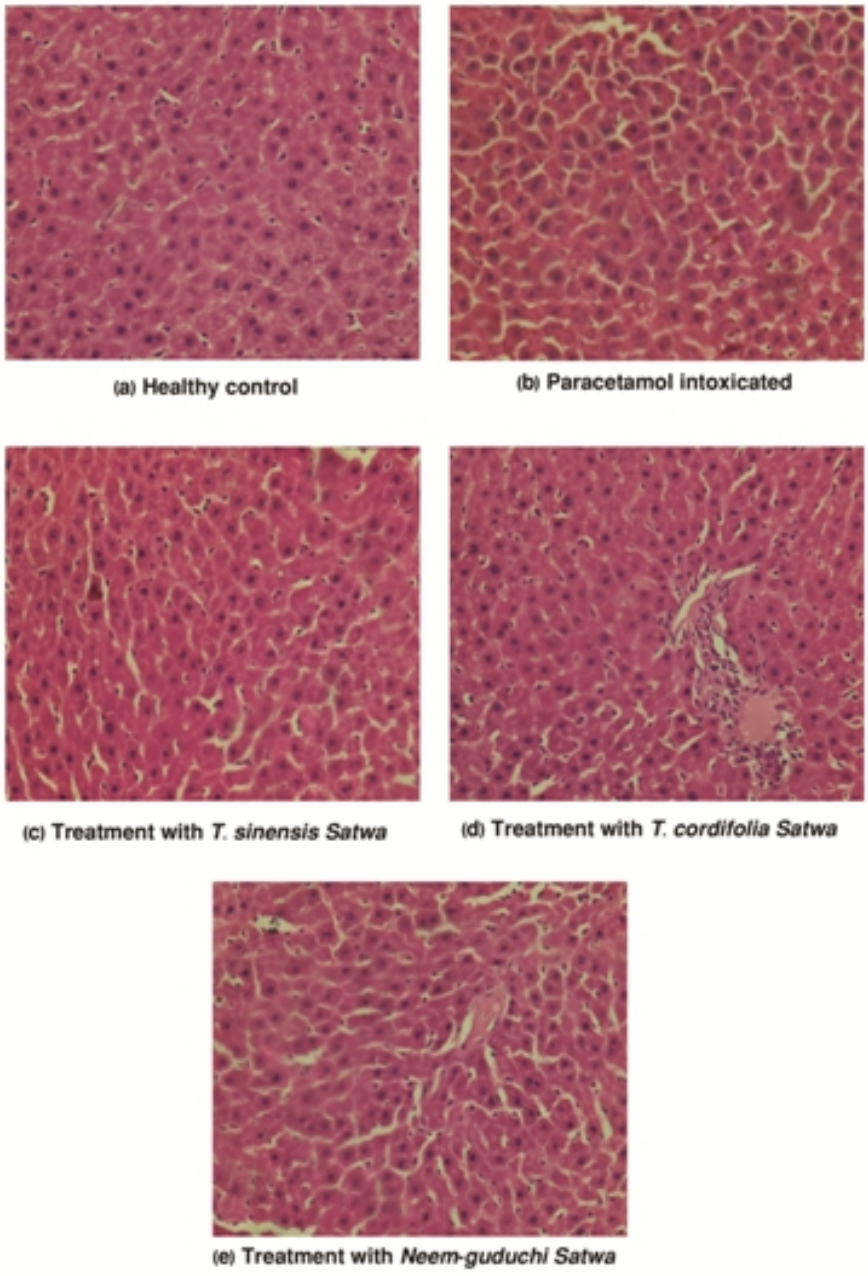

Fig. 1. Histology of liver tissues

a) Liver sections of healthy control group showing normal liver architecture. b) Paracetamol intoxicated group rats shows infiltration of macrophages, ballooning degeneration, lesions of necrosis, pyknosis and nuclear degeneration. c) Treatment with T. sinensis showing near-normal liver architecture. d) Treatment of T. cordifolia showing near-normal liver architecture. e) Treatment of Neem-guduchi showing disturbed cellular architecture with lesions of nuclear degeneration.

4. DISCUSSION

Serum biochemical markers are generally employed to assess liver function. The estimation of serum bilirubin is associated with normal liver function. On other hand, estimation of serum enzymes AST, ALT and ALP is the quantitative marker for the determination of type of liver diseases. In the present study, comparative hepatoprotective potential of *T. cordifolia, T. sinensis* and *Neem-guduchi Satwa* were evaluated by using paracetamol-induced hepatotoxicity. Paracetamol produces hepatic necrosis at higher doses. Several studies have demonstrated that induction of hepatocellular damage or necrosis by higher doses of acetaminophen in experimental animals and humans [29]. For screening of hepatoprotective agents, paracetamol-induced hepatotoxicity has been used as a reliable and reproducible method. Paracetamol is metabolized primarily in the liver and eliminated by conjugation with sulfate and glucuronide and then excreted through kidney. Protein-calorie malnutrition (PCM) is activated and converted by cytochrome P450 enzymes to toxic metabolite NAPQI (N-acetyl-p-benzoquinoneimine) that causes oxidative stress and glutathione (GSH) depletion [29,30]. Paracetamol and carbon tetrachloride (CCl_4) are well-known hepatotoxins, had been used to study hepatoprotective activity by several investigators [31-33]. An obvious sign of hepatic injury is leakage of cellular enzymes into plasma [34-36]. AST predominantly found in mitochondria of the hepatocytes. ALT is more specific to liver and thus is a reliable parameter for detecting liver injury. Serum ALP and bilirubin are also known to be associated with liver cell damage. The activities of ALT, AST and ALP and level of serum bilirubin are largely used as most common biochemical markers to evaluate liver injury [37]. Administration of paracetamol caused a significant elevation of enzymes level such as AST, ALT, ALP and bilirubin level and has been attributed to the damage structural integrity of liver, because they are cytoplasmic in location and released into circulation after cellular damages indicating development of hepatotoxicity [38,39].

The results of present study indicated that administrations *Satwa* of *T. cordifolia* and *T. sinensis* at dose 200 mg/kg, i.p. found to significantly reduce the increased activities of serum marker enzymes AST, ALT, ALP and total bilirubin level. Our study reveals comparative hepatoprotective effect *T. cordifolia, T. sinensis* and *Neem-guduchi* Satwa which is similar to the previous studies done to explore the hepatoprotective effect of *T. cordifolia* alone [40-45]. However, there is no report so far on possible hepatoprotective mechanism of aqueous stem extract of both species. We assumed that it could be mediated through the modulation of glutathione detoxification and/or suppressing free radicals. Furthermore, result of present study also exhibits *T. sinensis Satwa* have more hepatoprotective potential than *T. cordifolia Satwa*, which supports the view about this being potent alternative for guduchi. However, in the present study both *T. cordifolia* and *T. sinensis Satwa* found to have reversed to hepatotoxic activity at dose 400 mg/kg, o.p. that could be due to the toxic effect of *Satwa* at higher doses. *T. cordifolia* growing on *Neem* tree (*Azadirachta indica*) hence called *Neem-guduchi* was believed to be more medicinally potent than *T. cordifolia* growing on any other tree as emphesized in the ancient *Ayurvedic* literature [5,25]. However, result of present study revealed that *Neem-guduchi Satwa* did not significantly affect the paracetamol intoxicated elevated levels of ALT, AST and ALP and total bilirubin at selected doses. Thus, the result of present study does not support the claim of *Neem-guduchi* as far as hepatoprotective potential is concerned. The histological findings also supported the results of biochemical markers. Rats treated with *T. sinensis* and *T. cordifolia* showed almost normal hepatic cellular architecture similar to that of control. This confirmed the protection offered to hepatic structural integrity.

5. CONCLUSIONS

In conclusion, the result of hepatoprotective study indicated that *Satwa* of *T. sinensis* has comparatively higher hepatoprotective activity than *T. cordifolia*, although both formulations could have significant protection against paracetamol induced hepatic toxicity. Both the plants therefore may be used as *guduchi* as described in *Ayurvedic* literature. Our data on hepatoprotection however, could not support the claim about *Neem-guduchi*. Finally, it has been suggested that further comparative characterization of chemical constituents of each species is essential to reveal the potent Hepatoprotective components along with their proportionate combination.

CONSENT

Not applicable.

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.

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DISCLOSURE STATEMENT

Authors have declared that no competing financial interests exist.

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