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ANTIOXIDANT ACTIVITY OF HYDROCHLORIDE SALT OF TYLOPHORINIDINE AND TYLOPHORININE ISOLATED FROM AERIAL PARTS OF *TYLOPHORA INDICA*

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ABSTRACT
The extracts from aerial parts (leaves and stem) of *Tylophora indica* were evaluated for their antioxidant activity. The antioxidant activity of crude extracts was determined by using DPPH free radical scavenging method in which methanol and ethyl acetate extracts showed highest antioxidant activity (IC_{50} values 87 µg/mL and 81 µg/mL respectively). Methanol extract was further purified to isolated hydrochloride salt of tylophorinidine and tylophorinine, identified by analytical and spectral data. Tylophorinidine hydrochloride showed promising antioxidant activity with IC_{50} 24 µg/mL.

Keywords: *Tylophora indica*, Antioxidant, DPPH, Tylophorinidine and Tylophorinine.

INTRODUCTION

Tylophora indica belongs to the family Asclepiadaceae and has been known in ayurveda for more than a hundred years for its use in the treatment of respiratory diseases. The plant is found in plains, forests and hills of southern and eastern part of India. Clinical trials have been done on *Tylophora indica* extracts for evaluating their effectiveness in bronchial asthma[1-3]. The antiasthmatics, anticancer, anti allergic, hepatoprotective and immunomodulator activities of *Tylophora indica* extract have been reported[4-5]. *Tylophora indica* is known to contain 0.2-0.3% phenanthroindolizidine alkaloids[6-7] such as Tylophorine (1), Tylophorinidine (2), Septicine (3). Tylophoridicine E (4) is reported from *Tylophora atrofolliculata*[8]. Some naturally occurring N-oxide**s** of tylophorine such as Tylophoridicine C **(5),** Tylophoridicine F **(6)** have been isolated from *Tylophora atrofolliculata*[9] as shown in Figure 1.

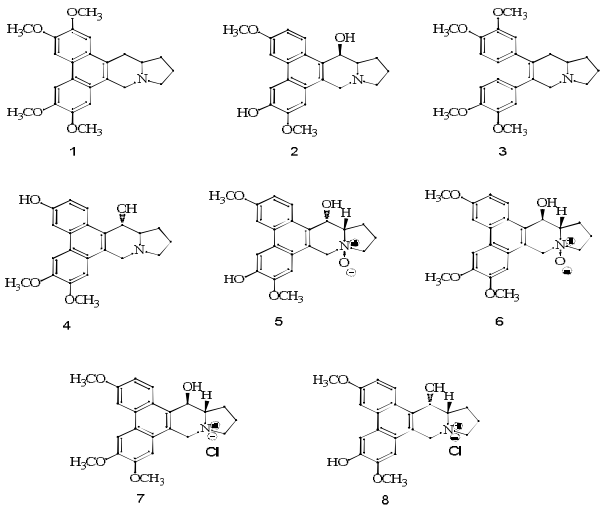

Figure 1: Phenanthroindolizidine alkaloids from *Tylophora*

Natural antioxidants protect us from Reactive oxygen species (ROS) by a pathway known as free radical scavenging. Many phenolic and polyphenolic natural compounds are known to act as good antioxidants. Antioxidant activity of *Tylophora indica* leaf extracts has been reported[10]. In this article, we report the antioxidant activities of different extracts from aerial parts (leaves & stem) as well the isolated hydrochloride salt of tylophorinine (**7**) and tylophorinidine (**8**) from methanol extract.

MATERIALS AND METHODS

HPLC analyses were performed on Waters Alliance 2695 instrument with PDA 2996 detector. Purification of extract was done on Waters Prep HPLC system with dual wavelength UV absorbance detector 2487. Lyophilization was done using Edwards Pirani 501 freeze dryer. ^{1}H and 2D NMR spectra were obtained on a Bruker Avance-500. Mass spectra were recorded on a Bruker micro TOF-Q mass spectrometer.

Solvents and Chemicals

Chemicals used in all the experiments were of analytical grade. Ascorbic acid was purchased by HiMedia laboratories Pvt. Ltd, Mumbai, India and DPPH (1, 1-diphenyl-2-picylhydrazyl) from Sigma, St. Louis, MO, USA. Trifluoroacetic acid was purchased from Spectrochem, Mumbai, India. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich, unless otherwise specified. All the solvents used in all the experiments were of analytical grade purchased from Fisher scientific.

Plant material

Aerial parts of *Tylophora indica* were collected from Victoria Garden of Medicinal Plants, Mumbai and authenticated by our in-house plant taxonomist. A voucher specimen No SKW-4625 was submitted in herbarium of Botany department of St. Xavier College Mumbai. Plant material was dried at 40 °C for one week

and then ground to a fine powder (1kg) by using pulverizer and 500 g powder was taken for sequential extraction.

Extraction of plant material

Preparation of Petroleum ether extract

500 g of plant crude was taken in a clean round bottom flask (5L), soaked in petroleum ether (2 × 2.5L, LR grade) and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper (201) and filtrate was concentrated to dryness at 45 °C on a rotary evaporator (Buchi). 3 g of oily, greenish crude petroleum ether extract was obtained and it was used as such for further studies. The residual precipitate was extracted further as described below.

Preparation of Dichloromethane extract

Residue obtained after extraction with petroleum ether was further soaked in dichloromethane (2 × 2.5L, LR grade) and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper (201) and filtrate was concentrated to dryness at 45 °C on a rotary evaporator. 3.9 g of thick, dark green gum was obtained. This was the dichloromethane extract which was used for further studies. The residual precipitate was extracted further as described below.

Preparation of Ethyl acetate extract

Residue obtained after extraction with dichloromethane was further soaked in ethyl acetate (2 × 2.5L, LR grade) and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper (201) and filtrate was concentrated to dryness at 45 °C on a rotary evaporator. 3 g of thick, dark green gum was obtained. This was the ethyl acetate extract which was used for further studies. The residual precipitate was extracted further as described below.

Preparation of Methanol extract

Residue obtained after extraction with ethyl acetate extraction was further soaked in methanol (2 × 2.5L, LR grade) and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper (201) and filtrate was concentrated to dryness at 45 °C on a rotary evaporator. 3.48 g of thick, dark green, gum was obtained. This was the methanol extract which was used for further studies. The residual precipitate was extracted further as described below.

Preparation of Aqueous extract

Residue obtained after extraction with methanol extraction was further soaked in distilled water (2 × 2L) and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper (201) and lyophilized to get 6.28 g of brown, crude aqueous extract and it was used for further studies.

HPLC analysis and preparative purification of Crude extracts

All the crude extracts were analyzed on reverse phase HPLC. 2 mg of each extract was dissolved in HPLC grade methanol, sonicated for few minutes and filtered through syringe filter (0.45µm, 25mm, Agela Technology, USA). HPLC analysis was carried out on Waters Alliance in reverse phase mode. The column used for analysis was Waters X-Terra, RP18, 150 × 3.9 mm, 5µ. The mobile phase used was linear gradient of 0.1% Trifluoroacetic acid in water and Acetonitrile. Methanol extract was further purified by preparative HPLC and the compounds having retention time of 7.27 and 7.67 min were isolated using preparative HPLC. Mobile phase for preparative purification comprised of a step gradient of Acetonitrile and 0.1% Trifluoroacetic acid in water and acetonitrile, Column used for purification was Waters X terra RP18, 150 x 19 mm, 5µ. Compound **8** was eluted in 14 % acetonitrile where as compound 7 was eluted in 15 % acetonitrile and was lyophilized to get a fine off-white powder. Comparative HPLC chromatogram of all the 5 extracts and the Compound **7** & **8** are shown in Figure 2.

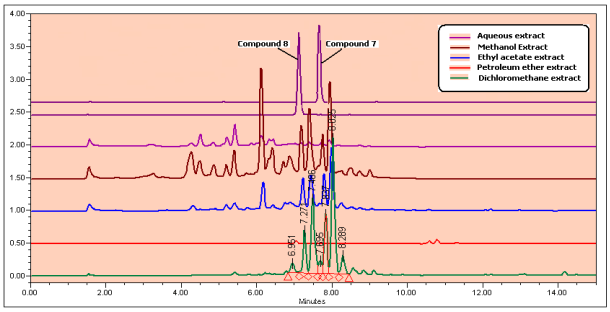

Figure 2: HPLC chromatogram of various extracts and Compound 7 and 8

***In-vitro* DPPH free radical scavenging assay**

Antioxidant activity of different crude extracts of *Tylophora indica* and compound **7** and **8** was determined by DPPH assay model[11]. DPPH assay was performed at various concentrations of all the samples (15.12, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) using analytical grade methanol. 75 µl of each concentration was taken in 96 well microtiter plates and 50µL of 150µM freshly prepared methanolic solution of DPPH was added to each well. Blank was prepared in a similar manner without the addition of test solution. Plates were wrapped with aluminum foil and incubated at 25ºC for 0.5 hour. After incubation, absorbance of reaction mixture was measured by using an ELISA plate reader at 517 nm. Ascorbic acid was taken as a positive control. DPPH radical scavenging activity (%) is expressed as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(Abs_{blank} - Abs_{sample})}{Abs_{blank}} \times 100$$

RESULTS AND DISCUSSION

Characterization

Methanol extract showed good antioxidant activity, which was further purified to isolate two pure compounds which were characterized by spectral analysis and found to be Tylophorinidine hydrochloride (**7**) and Tylophorinine hydrochloride (**8**). The spectral data is shown in table 1, which is in line with reported data[12]. The aliphatic protons showed downfield shift including C-9 (δ ~ 5.14 & 4.60), C-11 (δ 3.36-.340 & 3.82-3.84), C-13a (δ 2.23-2.26) and C-13a (δ 3.73) proton confirms that both the compounds are salt .

Table 1: Spectral data of isolated compounds 7 and 8

Tylophorinine hydrochloride (**7**)			Tylophorinidine hydrochloride (**8**)	
	1H	13C	1H	13C
C-1	8.20 d (*J* = 9 Hz)	126.6	8.14 d (*J* = 9.5 Hz)	126.6
C-2	7.34 d (*J* = 7.2 Hz)	116.5	7.29 d (*J* = 9.5 Hz)	116.7
C-3	----------	158.5	----------	158.4
C-4	8.15 br s	104.4	7.91 s	104.1
C-5	8.15 br s	105.1	8.09 s	108.6
C-6	---------	150.1	---------	149.5
C-7	----------	149.9	----------	147.9
C-8	7.26 s	105.1	7.25 s	104.6
C-9	5.14 d (*J* = 15.9 Hz) 4.60 d (*J* = 15.6 Hz)	51.4	5.13 d (*J* = 15 Hz) 4.58 d (*J* = 15 Hz)	51.5
C-11	3.36-3.40m & 3.82-3.84 m	54.2	3.36-3.40m & 3.82-3.84 m	54.5
C-12	2.01-2.05m and 2.23-2.26 m ,	20.9	2.01-2.06m and 2.23-2.26 m ,	20.9
C-13	2.23-2.26 m	23.4	2.23-2.26 m	23.4
C13a	3.73 m	66.2	3.71 m	66.2
C-14	5.51 s	61.0	5.48 s	61.1
OMe	3.98 s 4.02 s 4.05 s	56.4 56.2 56.0	3.99 s 4.00 s	55.8 56.3
$[\alpha]_D^{16}$: -13.0^0 (c 0.2 MeOH)			$[\alpha]_D^{16}$: $+30.0^0$ (c 0.2 MeOH)	
IR (KBr): 3404, 1686, 1036, 1136, 1201, 1263.			IR (KBr): 3404, 1686, 1036, 1136, 1201, 1263.	

Antioxidant activity

Antioxidant activities for all five extracts and the Compound **7** and **8** were determined by *in vitro* DPPH assay. All the extracts (with the exception of aqueous extract) showed promising antioxidant activity. The DPPH radical scavenging IC_{50} values of ethyl acetate and methanol extracts were 81 and 87 µg/mL respectively and these value are better than the reported antioxidant data (IC_{50} of 199.58µg/mL) for methanol extract from leaves of *Tylophora indica*[9]. Compound **7** showed moderate antioxidant activity where as compound **8** showed excellent antioxidant activity (IC_{50} 24 µg/mL), which is comparable to that of Ascorbic acid (IC_{50} 18 µg/mL) as shown in Figure 3 and Table 2.

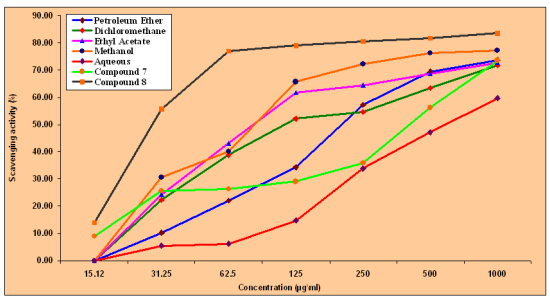

Figure 3: Antioxidant activity of different extracts and isolated compounds 7 and 8

Table 2: IC_{50} values of different extracts, isolated compounds 7, 8 and ascorbic acid

Sample	IC_{50} values
Petroleum Ether	200
Dichloromethane	110
Ethyl Acetate	80
Methanol	87
Aqueous	900
Compound 7	350
Compound 8	24
Ascorbic acid	18

CONCLUSION

Different extracts from aerial parts (leaves and stem) of *Tylophora indica* were evaluated for antioxidant activity. Antioxidant activity of methanol extract was determined as IC_{50} value of 87µg/mL and that of ethyl acetate extract was found to be IC_{50} value of 81µg/mL. We have isolated and characterized Tylophorinidine and Tylophorinine as their hydrochloride salts. Tylophorinidine hydrochloride exhibited promising antioxidant activity (IC_{50} of 24 µg/mL), which is comparable to that of ascorbic acid (IC_{50} of 18 µg/mL) where as Tylophorinine hydrochloride showed moderate antioxidant activity.

This signifies that compound **8** exhibits promising *In-vitro* antioxidant activity. Therefore it can prevent cells against oxidative damage and toxic effect which are caused by ROS. Further investigation is required to explore the *In-vivo* antioxidant activity of Tylophorinidine and it can be used as potential lead for therapeutic purpose.

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