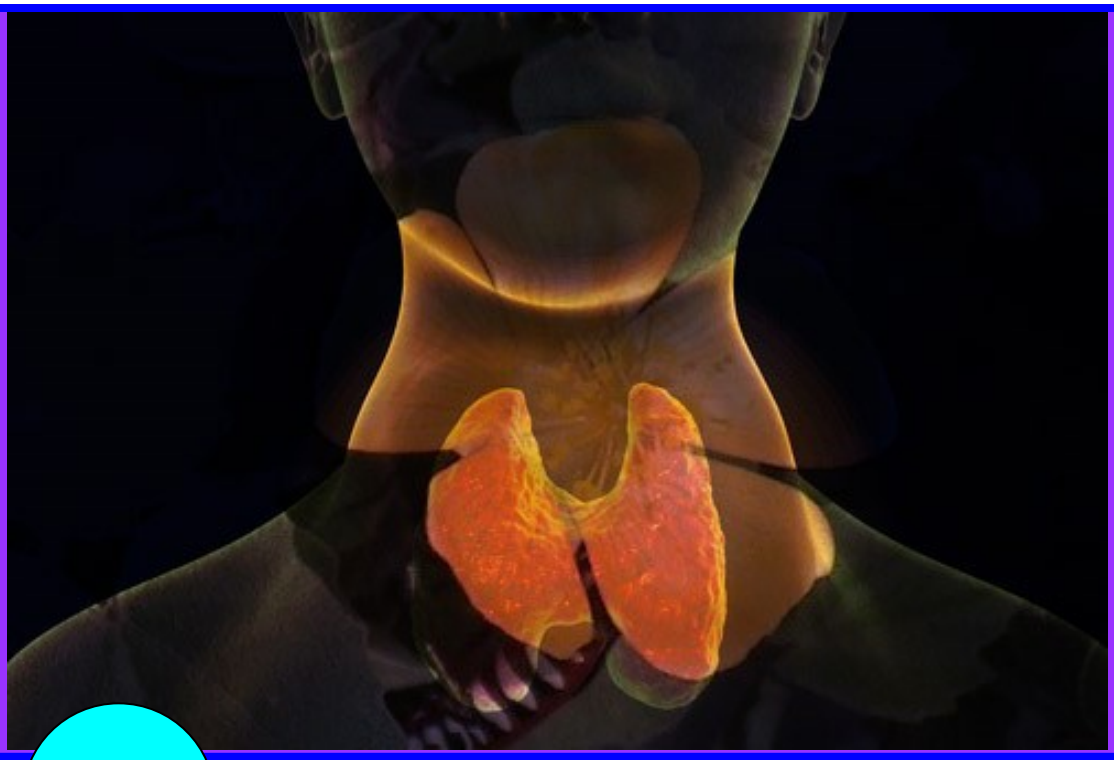

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Effect of *Abutilon indicum* in thyroxine-induced hyperthyroidism in rat

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Abstract

The study was designed to investigate the antithyroid activity of the crude methanolic (70%) extract of aerial parts of *Abutilon indicum* in male albino rats. The extract was prepared and analyzed for the presence of phytochemical constituents through preliminary chemical analysis, antioxidant assay and GC-MS. The *in vivo* antithyroid activities in thyroxine-induced hyperthyroidism were studied. Phytochemical analysis showed the presence of alkaloids, flavonoids and phenols, also verified by the data obtained from GC-MS. Thyroxine increased the levels of triiodothyronine (4.9 ± 0.1 ng/mL) and total thyroxine (9.4 ± 0.2 µg/dL); while, *A. indicum* at the doses of 300 and 500 mg/kg, showed significant decrease in the elevated levels of triiodothyronine (3.0 ± 0.1 and 2.6 ± 0.2 ng/mL) and thyroxine (7.7 ± 0.2 and 7.1 ± 0.2 µg/dL), respectively. Histopathological studies showed the restoration of filled follicular colloids in extract-treated animals. The results show that *A. indicum* exhibits dose-dependent antithyroid effects.

Introduction

Hyperthyroidism is commonly defined as the enhanced production of triiodothyronine and total thyroxine resulting in decreased levels of thyroid stimulating hormone in the blood leading to hypermetabolic activity.

The current medicines used in the treatment of hyperthyroidism, including carbimazole, methimazole, propylthiouracil, thiocyanate, perchlorate and fluoroborate, pose adverse effects such as aplastic anemia, angioedema and laryngeal edema (Reid and Wheeler, 2005; Truter, 2011).

The use of natural products, especially plants, for the treatment of endocrine dysfunction, can offer potential alternatives to conservative therapies for a variety of diseases as a variety of phytoconstituents including alkaloids, glycosides, polysaccharides, lectins, peptides, flavonoids and tannins have been reported to ameliorate endocrine disorders in various *ex vivo* and *in vivo* models (Panda and Kar, 2007). The most important medicinal plants which have been studied for antithyroid potential include *Aegle marmelos* (Panda and Kar, 2006), *Emblica officinalis* (Panda and Kar, 2003), *Foeniculum vulgare* (Luaibi, 2018), *Sonchus asper* (L.) Hill (Khan, 2012), *Ferula communis* L. (Chaachouay et al., 2019), *Trigonella foenum-graecum* and *Allium sativum* (Tahiliani and Kar, 2003), *Lycopus europaeus* L. (Vonhoff et al., 2006), *Melissa officinalis* (Mannaa et al., 2021), *Rosemarinus officinyroialis* (Al-Sharafi et al., 2020), *Salvia officinalis* (Mirazi et al., 2013).

Abutilon indicum L. (family: Malvaceae), locally known as Peeli booti, Kanghi and Kakhi, is a perennial shrub found in many countries of Asia (Kailasam, 2015). Almost all the parts of the plant are acknowledged to be beneficial in ethnobotanical surveys and are used traditionally for the treatment of various ailments. Roots are used as a diuretic, demulcent, in urethritis and chest infection, leaves in ulcer, urinary stones and are locally used for analgesia (in toothache, tender gums) and to reduce inflammation (of the bladder). The bark is used as febrifuge, astringent, anthelmintic and diuretic and seeds in piles, as a laxative, in gleet,

chronic cystitis and gonorrhea (Prachi et al., 2009; Singhai, 2013). Furthermore, it is traditionally being used in the management of various endocrine disorders including diabetes and infertility (Chakraborty et al., 2017; Krisanapun et al., 2011) making it a suitable candidate for scientific screening against hormonal disorders such as hyperthyroidism. Scientifically, *A. indicum* has been reported to possess antimicrobial and larvicidal (Edupuganti et al., 2015; Rahuman et al., 2008), anti-arthritic (Vallabh et al., 2009), anti-asthmatic (Paranjape and Mehta, 2008), anti-diarrheal, anti-ulcer (Chandrashekhar et al., 2004; Dashputre and Naikwade, 2011), anti-diabetic (Krisanapun et al., 2011), anti-inflammatory (Tripathi et al., 2012), antioxidant, hepatoprotective (Kalyani, 2011), immunomodulatory (Dashputre and Naikwade, 2010) and cytotoxic (Abdul et al., 2010) activities.

The objective of the study was to investigate the protective role of the crude extract of *A. indicum* against thyroxine-induced hyperthyroidism using *in vivo* animal model and to establish a scientific ground for its utilization, in thyroid dysfunction, by herbal practitioners and local folks in the southern Punjab of Pakistan.

Materials and Methods

Collection of plant material

The plant *A. indicum* was selected based on its traditional use against thyroid dysfunction. The aerial parts of *A. indicum* were collected from Lodhran District of Punjab, Pakistan. The plant sample was identified by the Botanist, Department of Life Sciences, the Islamia University of Bahawalpur, Pakistan, and deposited in the Herbarium of the Pharmacology Research Laboratory, Department of Pharmacology, Faculty of Pharmacy, Islamia University of Bahawalpur, Pakistan. Voucher number AI-AP-05-14-67 was issued for future reference.

Preparation of the crude extract

The shade-dried aerial parts of the plant (1 kg) were subjected to coarse grinding followed by maceration in 70% methanolic solution for three days thrice with occasional shaking at room temperature. Subsequently, filtration was performed with muslin cloth and then through filter paper and filtrate was concentrated using a rotary evaporator (Heidolph Laborata-4000) to thick, semi-solid paste. The crude extract of *A. indicum* was labeled, percentage yield was calculated and stored at -20°C for future use.

Chemicals

All the chemicals were of pure quality and analytical grade. Ascorbic acid, gallic acid, DPPH and sodium carbonate were purchased from the Sigma-Aldrich, carbimazole from the Pharmedic, thyroxine from the GlaxoSmithKline, ketamine HCl from the Global

Box 1: DPPH Method

Principle

DPPH• accepts hydrogen from an antioxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals. The anti-oxidant effect is proportional to the disappearance of DPPH• in the test sample.

Requirements

Ascorbic acid, DPPH (1,1-diphenyl-2-picrylhydrazyl), Extract of *A. indicum*, Methanol

Preparation of solutions

Ascorbic acid: Working solutions (125, 250, 500, 1000, 1500 and 2000 µg/mL) of ascorbic acid were prepared.

Extract: Working solutions (125, 250, 500, 1000, 1500 and 2000 µg/mL) of methanolic extract and its different fractions were prepared.

DPPH solution: The DPPH solution was prepared by dissolving 3 mg of it in 100 mL methanol.

Procedure

Step 1: The methanolic solution (1 ml of 0.2 mM) of DPPH was mixed with 1 mL each of the extract and L-ascorbic acid solutions of varying concentrations.

Step 2: DPPH solution with the addition of 1 mL methanol was used as control.

Step 3: All the solutions were kept in dark for 30 min.

Step 4: The absorbance was measured at 517 nm (purple color) using UV-spectrophotometer and adjusted it to less than one. The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant.

Calculation

The potential of plant extracts to scavenge DPPH free radicals was calculated by using the following equation.

%Inhibition = (*Ac- As/Ac*) x 100

Whereas, *Ac* is the absorbance of control; *As* is the absorbance of sample

Notes

1. Wear gloves while handling DPPH solution
2. Lab coat and goggles should be used
3. Work in fume hood
4. DPPH is stored in the freezer. It should be protected from light and the time out of the freezer should be minimized

Reference

Hayat et al., 2014

Reference (video)

Shah et al., 2019

Pharmaceuticals and xylazine from the Mylab, Pakistan.

Phytochemical analysis

Preliminary phytochemical analysis of the extract was performed for the identification of primary and secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, terpenes and sterols (Tiwari et al., 2011).

Determination of DPPH activity

%Inhibition = (*Ac- As/Ac*) x 100

Whereas, *Ac* is the absorbance of control; *As* is the absorbance of sample

Total phenolic contents

Folin–Ciocalteu method was used for the determination of total phenolic contents (Javed et al., 2020). The extract (200 µL) was added to 1mL of 10 times diluted Folin–Ciocalteu reagent. After 4 min, 800 µL of 7.5% sodium carbonate solution was added to the extract as well as gallic acid dilutions (curve calibration); i.e. 5, 10, 20, 40, 60, 80, 100, 300, 350, 400, 450 and 500 µg/mL. Test tubes were allowed to stand for 2 hours at room temperature and absorbance was measured in triplicate at 765 nm. The results were expressed as milligram of gallic acid equivalent (mg GAE)/g of the crude extract.

GC-MS analysis

GC-MS analysis of the crude extract was performed by dissolving the extract (50 mg/mL) in 50% aqueous methanol. The GC–MS system (Agilent Technologies, USA) consisted of: Agilent 7890B equipped with column HP-5 ms ultra inert (length 30 m, i.d. 250 µm, film thickness 25 µm). The mass spectrometer was an Agilent Technologies (5977A) system working at 70 eV; scan time 1.5 sec; mass range 50 to 600 amu. The mass spectrum and chromatograms were interpreted using NIST 05 spectral library (Gaithersburg, MD, USA) and from reported literature. The temperature of the injector was maintained at 250°C, having a split injection mode at a ratio of 10:1. The oven temperature was programmed from 110°C (1 min), then further increased to 200°C at a ramp rate of 10°C/min, then 5 °C/min to 280°C, ending with a 2 min of isothermal at 280°C, while the detector temperature was kept at 230°C. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min and the volume of injected sample was 1.0 µL of extract (Ahmed et al., 2017).

Experimental animals

Male Wistar albino rats weighing 150-250 g and Swiss albino mice of either sex, weighing 18-30 g were used in the experiments. The animals were kept in polycarbonate cages under standard conditions of temperature (23 ± 2°C), humidity along with 12 hours/12 hours light and dark cycle and were given standard rodents pellet diet and tap water *ad libitum* in the Pharmacology Research Laboratory.

Acute toxicity studies

Twenty-five mice of either sex having almost equal body weight were selected and divided into 5 groups each consisting of five animals and kept fasted overnight. The normal control group was given distilled water (10 mL/kg p.o) while other groups were given different doses of the crude extract; i.e. 1000, 3000, 5000 and 10000 mg/kg p.o. The animals were observed for changes in behavioral and physiological parameters including grooming, hyperactivity, convulsions, alertness, lacrimation, urination, corneal reflex, writhing reflex, righting reflex and gripping strength for 12 hours, on an hourly basis, and for the mortality for 48 hours and then for 14 days, on daily basis (Rasheed et al., 2016).

Induction of hyperthyroidism and treatment protocols

Hyperthyroidism was induced as described elsewhere with slight modifications (Azharuddin et al., 2015). At day 0, animals were anesthetized with ketamine and xylazine (10:1), blood samples were drawn for the determination of the levels of triiodothyronine, total thyroxine and thyroid stimulating hormone, and divided into six different groups each consisting of six rats. The normal control group was given distilled water 5 mL /kg (p.o.), while the rest of the animals were intoxicated with thyroxine at the dose of 600 µg/kg (p.o.) for 14 days to induce hyperthyroidism. After 14 days of intoxication, all the animals were screened for the biochemical markers; triiodothyronine, total thyroxine and thyroid stimulating hormone, and administered for the next 14 days with different treatments; i.e. disease control group (distilled water 5 mL/kg, p.o), standard group (carbimazole 30 mg/kg, p.o.) and treatment groups (crude extract at the doses of 100, 300 and 500 mg/kg, p.o). At the end of 28th day, the blood samples were collected by cardiac puncture and sera separated by centrifugation of blood for 30 min at 3,000 rpm. The levels of triiodothyronine, total thyroxine and thyroid stimulating hormone were determined in sera using ELISA kits (BioCheck, Inc, USA) as per manufacturer protocols.

Enzyme-linked immunosorbent assay

ELISAs for triiodothyronine, thyroxine and thyroid stimulating hormone were performed on serum sam-ples according to the protocol of the kit manufacturer (BioCheck, Inc).

Triiodothyronine levels were determined in the sera of animals from standard curve plotted using four-point logistic (4PL). Enzyme conjugate was prepared by diluting the 11x concentrate with conjugate diluent (1:10). The wells were poured with 50 µL of standard

solutions (0, 0.75, 1.5, 3.0, 6.0 and 10.0 ng/mL of triiodothyronine) and sample followed by the addition of 50 mL of triiodothyronine antibody reagent into each well and mixing for 30 sec. Afterward, 100 µL of enzyme conjugate was poured, mixed well for 30 sec and incubated for 60 min at 20-25°C. Further, the wells were emptied and cleaned with absorbent paper and washed with deionized water five times. Thereafter, 100 mL of TMB (3,3′,5,5′ tetramethylbenzidine) reagent was added into each well with gentle mixing for 5 sec and incubation at room temperature, in the dark, for 20 min. Subsequently, the reaction was stopped by the addition of stop solution to each well and the plate was read at 450 nm with a microtiter plate reader (Biotek, USA).

Total thyroxine levels were estimated with similar protocol with slight difference in standard concentrations (0, 2.0, 5.0, 10.0, 15.0 and 25.0 mg/dL) and volume (25 µL) used in the assay. For the determination of thyroid stimulating hormone levels, the standard concentrations of 0, 200, 500, 2500, 5000, 10000 and 20000 µIU/L were used while the volume of standard and samples was 50 µL.

Histological studies of thyroid gland

At the end of the study, thyroid gland from one animal of each group was dissected out and fixed in 10% formalin solution for histological examination. Tissues were sectioned and stained with hematoxylin and eosin (H&E), and examined under a light microscope equipped with a digital camera for photomicrography. The images were observed critically to observe the morphological changes in thyroid gland tissue among different groups.

Statistical analysis

All the results were expressed as mean ± standard error of the mean (S.E.M.) and data were analyzed using two-way ANOVA using Graphpad prism version 6.0 followed by Bonferroni Post-hoc test. The values were considered statistically significant if $p<0.05$.

Results

Percentage yield

One kg of the aerial parts of *A. indicum* gave the percentage yield of 11.5 (115 g).

Preliminary phytochemical analysis

The results of the phytochemical analysis showed the presence of various secondary metabolites including alkaloids, carbohydrates, coumarins, flavonoids, glycosides, phenols, phlobatannins, saponins and terpenes.

Determination of DPPH activity

The crude extract showed linear inhibition with the maximum up to 70% at the concentration of 1000 µg/g, when compared with ascorbic acid that exhibited maximum inhibition of 89% at 1000 µg (Table I)

Table I

DPPH assay of radical scavenging activity of the crude extract of *A. indicum*

	%Inhibition	
Concentration (µg/mL)	Ascorbic acid	Crude extract
50	64 ± 0.04	47 ± 0.03
100	67 ± 0.02	55 ± 0.03
200	72 ± 0.03	57 ± 0.02
400	77 ± 0.02	60 ± 0.01
600	80 ± 0.03	64. ± 0.04
800	84 ± 0.02	67 ± 0.02
1000	89 ± 0.02	70 ± 0.03

Results are expressed in mean ± SEM; Each sample in triplicates

Total phenolic contents

The total phenolic content of the crude extract was found to be 12.3 ± 0.0 mg GAE of the extract.

GC-MS analysis

GC-MS analysis of crude extract led to the identification of 33 compounds (Table II). The majority of isolated compounds, identified in *A. indicum* extract, were alkaloids, fatty acids, flavonoids, sterols and phenols. Various phenolic compounds include 4-(3-hydroxybutyl) phenol, 2-methoxy-4-vinylphenol, phenol, 2,6-dimethoxy-, benzeneethanamine and 4-(3-hydroxybutyl) phenol. The presence of volatile oils (4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-), fatty acids (palmitic and linoleic acid) and aliphatic hydrocarbons (3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester) were also verified.

Antithyroid effects of crude extract

Triiodothyronine levels

After intoxication, triiodothyronine values of crude extract, at the doses of 100, 300 and 500 mg, were 4.6 ± 0.1, 4.2 ± 0.2 and 4.7 ± 0.2 ng/mL, with significant increase in the levels of triiodothyronine from day 0 levels; i.e. 1.6 ± 0.0, 1.7 ± 0.0 and 1.7 ± 0.1 ng/mL, respectively. After the treatment for 14 days followed by 14 days of induction, crude extract (100 mg/kg) showed an insignificant decrease in triiodothyronine levels (4.4 ± 0.1), while at the doses of 300 and 500 mg/kg, significant dose-dependent decrease in the levels of triiodothyronine (3.0 ± 0.1 and 2.6 ± 0.2 ng/ml) was observed (Table III and Figure 1).

Total thyroxine levels

All the groups treated with thyroxine (600 µg/kg) showed significantly ($p<0.001$) elevated levels of thyroxine when compared with the normal control group, after 14 days of intoxication. The crude extract,

Table II

Gas chromatography-mass spectrometer (GC-MS) spectral analysis of the crude extract of *Abutilon indicum*

Compounds	Retention time	%Peak area	Molecular formula	Molecular weight	Nature
4-(3-Hydroxybutyl) phenol	6.832	2.16	$C_{10}H_{14}O_2$	166.22	Phenol
4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl-	7.599	12.67	$C_6H_8O_4$	144.12	Ketone
2-Methoxy-4-vinylphenol	8.508	8.87	$C_9H_{10}O_2$	150.17	Phenol
Phenol, 2,6-dimethoxy-	8.831	2.36	$C_8H_{10}O_3$	154.16	Phenol
Formic acid, 2,6-dimethoxyphenyl ester	9.497	4.73	$C_9H_{10}O_4$	182.17	Phenolic ester
Ethanone, 1-(2-hydroxy-5-methylphenyl)-	9.739	4.15	$C_9H_{10}O_2$	150.17	
Pyrazole, 4-ethyl-3,5-dipropyl-	10.668	9.12	$C_9H_{16}N_2O$	168.24	Alkaloid
2-(1-Methyl-2-propenyl)bicyclo[2.2.1]heptane	11.092	5.18	$C_{11}H_{18}$	150.26	
1H-Pyrrole, 2-(2,4,6-cycloheptatrienyl)	11.395	14.49	$C_{11}H_{11}N$	157.21	Alkaloid
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	12.051	8.79	$C_{10}H_{12}O_3$	180.2	Phenol
2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	12.758	4.68	$C_{11}H_{12}O_4$	208.21	
Fatty acid (palmitic)	13.535	3.75	$C_{16}H_{32}O_2$	256.42	Fatty acid
Oxacyclododecane-2,8-dione	13.777	3.08	$C_{11}H_{18}O_3$	198.26	
Ethyl 9,12,15-octadecatrienoate	14.454	1.86	$C_{20}H_{34}O_2$	306.48	
Fatty acid (linoleic)	14.676	4.85	$C_{18}H_{32}O_2$	280.4	Fatty acid
Phenol, 4,4'-(1-methylethylidene)bis-	14.999	1.82	$C_{25}H_{29}ClO_6S$	493	
N-(2-(4-hydroxyphenyl)ethyl] acetamide	15.504	3.44	$C_{10}H_{13}NO_2$	179.22	Alkaloid
2,6,10,14,18,22-Tetracosahexaen (squalene-triterpene)	16.029	2.67	$C_{30}H_{50}$	410.7	Terpene
4α-Methyl-1-methylidene-1,2,3,4,4a,9,10, 10a-Octahydrophenanthrene	16.301	2.43	$C_{14}H_{18}$	186.29	
Piperine	23.146	1.60	$C_{17}H_{19}NO_3$	285.34	Alkaloid

Table III

Effects of *A. indicum* on T_3, T_4 and TSH levels in rats

Compounds	Day 0			Day 14			Day 28		
	T_3 (ng/mL)	T_4 (µg/dL)	TSH (µIU/mL)	T_3 (ng/mL)	T_4 (µg/dL)	TSH (µIU/mL)	T_3 (ng/mL)	T_4 (µg/dL)	TSH (µIU/mL)
Control (distilled water 5 mL/kg)	1.5 ± 0.0	4.7 ± 0.1	0.6 ± 0.0	1.5 ± 0.0	4.9 ± 0.1	0.6 ± 0.0	1.6 ± 0.1	4.9 ± 0.1	0.6 ± 0.0
Intoxicated (thyroxine 600 µg/kg, p.o.)	1.6 ± 0.0^{ns}	5.2 ± 0.1	0.6 ± 0.0	4.9 ± 0.1^{a}	9.4 ± 0.2^{a}	0.1 ± 0.0^{a}	4.8 ± 0.1^{a}	9.4 ± 0.2^{a}	0.1 ± 0.0^{a}
Standard (carbimazole 30 mg/kg, p.o.)	1.7 ± 0.0	5.2 ± 0.1	0.7 ± 0.0	5.3 ± 0.2^{e}	9.3 ± 0.1^{e}	0.2 ± 0.0^{e}	1.7 ± 0.1^{d}	5.1 ± 0.1^{d}	0.7 ± 0.0^{d}
Intoxicated plus crude extract (100 mg/kg, p.o.)	1.6 ± 0.0	5.1 ± 0.1	0.6 ± 0.0	4.6 ± 0.1^{e}	9.1 ± 0.3^{e}	0.2 ± 0.0^{e}	4.4 ± 0.1^{ns}	8.7 ± 0.4^{ns}	0.2 ± 0.0^{ns}
Intoxicated plus crude extract (300 mg/kg, p.o.)	1.7 ± 0.0	5.1 ± 0.1	0.7 ± 0.1	4.2 ± 0.2^{e}	9.1 ± 0.2^{e}	0.2 ± 0.0^{e}	3.0 ± 0.1^{b}	7.7 ± 0.2^{b}	0.4 ± 0.0^{b}
Intoxicated plus crude extract (500 mg/kg, p.o.)	1.7 ± 0.1	5.2 ± 0.1	0.7 ± 0.0	4.7 ± 0.2^{e}	9.1 ± 0.1^{e}	0.2 ± 0.0^{e}	2.6 ± 0.2^{c}	7.1 ± 0.2^{c}	0.5 ± 0.1^{c}

Values are mean ± SEM; $^{a}p<0.001$ highly significant, when compared with normal control; $^{b}p<0.05$ significant, $^{c}p<0.01$ more significant; $^{d}p<0.001$ highly significant; ns: non-significant, when compared with intoxicated group (thyroxine 600 µg/kg, p.o.); $^{e}p<0.001$ highly significant, when compared with day 14 from day 0 within the respective group

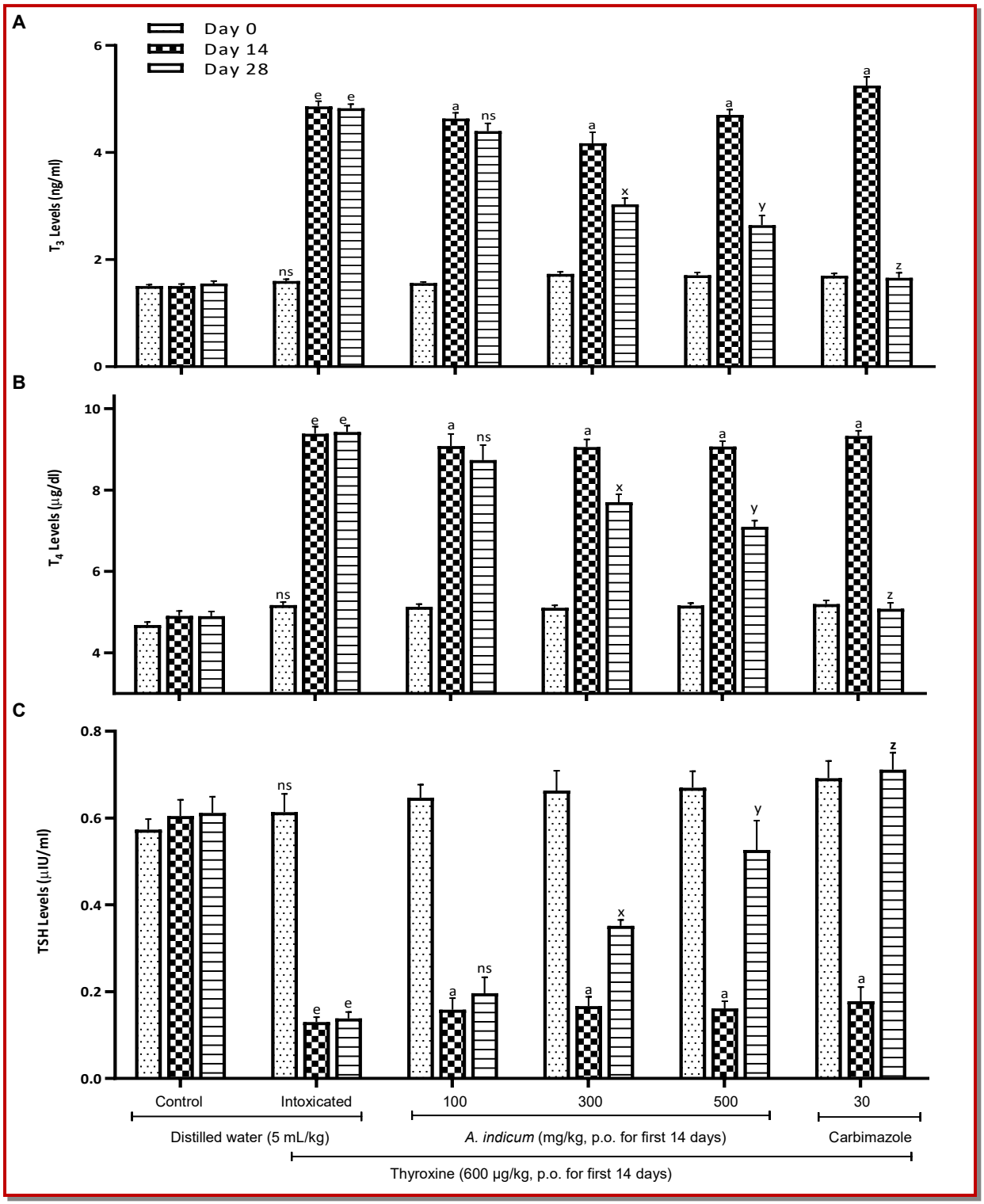

Figure 1: The dose-dependent levels of T_3, T_4 and TSH indicating the antithyroid effects of different doses of crude extract of *A. indicum* and carbimazole, after intoxication with thyroxine, at 600 µg/kg for 14 days in male albino rats. Values are mean ± SEM. $^{e}p<0.001$ highly significant, when compared with normal control (D.W 5 mL/kg). $^{x}p<0.05$ significant, $^{y}p<0.01$ more significant; $^{z}p<0.001$ highly significant; ^{ns}non-significant, when compared with intoxicated group (thyroxine 600 µg/kg, p.o.). $^{a}p<0.001$ highly significant, when the values are compared those at day 14 from day 0 within the respective group

at 100 mg/kg, when given for 14 days, showed non-significant ($p > 0.05$, ns) decrease in thyroxine levels (8.7 ± 0.4 µg/dL) from intoxicated group (9.4 ± 0.2 µg/dL). The levels of thyroxine, at 300 mg/kg, showed a significant decrease; 7.7 ± 0.2 ($p<0.05$). The effects of crude extract, at 500 mg/kg, were more profound and significant ($p<0.01$); i.e. 7.1 ± 0.2 µg/dL (Table III and Figure 1).

Thyroid stimulating hormone levels

The effects of intoxication on thyroid stimulating hormone levels were found to be significant as compared to normal control (Table III). Treatment of animals with *A. indicum* at the dose of 100 mg/kg showed non-significant change in thyroid stimulating hormone levels (0.2 ± 0.0 µIU/mL) as compared to its respective intoxicated values (0.2 ± 0.0 µIU/mL). The crude extract, at the dose of 300 mg/kg, increased the levels of thyroid stimulating hormone (0.4 ± 0.0 µIU/mL) as compared to the intoxicated group (0.1 ± 0.0), while 500 mg/kg group showed an increase in thyroid stimulating hormone; i.e. 0.5 ± 0.1 µIU/mL from the day 14 levels of thyroid stimulating hormone 0.2 ± 0.0 µIU/mL (Table III and Figure 1).

Acute toxicity assay

70% aqueous methanolic extract of *A. indicum* was found to be safe up to the dose of 10 g/kg and no mortality was observed.

Histological studies of the thyroid glands

Figure 2A shows the histology of the normal control thyroid gland follicles with colloids and increased cytoplasmic concentration. Figure 2B indicates hyperthyroid gland with an increased number of empty colloids and follicles are smaller in size and Figure 2C shows standard group (carbimazole-treated) with recovered thyroid gland to normal and increased number of colloids. The crude extract, at the dose of 100 mg/kg, (Figure 2D) showed the empty colloids as it could not decrease the effects of thyroxine on the thyroid gland significantly, while the treatment with 300 mg/kg (Figure 2E) and 500 mg/kg (Figure 2F) showed the increased number of filled colloids with fewer empty spaces, thus showing a significant and dose-dependent increase in colloids.

Discussion

The results of phytochemical analysis of crude extract showed the presence of alkaloids, coumarins, glycosides, flavonoids and phenols. The phytochemical constituents are responsible for various pharmacological activities including antibacterial, antimalarial, anti-inflammatory, antiproliferative, hepatoprotective and anti-tubercular (Imo and Uhegbu, 2015). Flavonoids are thought to be one of the most important medicinal phytoconstituents which is due to their protective effects against oxidative stress-mediated organ damage (Evans et al., 1997). The possible mechanisms of flavonoids and polyphenols, as an antioxidant, mainly include free radical scavenging activity as well as their ability to affect the signaling pathways and gene expression (Soobrattee et al., 2005).

Estimation of total phenolic contents provides information about the presence of phenols in the crude extract while 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method is used for the evaluation of antioxidant activity. DPPH molecule contains the free radical, capable of accepting electron, in the presence of antioxidant these radicals would be scavenged and absorbed. The radical scavenging activity of crude extract on the free radicals could be attributed to the hydrogen donation ability of polyphenols present in the crude extract (Kumarappan et al., 2012).

Crude extract was further characterized by the GC-MS analysis for the identification of major phytoconstituents and their pharmacological relevance, also supported by available literature on *A. indicum*. GC-MS results of crude extract showed the presence of various compounds that are previously identified in other studies including linoleic acid (Meier et al., 2000), 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 2-methoxy-4-vinylphenol that possess antioxidant, anti-dopaminergic, anti-inflammatory and antibacterial activity (Chhabra and Gupta, 2015; Gomathi et al., 2015). Phenol 2,6-dimethoxy-, ethanone, 1-(2-hydroxy-5-methylphenyl)-, octadecanoic acid ethyl ester, (Lim, 2014) phenylalanine (Jeong et al., 2011; Kuo et al., 2008). The other compounds which were separated during the GC-MS characterization were formic acid, 2,6-dimethoxyphenyl ester, palmitic acid (Barbara et al., 2002), 2,6,10,14,18,22-tetracosahexaen 4α-methyl-1-methylidene-1,2,3,4,4a,9,10,10a-octahydrophenanthrene, piperine and 9-octadecenoic acid, 1,2,3-propanetriyl ester and these compounds have been reported to possess anti-cancer, gastroprotective and hepatoprotective potential. Moreover, these compounds have also been found to exhibit gastroprotective, anti-inflammatory and analgesic (Hamdi et al., 2018), anti-asthmatic, hepatoprotective and also effective in endocrine diseases. The presence of polyphenolic compounds in the crude extract is verified by chemical analysis, antioxidant assay and GC-MS. The polyphenols mainly act as primary antioxidants and may counteract the free radical damage in the human body and have the potential therapeutic use in thyroid dysfunction (Gomathi et al., 2015; Harada et al., 2002).

In the thyroid gland, oxidation of iodide to iodine occurs in the presence of H_2O_2, which is further catalyzed by thyroid peroxidase (TPO) leading to the iodination of iodotyrosines and production of triiodothyro-

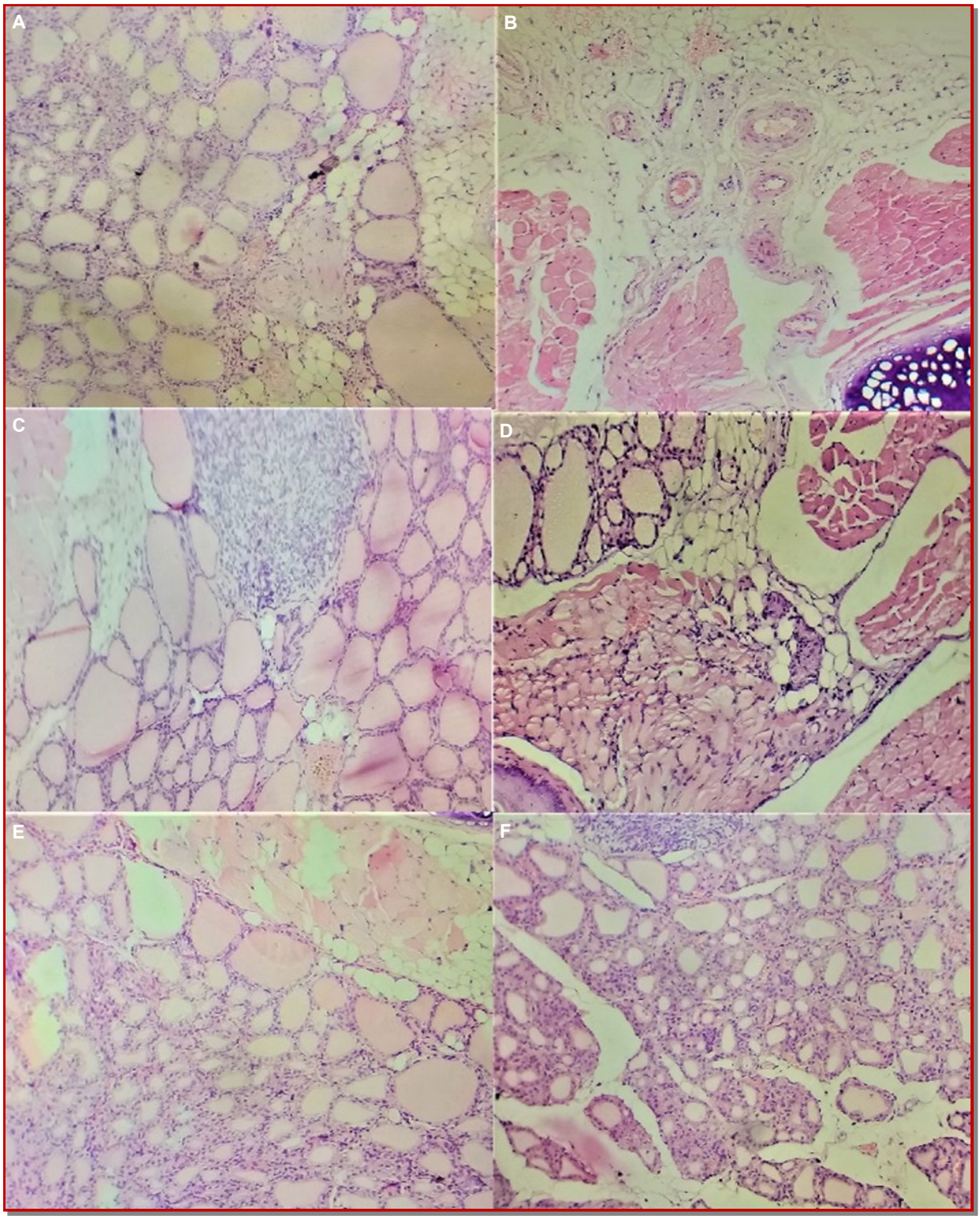

Figure 2: Histological examination of thyroid gland at 14 day of different treatments: **A)** control group (distilled water, 5 mL/kg); showing filled thyroid colloids follicles, **B)** thyroxine-intoxicated group (distilled water, 5 mL/kg); depicting increased number of empty spaces as result of hyperthyroidism, **C)** carbimazole-treated group; representing the follicular colloids recovery in the intoxicated animals, **D)** *A. indicum* 100 mg/kg: showing the inability to recover thyroidal follicular colloids in comparison with intoxicated group, while, **E)** & **F)** *A. indicum*, at the doses of 300 and 500 mg/kg respectively, displaying dose-dependent recovery of thyroid follicular colloids

nine and thyroxine. Thyroid peroxidase catalyzes the conversion of iodide into iodine and at this stage, H_2O_2 is required, which is synthesized by the NADPH oxidation to NADP. H_2O_2 is the frequently occurring oxidant that exists in the body and is involved in the production of iodide ions; resulting in reduced H_2O_2 damaging oxidative processes in the body. H_2O_2, in the thyroid, is activated by NADPH oxidase induction, which is stimulated by thyroid stimulating hormone and superoxide dismutase (SOD). SOD increases the rate of iodination in the thyroid. The growth of follicular thyroid is regulated by thyroid stimulating hormone; by the intracellular signaling leading to the activation of cAMP and phosphatidyl-inositol cascade (Corvilain et al., 1991).

H_2O_2 and reactive oxygen species (ROS) cause oxidative damage in thyroid gland, which is usually protected by the selenium containing enzymes that are antioxidant; i.e. the glutathione peroxidase (GPx) family. Whenever there is decreased iodine level in blood and increased thyroid stimulating hormone production, the elevated production of H_2O_2 results causing severe oxidative damage to the thyroid. Deficiency of GPx, because of decreased selenium levels and thus fail to remove H_2O_2, may further lead to increased oxidative stress and thyroidal damage (Birringer et al., 2002).

In the case of hyperthyroidism, there is increased iodide peroxidation and production of thyroid hormones; possibly because of increased SOD activity which leads to excessive production of H_2O_2 leading to the thyroidal gland damage as there are low levels of thyroid stimulating hormone but higher levels of the thyroid stimulating immunoglobulins, leading to the excessive production of H_2O_2 resulting in the thyroidal damage. The elevated lipid and protein peroxidation and decreased cellular and extracellular antioxidant activity assure the oxidative stress in Graves' hyperthyroidism (Kohn et al., 2001; Vassev et al., 2000).

The results show that the crude extract of *A. indicum* can decrease the levels of triiodothyronine and thyroxine, which may be due to its antioxidant potential, as oxygen consumption is directly affected by thyroid hormones resulting in the enhanced production of superoxide radicals. The scavenging of radicals, because of antioxidant activity, may be responsible to treat hyperthyroidism as there are many evidences in which provision of antioxidant therapy (Mono et al., 1997), in combination with antithyroid drugs, showed better results in the management of hyperthyroidism with fewer adverse drugs reactions. For example, in a study, 24 hyperthyroidism patients were treated with propylthiouracil (for 5 days) and ascorbic acid (for 1 month); and the results showed an increase in the antioxidant defense system (Vrca et al., 2004). The effects of crude extract of *A. indicum* against hyperthyroidism may be due to the presence of flavonoids and phenols which have been reported to possess antioxidant activity (Soobrattee et al., 2005). The other possible mechanism involves the blocking of the TPO-mediated iodination of tyrosine residues in thyroglobulin, a key step in the synthesis of triiodothyronine and total thyroxine. The increased concentration of thyroid stimulating hormone may be due to the constituents that are involved directly or indirectly in the feedback mechanism or to the stimulation of hypothalamic cells to secrete thyrotropin-releasing hormone. TRH is secreted into capillaries of the pituitary portal venous system and in the pituitary gland and stimulates the synthesis and release of thyroid stimulating hormone and in combination with antioxidants with antithyroid drugs have been studied to increase the antioxidant defense system and thus reduce the time of exposure of thyroid gland to the oxidative stress (Katzung and Trevor, 2015; Manna et al., 2013).

The histological studies of animals have reported the thyroid gland to have numerous follicles with the occasional presence of colloids while their thickness may vary from dense to thin lining. The follicles have round nuclei surrounded by the cytoplasm (Azharuddin et al., 2015). The hyperthyroidism model has been reported to exhibit follicles that have an irregular shape and numerous follicular cells lined with basophilic nuclei containing empty luminal colloids. On contrary, carbimazole treatment has been reported to show the variable size of follicles with abundant colloid (Rajab et al., 2017). The results showed that the groups treated with the crude extract of *A. indicum* have thyroid gland follicles with filled colloid and large size of the follicles. Therefore, extract of *A. indicum* caused significant recovery of the thyroid gland in morphological changes against thyroxine induced-hyperthyroidism.

Conclusion

The aqueous methanolic extract of *A. indicum* has antithyroid potential as evident from the restoration of biochemical parameters including thyroid stimulating hormone, triiodothyronine, total thyroxine, and the histological studies showing the recovery of glandular tissues filled with follicular colloids.

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Ethical Issue

The study was conducted according to the guidelines of Phar-

macy Research Ethics Committee of the Faculty of Pharmacy, The Islamia University of Bahawalpur and approved under the number 57/S-2019/PREC.

Conflict of Interest

Authors declare no conflict of interest

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