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Evaluation of chemical profile and antioxidant potential of *Trichodesma indicum* (L.) R.Br.

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Abstract

The present study was conducted to evaluate the antioxidant activity and chemical composition of aqueous methanolic extract of *Trichodesma indicum* (L.) R.Br. The extract was tested for antioxidant activity using various *in vitro* models viz., DPPH, ABTS, NO, FRAP, total antioxidant activity and reducing power. The total phenolic and flavonoid contents were found to be equivalents to 97.83 ± 0.08 µg of gallic acid and 45.45 ± 0.19 µg of rutin /mg of dried aqueous methanolic extract, respectively. Among various antioxidant assays performed, maximum inhibition was observed in the ABTS (IC_{50} 17.13 ± 0.23 µg) followed by DPPH (IC_{50} 80.64 ± 0.29 µg) and NO (IC_{50} 4.12 ± 0.02 µg) assay. GC-MS analysis revealed the presence of over 38 compounds, the prevailing compounds were hexadecanoic acid, β-sitosterol, ethyl iso-allocholate and 9,12,15,-Octadecatrienoicacid. The HPLC analysis further confirmed the presence of rutin, salicylic acid and benzoic acid. This confirms that *T. indicum* could be the good source of natural antioxidant for industrial and pharmaceutical preparations.

Keywords: Antioxidant, hydro- methanolic extract; *Trichodesma indicum,* GC-MS, HPLC

Introduction

Plants have remained as an excellent source of health promoting agents for mankind since thousands of years, and still today, they appear to have a huge share in the primary health care system. Hundreds of plants found to have various therapeutic effects, and majority of these effects have been attributed to pathological oxidative stress minimizing ability. *In vitro* antioxidant assessment methods are often used to screen plants for their antioxidant properties. Almost all plants have some extent of antioxidant activity, even if, screening of plants for their antioxidant activity efficacy remains an interesting and useful field of research, especially for finding new natural antioxidant- entities, and/or to prove that one source of antioxidant is better in comparison with other [1,2].

Trichodesma indicum (L.) R.Br. belongs to family Boraginaceae, commonly found as a weed in the tropical and subtropical regions of the world [3]. In Ayurveda, the plant is prescribed for the treatment of eye diseases, arthralgia, inflammations, dyspepsia, diarrhea, dysentery, leprosy and skin diseases [4,5].The root paste is reported to be useful in reducing swellings, particularly of the joints and the extract is given to children in dysentery and fever [6,7]. Chemically and pharmacologicaliy, *T. indicum* is less studied plant species, though there are few reports on chemical constituents [8,9] and bioactivity [10-17]. In this study, we investigated the *in-vitro* antioxidant activity of the hydro-methanolic extract of aerial part of *T. indicum* using various models. Furthermore, the extract was chemically characterized to identify the active constituents.

Materials and Methods

Sample collection and Extraction

The aerial (above ground) part of *T. indicum* was collected from Baleshwar range of Sangamner tehsil located in the Ahmednagar district (MS), India. The collected plant specimen was authenticated at the Department of Botany, Sangamner College, Sangamner, by using floras [18, 19]. Tap water was used to wash plant material. Afterward, it was dried at room temperature on the laboratory bench for 15 days, and ground to fine powder using an electric mill. Dried powdered material (100 g) was extracted with aqueous methanol (20:80, v/v) by adapting cold extraction method [20, 21]. Briefly, extraction mixture was kept for 48 hrs with frequent shaking at room temperature, filtered and dried using rotavapor at 40 ºC. The procedure was repeated thrice. Finally, the dried extract was stored in the refrigerator until use.

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Phytochemical screening
Qualitative Phytochemical analysis

The extract was analyzed for the preliminary detection of classes of phytoconstituents such as alkaloids, cardiac glycosides, caumarins, flavonoids, phenolic compounds, reducing sugar, saponins, steroids, tannins and terpenoids using prescribed methods [20, 22].

Quantification of total flavonoids

The total flavonoid content of the extract was determined using the method by described Zou *et al.* [23]. In brief, 0.1 ml of sample solution (1mg/ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min of incubation, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, followed by addition of 2 ml of 4% NaOH solution to the mixture. The final volume was adjusted to 5 ml with distilled water and mixed thoroughly. The absorbance of the mixture was recorded at wavelength 510 nm. Total flavonoid content was expressed as µg rutin/mg dry weight, through the calibration curve of rutin (10-100 µg/ml). All samples were analyzed in triplicates.

Quantification of total Phenolics

The total phenol content of the extract was analyzed using Folin–Ciocalteu method adapted by Bhalerao *et. al.* [24] and Cliffe *et. al,* [25]. Briefly, the extract (0.2 ml of 1 mg/ml) was mixed with 2.5 ml of distilled water. After 0.5 ml of the Folin–Ciocalteu reagent and 1.0 ml of Na_2CO_3 reagent were added to the mixture, and incubated at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically (Systronic UV-VIS, India) at wavelength 765 nm. The total phenol content was expressed in microgram gallic acid equivalents per milligram of extract. Triplicate measurements were taken and data were presented as mean ± standard deviation (Mean±SD).

In vitro antioxidant activity

As per the report there is no single testing method, which capable of providing a comprehensive picture of the anti-oxidant profile of a given plant samples containing different antioxidant components [26]. Accordingly, multiple methods are often collectively used to describe antioxidant activity of plant extracts [27]. Herein, we have used six different *in-vitro* assay models for evaluation of the antioxidant activity.

DPPH radical scavenging assay

The DPPH (2,2 diphenyl-1-picrylhydrazyl) free radical scavenging capacity of extract was measured by using standard protocol [28] with some slight modifications. Briefly, the reaction mixture contained 300 µl of extract of varying concentrations (10–300 µg/ml) and 2ml of DPPH solution. After 10 minutes, the change in absorbance was recorded at 517 nm in a spectrophotometer against a blank. L-ascorbic acid was used as a positive control. The DPPH radical scavenging capacities were expressed as ascorbic acid equivalent antioxidant capacity in m mol/g of extract. The % DPPH scavenging activity was calculated by the equation:

% DPPH scavenging activity = $A_{control}$ $A_{sample}/A_{control}$ 100

Where $A_{control}$ is the absorbance of the control (DPPH + methanol) and A_{sample} is the absorbance of the sample (DPPH + sample).

ABTS radical scavenging assay

ABTS (2,2-azinobis [3,ethyl-benzothiazoline-6s-sulphonic acid]) radical scavenging activity was determined by using standard described protocol [29]. The ABTS radical cations are produced when ABTS (7 mM) reacts with potassium persulfate (2.45 mM) at room temperature in the dark for 16 hrs. The solution thus obtained was further diluted with phosphate buffer saline to give an absorbance of 1.000. Different concentrations of the test sample in 50 µl were added to 950 µl of ABTS working solution to give a final volume of 1 ml. The absorbance was recorded immediately at 734 nm. Ascorbic acid was used as a reference standard and inhibiting concentrations of extracts were tested at 10, 20,40,60,80 and 100 µg/ml. Reference standard (ascorbic acid) was also tested with similar concentrations and percent inhibition was calculated by following equation:

% inhibition = ($[A_{blank} - A_{sample}]/A_{blank}$) 100.

Where A_{blank} is the absorbance of ABTS radical + methanol used as control; A_{sample} is the absorbance of ABTS radical + sample extract/standard.

Nitric oxide radical scavenging assay

The nitric oxide scavenging activity of extract on nitric oxide radical was measured according to the method described by Ilavarasan *et. al.*[30]. Sodium nitroprusside (1ml, 5mM) in phosphate buffer saline (PBS) (0.1 M, 7.4 pH) was mixed with 3 ml of different concentration of the extract and incubated at 25 C for 150 min. Afterward, 0.5 ml of the this sample was mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthyl ethylene diamine dihydrochloride) and absorbance was measured at 546 nm.

Reducing power assay

The reducing power of extracts was evaluated according to the method described by Yen and Chen [28] with slight modification. Briefly, different amounts of extracts (100 –700µg/ml) were incubated with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide at 50 C for 20 min. The

reaction was terminated by adding 2.5 ml of 10% TCA solution and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) was mixed with 2.5 ml of distilled water and 1.0 ml of 0.1% ferric chloride ($FeCl_3$) solution and absorbance was measured at 700 nm after incubation at room temperature for 10 min. Quercetin and Butylated Hydroxy Toluene (BHT) (5-50 μg/ml) were used as positive control and experiment was performed in triplicate.

Ferric reducing activity

The FRAP (Ferric reducing antioxidant power) assay was conducted according to standard protocol [31]. The method is based on the reduction of a ferric 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) by antioxidants to the ferrous form (Fe^{2+}-TPTZ). FRAP reagent was prepared freshly by mixing 2.5 mL of TPTZ solution (10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) in 25 mL of acetate buffer (300 mM and pH 3.6). The light blue Fe^{3+}-TPTZ reagent changes to dark blue after contact with an antioxidant, due to the formation of Fe2+-TPTZ. Absorbance was monitored at 593 nm for two different concentrations (100 and 200 μg/mL) of extracts in FRAP reagent. All the results were based on three separate experiments and antioxidant capacity was expressed as μM $FeSO_4$/mg of dry extract. Quercetin and BHT were used as positive control.

Total Antioxidant Capacity

The aqueous extract (0.4 ml, 1mg/ml) was combined in test tube with 4 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95 C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm against blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid [32,33].

FT-IR analysis for phytochemical screening

FT-IR (Fourier Transform Infrared Spectroscopy) analysis of aqueous methanol extract was performed using Chemito 410 Spectrophotometer to confirm class of phytochemicals. The dried extracts was ground into a fine powder using an agate mortar along with a standard KBr pellet and examined with the FT-IR spectrometer in the region of 4000–400 cm ^{1} and the peak values were recorded [34].

GC-MS analysis

The extract was dissolved in methanol and analyzed by gas chromatography (GC) coupled with a mass spectrometer (MS) using a THERMO GC (TRACE 1300) with a fused silica capillary column, PE-5 (50m 0.32mm, film thickness 0.25μm) and a triple quadrapole Thermo MS (TSQ 8000) mass spectrometer. A sample of 5.0 μl was injected in the split mode with split ratio 10:1. An electron ionization (EI) system, with electron energy of 70 eV and emission current 200 μA was used for GC-MS detection. Helium was used as a carrier gas at a flow rate of 1 ml/min and ionization temperature was kept at 200 ^{o}C. The GC-MS was equipped with Dyna Max XR detection system having discrete dynode electron multiplier and electrometer. The mass scanning range was varied over 40-550 Da and for run time of 40 min. The components of the extract was identified by their retention time and compared with mass spectrum data from the National Institute Standard and Technology (NIST) library available with the GC-MS system.

HPLC analysis

To quantify phenolic components, HPLC analysis of the extract was carried out using Younglin's HPLC (Acme-9000) instrument (Kyounggi, Korea) equipped with UV detector. Column used was C18 (Cosmosil RP 150 mm X 4.6 mm i.d., 5 μm particle size). The injection volume was 20 μl. Mobile phase acetonitrile and water (60: 40) was used, and detection was carried out at wavelength 230 nm. The isocratic flow was kept constant at 10 ml/min. For the identification and quantification of phenolics from extract, stock solutions of rutin, quercitin, gallic acid, vanillic acid, benzoic acid, cinnamic acid and salicylic acid were prepared in methanol (1mg/10 ml). The hydro-methanolic extract of T. indicum was prepared at concentration of 10 mg/mL in HPLC grade methanol. Both sample and standards were prepared fresh and used for analysis immediately.

Results and discussion

The results of the preliminary phytochemical screening of hydro-methanolic extract of *T. indicum* revealed the presence of steroids, phenolics, saponins, tannin and flavonoids (Table1). These secondary metabolites revealed biological and pharmacological activities, such as tannins which are known to have anti-inflammatory and anticancer property [35,36]; flavonoids have reported to be antioxidant, anti-inflammatory and anticancer property [37], saponins possesses antimicrobial property and also maintain the blood cholesterol level [38].

Table 1: Preliminary phytochemical constituents of *T. indicum*

Phytochemical constituents	Inference
Alkaloid	--
Anthraquinones	--
Anthocyanosides	--
Flavonoids	+
Glycosides	-
Reducing sugar	+
Phlobatannins	+
Simple Phenolics	+
Saponins	+
Steroid	+
Tannins	+
Terpenoids	-

Total phenolic and total flavonoid contents

These days, phenolics and flavonoids have gained much attention as a source of natural antioxidant because of their efficient radical scavenging, metal chelating, hydrogen donating and redox properties [39, 40]. The total phenolic and flavonoid contents were estimated to be equivalents to 97.83 ± 0.08 µg of gallic acid and 45.45 ± 0.19µg of rutin /mg of dried hydro-methanolic extract, respectively. Our reports indicated more amounts of phenols and flavonoids than earlier reports [16]. These results confirmed that hydro-methanolic extract of *T. indicum* to be comparatively rich in total phenols than flavonoids.

DPPH radical scavenging activity

The scavenging potential of hydro-methanolic extract on DPPH radical is exemplified in line graph (Figure1) and compared with ascorbic acid. Results showed that scavenging activity of extract on DPPH radicals was concentration dependent i.e. increases with increasing concentration (10-300µg/ml). The percentage of inhibition was observed from 8.78 (at 10 µg/ml) to 77.76 (at 300 µg/ml) for the extract which was close to the standard, ascorbic acid. The observed IC_{50} value was 80.64 ± 0.29 µg/ml. In earlier study, reported IC_{50} was 135.00 µg/ml for 70% ethanol extract of *T. indicum* [12]. This finding confirmed that 80% menthol has more extraction efficiency of antioxidant components than 70% aqueous ethanol. The observed variation might be due to the environmental conditions [41].

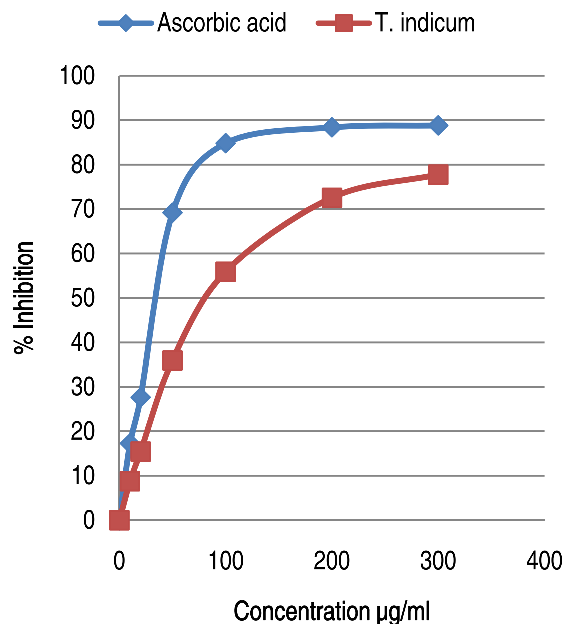

Figure 1: DPPH scavenging activity of hydro-methanolic extract of *T. indicum*

ABTS radical scavenging activity

The ABTS assay commonly used to study antioxidant capacity in plants based on the capacity of the extracts to scavenge the radical cation $ABTS^{\bullet+}$ was generated during the application of this method [42, 43]. The results of ABTS scavenging activity of the extract and standard are shown in the figure 2. The extract exhibited strong scavenging activity than standard ascorbic acid. The IC_{50} values of the extract and standard was 17.13 ± 0.23 µg/ml and 21.48 ± 0.68 µg/ml, respectively, which confirmed total antioxidant capacity of the extract was nearly same to the standard, ascorbic acid. Previous research study [44] reported that methanolic extract of whole plant of *T. indicum* exhibited radical scavenging potential with IC_{50} 130.04µg/ml which is very low as compared to present study in hydro-methanolic extract. This could be due to the solubility of phenolic compounds in more polar solvents.

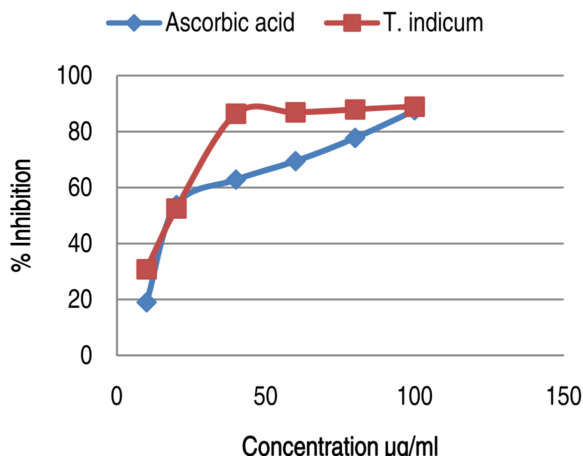

Figure 2: ABTS scavenging activity of hydro-methanolic extract of *T. indicum*

Nitric Oxide assay

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages and neurons. Excess production of nitric oxide may cause several diseases [40]. The extract showed greater scavenging activity than referenced standard, ascorbic acid. The resultant IC_{50} values of extract and standard were 4.12±0.02 µg and 17.11 µg respectively (Figure 3). The flavonoids content of the extract might be responsible for the scavenging of nitric oxide radicals. We found more nitric oxide scavenging activity of *T. indicum* than in previously reported [12, 44].

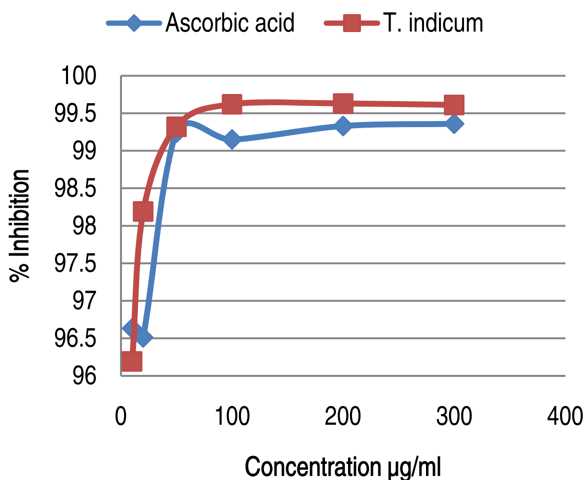

Figure 3: Nitric oxide scavenging activity of hydro-methanolic extract of *T. indicum*

Ferric reducing activity

The FRAP method is often used to ascertain antioxidant activity to plant materials, in which the capacity of the extracts to reduce ferric complexes to the ferrous form was measured [45]. The estimated FRAP value for the sample was 44±0.22mg/gm.

Reducing power assay

The reducing power assay of the aqueous methanolic extract of *T. indicum* was compared with standard BHA and quericitin. The extract showed promising reducing power ability, which reflected its antioxidant potential and increased with the increase in concentration. In the reducing power assay antioxidants from sample reduces Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm [46].The results showed that the reducing power of the hydro-methanolic extract was concentration-dependent (0.1-0.5 mg/ml). At 0.5 mg/ml extract showed strong reducing power (0.248±0.02), comparison to that of reference standard quercetin (1.024± 0.018) and BHA (0.249± 0.06) at 25µg/ml.

Total antioxidant capacity

The total antioxidant capacity by phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH [46]. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid. The total antioxidant capacity of the extract was 32.8±0.15 %.

FT-IR analysis

The FT-IR analysis was carried out to support the results of preliminary phytochemical screening. The basic idea was to identify the functional groups which are specific to the classes of the phytochemicals present in the extract.

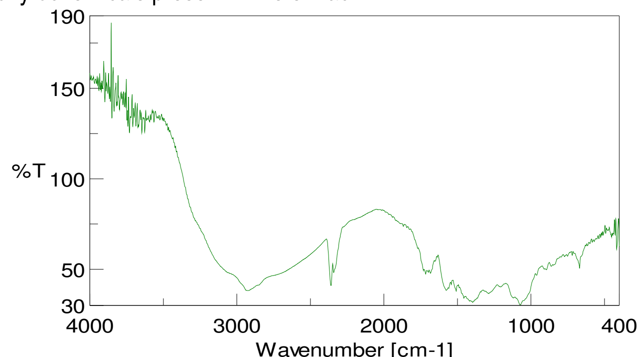

Figure 4: FTIR spectrum of hydro-methanolic extract of *T. indicum*

The results of FT-IR spectrum and peak values along with functional groups were documented in the figure 4 and table 2. The

FT-IR analysis of the extract predominantly revealed the presence of alcohols, phenols, alkanes, alkynes, alkyl halides, aldehydes, carboxylic acids, aromatics, nitro compounds and amines in the extract.

Table 2: FTIR spectral peak values and functional groups obtained for the hydro-methanolic extract of *T.indicum*

	Functional groups	Frequency range (cm^{-1})	Visible Intensity
	Carboxylic acid		
	O–H bend	2923.56	m
	C=O stretch	1716.34	s
	esters		
	C=O stretch	1716.34	s
	Nitro Compounds		
	N-O symmetric & asym. stretch	1508.06	s
	N–O symmetric stretch	1339.32	m
	Aromatic compounds		
	C–C stretch (in–ring)	1417.42	m
	Alkane		
	C–H bend	1455.99	m
	Aromatic amines		
	C-N stretch	1287.25	s
	Alkyl halide		
	C–H wag (–CH_2X)	1208.18	m
	C-Cl stretch	669.178	m
	C–Br stretch	598.789	m
	Ethers		
	C–O stretch	1072.23	s
	Aliphatic amines		
	C–N stretch	1118.51	m
	Alkenes		
	=C–H bend	961.341	s
	–C=C– stretch	1653.66	m
	Amine		
	N–H wag	894.804	s, b
	N–H bend	1636.3	m
	Alkynes		
	–C C–H: C–H bend	615.181	b,s
	Carbonyls		
	C=O stretch	1683.55	s
	Phennols		
	O–H stretch, free hydroxyl	3615.88	s, sh
	O–H stretch, H–bonded	3499.2	s, b
	Ketons		
	C=O stretch	1683.55	s

GC-MS analysis

The GC–MS analysis chromatogram and the different types of compounds present in the hydro-methanolic extract of *T. indicum* are shown in the figure 5 and Table 3.

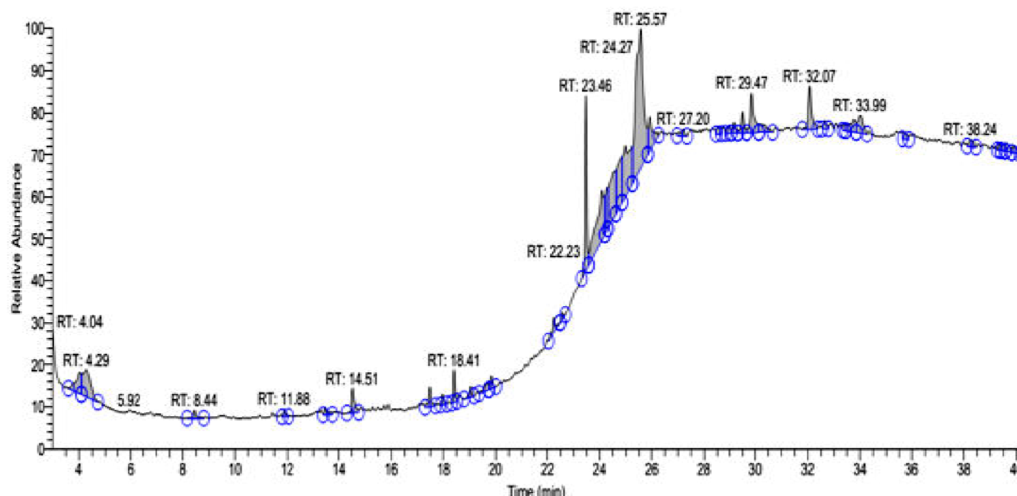

Figure 5: GC-MS chromatogram of hydro-methanolic extract of *T. indicum*

The steroidal compound 9,19-Cyclolanostan-3ol,24,24-epoxymethano acetate was the major compound found the in extract. Some of the identified phytochemicals such as hexadecanoic acid, β-sitosterol, ethyl iso-allocholate, 9,1215,-Octadecatrienoic acid reported to have antioxidants activity [47-52]. These molecules may contribute to the observed activity of hydro-methanolic extract of *T. indicum.*

Table 3: GC-MS Chromatogram of hydro-methanolic extract of *T. indicum*

RT	Name of compound	Molecular formula	Molecular weight (g/mol)	% peak area
4.29	Cyclohexanol	$C_6H_{12}O$	100.15	4.54
8.44	Benzoic acid	$C_{16}H_{30}O_4Si_3$	370.664	0.50
11.88	bis(1,1,3,3-tetramethylbutyl) Thiocyanic acid	$C_{16}H_{34}S_2$	290.57	0.36
13.48	2,4-bis(1,1-dimethylethyl)- Phenol	$C_{14}H_{22}O$	206.33	0.50
14.51	Phthalic acid	$C_{13}H_{16}O_4$	166.14	1.53
17.46	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	1.09
17.97	Hexadecanoic acid	$C_{17}H_{34}O_2$	270.45	0.50
18.41	butyl hex-3-yl ester	$C_{18}H_{26}O_4$	306.4	1.25
19.05	Ethyl isoallocholate	$C_{26}H_{44}O_5$	436.62	0.47
19.83	9,10-Secocholesta-5,7,10(19)-triene-1,3β,25-triol	$C_{27}H_{44}O_3$	416.63	0.77
22.23	9,12,15-Octadecatrienoic acid	$C_{27}H_{52}O_4Si_2$	278.42	1.05
22.55	1-mono linoleoyl glycerol trimethyl silyl ether	$C_{27}H_{54}O_4Si_2$	498.88	0.30
23.46	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390.55	6.51
24.08	Hexasiloxane	$C_{12}H_{38}O_5Si_6$	430.94	9.11
24.27	Octasiloxane	$C_{16}H_{50}O_7Si_8$	579.24	2.34
25.57	9,19-Cyclolanostan-3ol,24,24-epoxymethano acetate	$C_{33}H_{54}O_3$	498.78	23.12
25.92	Piperidine	$C_{23}H_{43}NO$	85.15	3.05
29.15	9 12 15 octadecatrienoic acid	$C_{27}H_{52}O_4Si_2$	496.87	0.81
29.47	Stigmast-5-en-3-ol	$C_{47}H_{82}O_2$	679.15	1.31
29.85	1,2-Dimethoxy-4-(1-methoxy-1-propenyl) benzene	$C_{12}H_{16}O_3$	208.25	3.38
30.17	Hexa-t-butyl selenatrisiletane	$C_{24}H_{54}SeSi_3$	505.90	1.42
32.07	1,10-Phenanthroline,2,9-dimethyl-2-Propenoic acid	$C_{14}H_{12}N_2$	208.25	3.34
32.65	psi psi-carotene	$C_{42}H_{64}O_2$	600.95	0.57
33.99	Ç-Sitosterol	$C_{29}H_{50}O$	414.70	1.97

HPLC analysis

The HPLC fingerprint of hydro-methanolic extract of *T. indicum* revealed 11 different peaks as shown in the chromatogram (Figure 6).

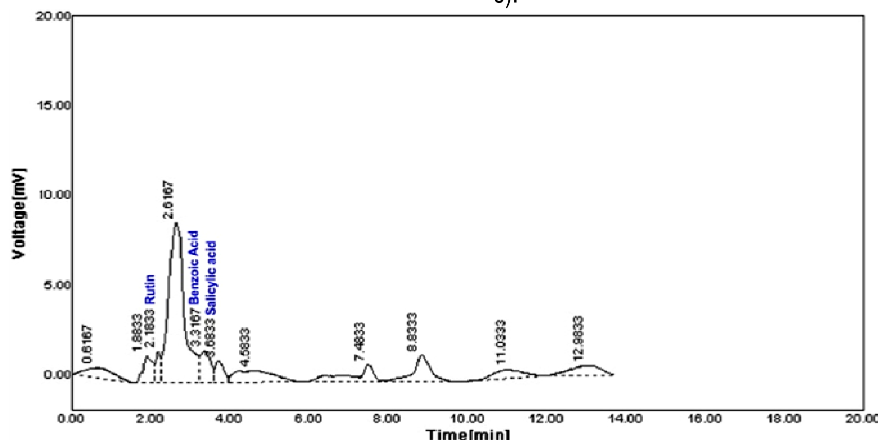

Figure 6: HPLC chromatogram of *T. indicum*

The peaks at retention time 2.18, 3.31 and 3.68 were identified as rutin, benzoic acid and salicylic acid respectively. The phenolic compounds were identified from the extract by matching their retention times against those of available standards. The amount of rutin, benzoic acid and salicylic acid was calculated as 0.75, 0.51 and 0.92 μg/mg of hydro-methanolic extract of *T. indica* respectively. Present findings however, obtained in this work compared with earlier literature revealed the other phenolic compounds such as gallic acid and catechin [13].

Conclusion

In this study, *in-vitro* antioxidant activities and chemical composition (FT-IR, GC-MS and HPLC) of hydro-methanolic extract obtained from aerial part of *T. indicum* was determined. The extract exhibited potent antioxidant and free radical scavenging activity when compared with reference standard. The GC-MS and RP-HPLC confirmed the presence of 9,19-Cyclolanostan-3ol,24,24-epoxymethano acetate, salicylic acid, benzoic acid and rutin. All these compounds are responsible for the antioxidant property. The results of the present study confirmed that the aerial part of *T. indicum* revealed strong antioxidant property and could be used is a good source of natural antioxidants which may have commercial applications especially in the food, cosmetical and pharmaceutical preparations for the implications of human health. Moreover, further studies are warranted to isolate and characterize the active phenolics present in hydro-methanolic extract of *T. indicum* in order to provide more convincing evidence to use this plant as a source of natural antioxidant.

Author's contributions

MSK conceived, designed the experimental work and wrote the manuscript. SNV prepared crude methanol plant extract and measurement of total phenolic and flavonoid contents. In-vitro experiments, statistical data and results analysis done by ADP. All the authors read and approved the final submitted manuscript. None of the authors have any conflict of interest.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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