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Preliminarily phytochemical screening and *in vivo* safety evaluation of ethanolic extract of *Hemidesmus indicus* (Linn.)

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ABSTRACT

Objectives: Phytochemical and safety evaluation of *Hemidesmus indicus* (Linn.) of Apocynaceae family was performed to establish the justification of its therapeutic significance in traditional medicine.
Material and Methods: Phytochemical analysis of leaf, stem, and root was done according to standardized protocols. For determination of no-observed-adverse-effect-level female rats were randomized into five groups of six animals, each administered with root extract at 100, 200, 400, and 800 mg/kg for 7 days. Blood was collected by a retro-orbital puncture for serum biochemical parameters. Choleretic activity was done by intra-duodenal administration of *H. indicus* root extract (400 mg/kg).
Results: *Hemidesmus indicus* revealed the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, carbohydrate, glycosides, proteins, flavonone, flavone, and flavonol, polyphenolic contents, and hydrogen peroxide scavenging activity. Root extract showed the highest amount of phytochemicals in comparison with leaf and stem. No significant difference was observed in alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin, albumin, urea, uric acid, creatinine, triglycerides, and cholesterol after treatments. The choleretic activity of *H. indicus* showed an insignificant upsurge in bile flow and bile solids.
Conclusions: *Hemidesmus indicus* possesses phytochemicals responsible for its antioxidant and choleretic activity. Furthermore, administration of *H. indicus* root extracts up to 800 mg/kg is safe for *in vivo* studies in rats.

INTRODUCTION

Plants have been a source of many important medicaments since ancient time for indigenous people (Fabricant and Farnsworth, 2001). A significant proportion of the population (approx. 80%) in developing countries still depends on the traditional remedies to treat several diseases (Pal and Shukla, 2003). *Hemidesmus indicus* (Linn.) of Apocynaceae (subfamily Asclepiadaceae) is known as Indian sarsaparilla in English, *Anantmul* in Hindi, and *Ananta* in Sanskrit language. It is well distributed throughout India and frequently used in Indian traditional medicine (Nair *et al*., 2014). It is a thick, woody plant with brown and fragment bark. It is popularly used for the treatment of blood diseases, dyspnea, dyspepsia, loss of flavor, dyspnea, cough, menstrual problems, fever, diarrhea (Mary *et al*., 2003), and cancer (Ferruzzi *et al*., 2013; Fimognari *et al*., 2011; Samarakoon *et al*., 2012; Zarei and Javarappa, 2012). *Hemidesmus indicus* has a potential hepato-protective, nephron-protective, and anti-inflammatory activity (Kotnis *et al*., 2004; Qureshi *et al*., 1997).

Safety of plant-based medicines has frequently been questioned due to reported toxic effects in test animals (Park *et al*., 2010). Safety evaluation is important for hazard identification and standardization of novel drugs. Regulatory safety assessment for herbal products relies on a range of adverse drug reactions and published toxicity reports (De Smet, 1995). The choleretic agent is a drug which stimulates bile flow and increases solid contents in bile. Choleretic effects of the herbal medicine and its component have been a good source of scientific information for their

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clinical use in wide variety of liver diseases (Hoefler *et al*., 1987; Wang *et al*., 2016).

Hemidesmus indicus may represent an interesting botanical drug (Turrini *et al*., 2015), however, scientific basis for its safety has not been reported. Thus, the aim of this investigation was a qualitative and quantitative analysis of *H. indicus* leaf, stem, and root extract followed by safety evaluation of the root extract. Since *H. indicus* possesses potent hepatoprotective activity, the present investigation will also explore its therapeutic effect relating to its choleretic activity in rats.

MATERIALS AND METHODS

Collection and extraction

The whole plant was collected from Bilaspur district, Chhattisgarh in India. The plant was identified by an eminent botanist, and voucher specimen No. GG/C/APO/101 was deposited in the herbarium of Department of Botany, Guru Ghasidas University. Fresh leaves, stems, and roots were separated from the plant and washed in distilled water to remove dust. Then it was dried in shade at room temperature. Dried plant parts were subsequently sieved separately to obtain a fine powder. Then 70% hydroethanolic extracts of the fine powder was prepared using an accelerated solvent extractor (DIONEX. ASE-150) at 20°C and 15 atm pressure (Azwanida, 2015). Standardized modern analytical methods were used to ensure quality control of plant extract (Fibigr *et al*., 2018). The extracts were dried at room temperature and their residues were weighed and recorded. The yield of leaves, stem, and root of *H. indicus* extracts was found to be 16.7% w/w, 19.2% w/w, and 20.7% w/w, respectively. The extracts were dried at room temperature and stored in a refrigerator at 4°C for their future use in phytochemical and toxicity analysis.

Qualitative phytochemical analyses

A small portion of *H. indicus* ethanolic extract was used for qualitative phytochemical screening of alkaloids, flavonoids, tannins, saponins, terpenoids, carbohydrate, glycosides, and proteins according to standardized protocols given by Harbone (1973) and Trease and Evans (1989).

Quantitative phytochemical analyses

Determination of hydrogen peroxide (H_2O_2) scavenging activity

Determination of H_2O_2 scavenging activity was done by the method of Ruch *et al*. (1989). Absorbance was recorded in UV/VIS spectrophotometer at 230 nm. A standard curve prepared using a different concentration of ascorbic acid. The results were expressed as H_2O_2 scavenging activity/µg of extract.

Determination of total polyphenolic content

The polyphenolic content was determined by the Folin–Ciocalteu assay of Slinkard and Singleton (1997). Absorbance of the test sample was recorded at 725 nm. Total polyphenolic content was determined by plotting a standard curve using different concentrations of gallic acid. The results were expressed in mg/g of extract.

Determination of flavone and flavonol content

Total flavone and flavonol content were determined by the aluminium chloride method of Cvek *et al*. (2007). Absorbance was measured at 415 nm. The total polyphenol content was determined by plotting a standard curve using different concentrations of quercetin. The results were expressed in mg/g of extract.

Determination of flavonones

The flavonone content was determined by using the 2,4-Dinitrophenylhydrazine method of method of Cvek *et al*. (2007). Absorbance was measured at 495 nm. The flavonone content was determined by plotting a standard curve using different concentrations of pinostrobin. The flavonone content was expressed in mg/g of extract.

Determination of flavonoid content

Total flavonoid was determined by measuring a complex of flavonoid and aluminum at 510 nm by the method of Cvek *et al*. (2007). Flavonoid content was determined by plotting a standard curve using different concentrations of naringenin. Flavonoid content was expressed in mg/g of extract.

In vivo safety evaluation

Animals and chemicals

A total of 48 female *Wistar* rats (10–12 weeks old; 180 ± 20 g) were procured from the Defense Research and Development Establishment, Gwalior, India. All animal procedures were approved by the Institutional Animal Ethics Committee (994/Ere/GO/06/CPCSEA). Rats were kept in contamination-free environment at (25°C ± 2°C) temperature, 12 hours dark–light cycle, and relative humidity of 60%–70%. A standard pelleted diet with free access to tap water was provided to rats. Animals were acclimatized for 10 days before the commencement of experiments. Pure and analytical grade chemicals acquired from standard chemical suppliers were used in the present study.

No-observed-adverse-effect-level (NOAEL)

For estimation of NOAEL rats were randomly assigned to five groups containing six animals. Group 1 served as control (gavaged with normal saline), Groups II–V were administered with *H. indicus* root extract at 100, 200, 400, and 800 mg/kg body weight doses, respectively, for 7 days (Sandhiya and Ubaidulla, 2017). All the animals were weighed and partially anesthetized with sodium pentobarbitone after 24 hours of last treatment. Blood was collected by retro-orbital puncture and allowed to clot in test tubes for 30 minutes at room temperature. Clotted blood was centrifuged for 20 minutes at 375 g for serum isolation and stored at −20°C (Riley, 1960). Various parameters of liver function tests, i.e., alanine aminotransferase, aspartate aminotransferase alkaline phosphatase, bilirubin, triglycerides, cholesterol and kidney function tests, i.e., urea, uric acid, and creatinine were performed using standard commercial Kits (Erba Diagnostics, Germany) according to the manufacturer's instructions.

Table 1. Qualitative phytochemical evaluation of *H. indicus* ethanolic extract.

Sl. no.	Phytochemical	Leaf	Stem	Root
1	Alkaloids	Present	Present	Present
2	Flavonoids	Present	Present	Present
3	Tannins	Present	Present	Present
4	Saponins	Present	Present	Present
5	Terpenoids	Present	Present	Present
6	Carbohydrate	Present	Present	Present
7	Glycosides	Present	Present	Present
8	Proteins	Present	Present	Present

Table 2. Quantitative phytochemical evaluation of *H. indicus* ethanolic extract.

Phytochemical (per gram of extract)	Leaf (mg/g)	Stem (mg/g)	Root (mg/g)
Polyphenols	45.5	46.7	57.7
Flavonoids	12.2	22.3	27.3
Flavonones	4.80	3.69	7.16
Flavones and flavonols	0.32	0.62	0.69

Choleretic activity

For the estimation of choleretic activity, female rats were divided into three groups of six animals each. Group 1 served as control, administered with normal saline at 2 ml/kg, id, Group 2 received dihydrocholic acid (DHC) as standard (50 mg/kg, id), and Group 3 administered with *H. indicus* (400 mg/kg, id). Animals were anesthetized with urethane (25%, 6 ml/kg, ip) after 12 hours fasting period. The bile duct was surgically exposed by midline incision (25 mm) and cannulated close to the duodenum with PE-10 tubing. The rectal temperature of anesthetized rats was maintained at 37°C. Bile was collected for an hour separately in all three groups and at the end of the first hour, rats were administered with normal saline (Group 1), DHC (Group 2), and root extract of *H. indicus* (Group 3). Bile was collected for 2–5 hours followed by administration of normal saline, standard, and extract. Total bile volume was measured after the end of the fifth hour and kept for drying at room temperature. Bile solid content was determined by evaporating samples to dryness and weight of residue was recorded (Klaassen and Plaa, 1969).

Statistical analysis

Results were expressed as the mean ± standard error of six animals used in each group. The Student's *t*-test was used to compare mean values of different parameters obtained in various groups and $*p \leq 0.05$ was considered as significant. Data were subjected to statistical analysis by using Microsoft Excel 2013 (15.0.4420.1017) 32-bit, India through one-way analysis of *F* variance (ANOVA).

RESULTS AND DISCUSSION

Qualitative and quantitative phytochemical evaluation

Antioxidant properties of plants due to the presence of phytochemicals have gained much interest in pharmaceutical research (Saha *et al*., 2018). Presence of polyphenolic compounds in plants contributes to medicinal properties. Phenolic OH group is vital for both antioxidant activity and free radical kinetics, it may contribute directly in entrapments of singlet and triplet oxygen radicals (Fergusion *et al*., 2006; Priyadarsini *et al*., 2003). *Hemidesmus indicus* is a well-known medicinal plant with a high concentration of bioactive components (Ananthi *et al*., 2010). Phytochemicals present in root extract of *H. indicus* possess free radical hunting property (Ravishankara *et al*., 2002; Saravanan and Nalini, 2007). Preliminary qualitative phytochemical evaluation of *H. indicus* leaf, stem, and root extract revealed the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, carbohydrate, glycosides, and proteins (Table 1). The amount of total polyphenolic content in *H. indicus* was found to be 45.5, 46.7, and 57.7 mg/g of extract in leaf, root, and stem, respectively. Total flavonoid content was observed to be the highest in root extract of *H. indicus* (27.3 mg/g of extract) followed by the stem (22.3 mg/g of extract), and leaf (12.2 mg/g of extract). Total flavonone was found to be 4.80, 3.69, and 7.16 mg/g of extract in leaf, stem, and root, respectively. Total flavone and flavonol content in leaf were very less (0.32 mg/g of extract) as compared with the stem (0.62 mg/g of extract) and root (0.69 mg/g of extract). The *H. indicus* extract showed the highest concentration of polyphenolic content followed by flavonoids, flavonones, flavones, and flavonols in root as compared with leaf and stem, respectively (Table 2). Thus, a root extract of the plant may hold better therapeutic applications (Kundu and Mitra, 2014).

The high concentration of phenolic compounds in the plant might be responsible for its strong antioxidant property. Secondary metabolites of plants are reported to have many biological and therapeutic properties (Benedec *et al*., 2013; Charalampos *et al*., 2013; Narender *et al*., 2012; Vishnu *et al*., 2013). The presence of phytochemicals such as phenols, tannins, and flavonoids in *H. indicus* extract might give approval to its local usage in the treatment of various diseases (Gopi and Setty, 2010). Traditionally, tannins have been used for detoxification and treatment of wound healing, diarrhea, and hemorrhage (Afolayan and Mabebie, 2010; Okwu and Emenike, 2006). The methanolic extracts of the plant are reported to protect plasmid DNA from strand breaks and reduced damage due to lipid peroxidation in microsomes of rat liver (Shetty *et al*., 2005). Flavonoids are an important secondary metabolite of plants and well-known for their antioxidant activity (Shi *et al*., 2006). The ethnomedicinal usage of *H. indicus* extract for management of oxidative stress-induced diseases might be due to the high concentration of flavonoids (Ammar *et al*., 2008; Kostova, 2005). Flavonoids help to preserve membrane permeability of cell by inhibition of ATPase and phospholipase A2 enzymes (Li *et al*., 2003). Free radicals are inactivated by flavonoids by the formation of stable and less toxic product (Dureja and Dhiman, 2012; Korkina and Afanas'ev, 1997). Thus, the use of *H. indicus* in the treatment of free radical associated diseases, i.e., arthritis and rheumatic disease is well justified (Mehta *et al*., 2012). Terpenoids are known to have anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory, antiviral, antimicrobial, antifungal, antiparasitic, and immunomodulatory effect (Rabi *et al*., 2009; Wagner and Elmadfa, 2003). Saponins possess blood coagulating property (Okwu, 2004). Interestingly, both terpenoids and saponins are present in *H. indicus* (Aneja *et al*., 2008; Austin, 2008;

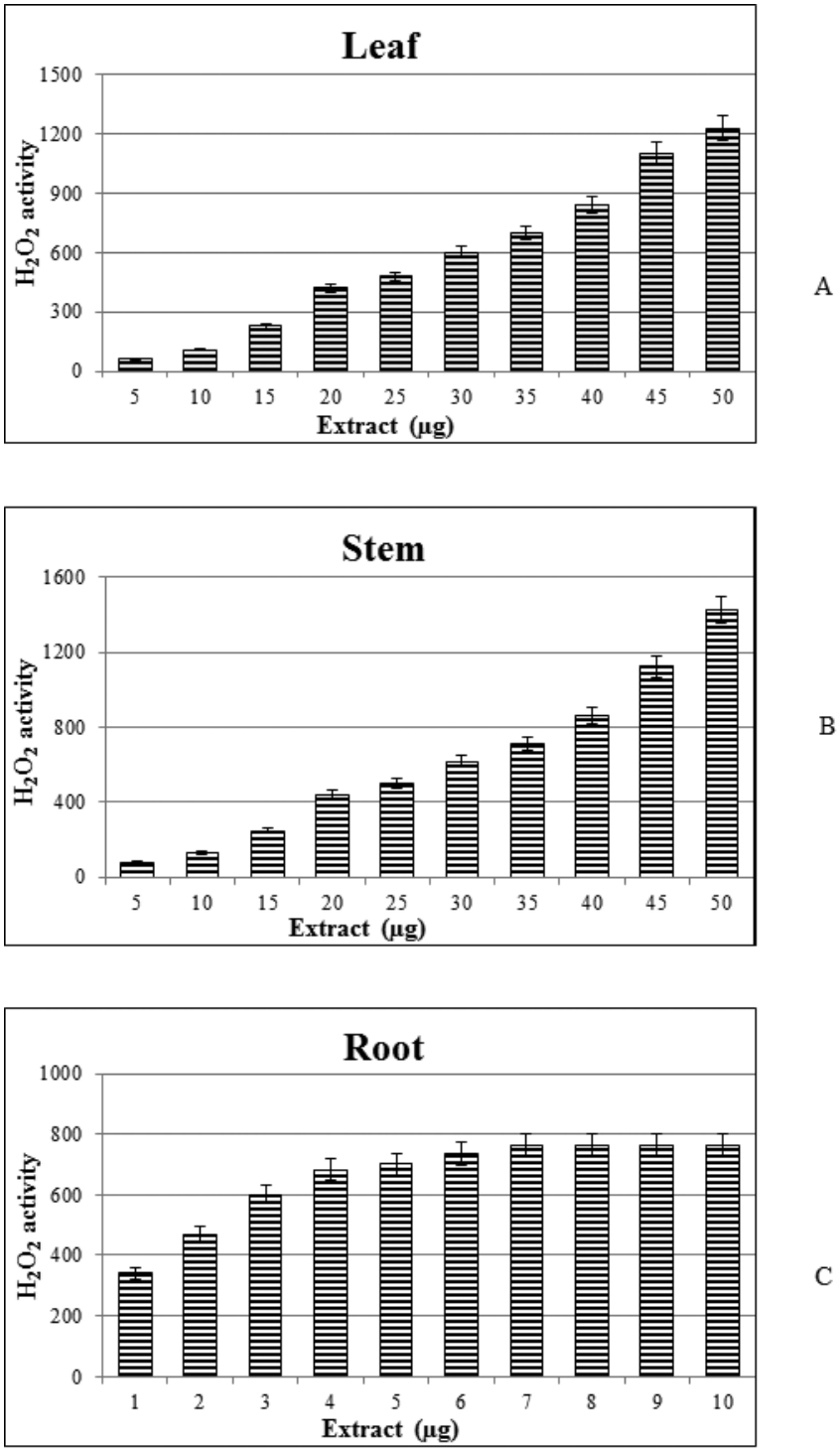

Fig 1. Hydrogen peroxide scavenging activity of *H. indicus* leaf (A), stem (B) and root (C) extract. (Data are expressed as mean ± SE at 5%, n = 6).

George *et al*., 2008). Phytochemicals like carbohydrates and glycosides were also observed in the plant extract (Austin, 2008; Sigler *et al*., 2000), which are known to improve the immune system (Theis and Lerdau, 2003). The extract of *H. indicus* contains proteins, the building blocks of life which help to repair and maintain cells (Ojala *et al*., 2000). Previous reports suggests that alcoholic extract of its roots possesses antihepatotoxic, renoprotective, anti-inflammatory, antithrombotic, antidiarrheal, antinociceptive, and antienterobacterial activities (Baheti *et al*., 2006; Das and Devaraj, 2006; Das *et al*., 2003; Kotnis *et al*., 2004; Mary *et al*., 2003; Prabakan *et al*., 2000; Saravanan and Nalini, 2008; Verma *et al*., 2005).

Table 3. Serum biochemical values of rats treated with *H. indicus* root extract for a week.

Concentration of extract (mg)	ALT (IU/l)	AST (IU/l)	ALP (IU/l)	Bilirubin (mg/dl)
Control	32.2 ± 2.82	75.4 ± 3.89	430 ± 32.2	0.23 ± 0.01
100	34.1 ± 2.82	76.7 ± 4.78	428 ± 29.6	0.26 ± 0.02
200	31.8 ± 2.39	79.5 ± 4.48	418 ± 29.6	0.23 ± 0.01
400	34.2 ± 3.05	85.5 ± 4.75	370 ± 20.4	0.20 ± 0.01
800	37.5 ± 2.79	90.2 ± 6.45	395 ± 21.8	0.24 ± 0.01
F variance	0.71^{ns}	1.68^{ns}	1.52^{ns}	0.46^{ns}

Data are expressed as mean ± SE; $n = 6$; *Significant p value Therapy *vs*. control at 5%; ns = not significant F variance at 5%. ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase.

Hydrogen peroxide scavenging activity

The H_2O_2 penetrates plasma membranes of the cell where it converted into highly reactive hydroxyl radical (Gulcin *et al*., 2003). Medicinal plants are the best-known scavenger of free radicals with negligible adverse effects (Naidoo *et al*., 2016). Scavenging of hydrogen peroxide by *H. indicus* leaf, stem, and root extracts might be attributed to the presence of polyphenolic compounds, which contribute electron to hydrogen peroxide, thus reducing it to water. All the three parts of plant extract efficiently scavenged hydrogen peroxide in a concentration-dependent manner, however, the root extract showed better scavenging activity as compared with leaf and stem due to the presence of the highest amount of flavonoid contents [(Fig.s 1(A)–(C)].

No-observed-adverse-effect-level

The no-observed-adverse-effect-level is an essential part of non-clinical drug hazard evaluation and is defined as adverse reactions occurring instantaneously or in a short period of time after administration of any drug (Dorato and Engelhardt, 2005). Determination of serum biochemical parameters could be used to reveal the harmful effect of foreign substances, including plant extracts. It includes the evaluation of possible variations in enzymes, metabolic products, and functioning of organs (Olson *et al*., 2000). The liver plays a vital role in the metabolism and detoxification of harmful substances. Therefore, the liver is a potent target organ of toxic insult from drugs (Shah *et al*., 2011). In the present study, 1- week administration of *H. indicus* root extract at 100, 200, 300, 400, and 800 mg/kg brought no significant change in the level of liver-related enzymes, which is an indication of no cellular damage by the plant extract. An elevated level of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase in serum may designate cellular outflow and damages functional integrity of the cell membrane in the liver as a result of metabolism of toxic substances (Ozer *et al*., 2008). Ethanolic root extract of *H. indicus* at all doses (100, 200, 300, 400, and 800 mg/kg) did not show significant alteration in total bilirubin (Table 3). The concentration of bilirubin reveals the amount of conjugated and unconjugated bilirubin. Total bilirubin levels are mostly increased in various types of hepatic diseases, i.e., blockades in a hepatobiliary system such as gallstones or tumors, cirrhosis, hepatitis (Burtis and Ashwood, 1999). Bilirubin content not significantly altered after *H. indicus* treatment might be due to the tendency of the plant extract to enhance the excretion of bilirubin through bile. Enhancement of non-essential fatty acids occurs during oxidative stress and disturbed lipid metabolism, which in turn upsurges triglycerides and cholesterol levels in serum (Bhadauria *et al*., 2008). Administration of *H. indicus* root extract slightly altered triglycerides and cholesterol content showing no adverse effect on the metabolic activity of the liver.

The kidney is responsible for conserving homeostatic balance of body by reabsorbing essential material and eliminating waste products (James *et al*., 2010). Kidney functions are generally determined by assessment of urea, uric acid, and creatinine level in blood. Creatinine is formed in muscles by metabolism of creatine. It is synthesized in the liver, passes into blood circulation, and is taken up by skeletal muscle (Gowda *et al*., 2010). However, the retention of creatinine in blood is an indication of renal disease. Creatinine level was slightly elevated at a dose of 800 mg/kg but the increase was not significant as compared with control, which is a clear signal of effective removal of the extract by the kidneys without any adverse effect (Eaton and Pooler, 2009). Tubular necrosis, liver ailments, and the incomplete absorption of proteins may cause increased serum urea (Burti *et al*., 2006). Uric acid is the metabolic product of purines, excreted into urine. The increased level of uric acid reduces renal blood flow and hampers the glomerular filtration rate. In the present study, there was no substantial change in urea and uric acid levels at all the doses of *H. indicus* indicating that the root extract is harmless to use as a therapeutic agent up to 800 mg/kg dose (Table 4).

Table 4. Serum biochemical values of rats treated with *H. indicus* root extract for a week.

Concentration of extract (mg)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)
Control	28.1 ± 1.96	10.2 ± 0.68	28.6 ± 2.33	1.43 ± 0.08	0.68 ± 0.04
100	31.8 ± 1.98	10.3 ± 0.56	33.2 ± 2.09	1.21 ± 0.08	0.64 ± 0.04
200	32.6 ± 2.10	10.6 ± 0.55	35.1 ± 2.56	1.18 ± 0.08	0.66 ± 0.05
400	30.4 ± 1.84	9.29 ± 0.51	34.3 ± 2.82	1.28 ± 0.11	0.65 ± 0.05
800	33.0 ± 2.05	9.62 ± 0.52	36.5 ± 3.19	1.32 ± 0.11	0.75 ± 0.06
F variance	1.83^{ns}	1.03^{ns}	1.57^{ns}	1.26^{ns}	0.86^{ns}

Data are expressed as mean ± SE; $n = 6$; *Significant p value Therapy *vs*. control at 5%; ns = not significant F variance at 5%.

Table 5. Effect of *H. indicus* root extract on the choleretic activity of rat.

Treatment	Bile flow (ml)			Bile solid (mg)		
	1 hour	2–5 hours	Ratio	1 hour	2–5 hour	Ratio
Control	0.65 ± 0.03	1.68 ± 0.10	1:2.58	19.9 ± 1.68	49.0 ± 3.08	1:2.46
DHC (50 mg/kg)	0.56 ± 0.03	2.05 ± 0.17	1:3.66	18.2 ± 1.41	58.3 ± 4.85	1:3.20
H. indicus (400 mg/kg)	0.62 ± 0.04	1.79 ± 0.10	1:2.89	20.1 ± 1.52	55.9 ± 3.73	1:2.78
F variance	1.73^{ns}	2.50^{ns}		0.76^{ns}	1.78^{ns}	

Data are expressed as mean ± SE; $n = 6$; *Significant p value Therapy *vs*. control at 5%; ns = not significant F variance at 5%. DHC = dihydrocholic acid (standard).

Choleretic activity

The liver is involved in secretion and stimulation of normal bile flow and determination of choleretic activity of any therapeutic agent is a worthy pointer for safety evaluation (Bhadauria *et al*., 2007). Cholic acid is an essential bile acid found in mammals, thus in the present study, dehydrocholic acid was used as a standard. The decrease in bile flow was observed from the first hour and second– to the fifth hour. It was found that DHC (50 mg/kg) and *H. indicus* (400 mg/kg) treated rats had more bile outputs during 2–5 hours period as compared with control. *Hemidesmus indicus* administration increased bile volume during 2–5 hours which indicated its choleretic activity. The excretion of total solids was observed for five consecutive hours. *Hemidesmus indicus* increased excretion of bile solid content during 2–5 hours comparable with the standard drug DHC (Table 5). Present study showed an upsurge in bile flow and bile solids under the influence of *H. indicus* is suggestive of its stimulatory action on secretary activity of hepatic cells. Phenolic compounds have been associated with choleretic activity in animals (Mortier, 1972). Their metabolites are secreted in bile as an organic anion coupled with Na^+ or K^+ and water is passively excreted (Takeda and Aburada, 1981). Phytochemical analysis in the present study confirmed the presence of terpenes, polyphenol, and flavonoids could be involved in the choleretic activity (Hoefler *et al*., 1987). Present study is in corroboration with findings of other plant extracts (Chandan *et al*., 2008; Mittal *et al*., 2012).

CONCLUSION

Hemidesmus indicus may be a potent therapeutic agent due to the presence of a plethora of secondary metabolites such as flavonoids, tannins, alkaloids, carbohydrates, polyphenols, saponins, glycosides, terpenoids, and proteins. The strong correlation between the contents of total polyphenols, flavonoids, flavonones, flavone and flavonols, and radical scavenging activity indicates that these phytochemical constituents are responsible for the antioxidant potential of the plant. Root extract possesses the highest amount of bioactive components as compared with stem and leaf. NOAEL of *H. indicus* root extract is 800 mg/kg dose. The antioxidant and choleretic activity of the plant provides a satisfactory rationale for its hepatoprotective activity. *Hemidesmus indicus* may be helpful in improving the adherence to hepatotoxic drugs and its clinical outcome.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

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