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Evaluation of antimicrobial and antioxidant activities from *Toona ciliata* Roemer

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Abstract

Background: In the present study, the different solvent extracts viz., petroleum ether, chloroform, ethyl acetate and methanol of the medicinal plant *Toona ciliata* (leaf and flower) were evaluated for phytochemical analysis, antimicrobial and antioxidant activities.

Methods: A qualitative phytochemical study was conducted to know the presence or absence of phytoconstituents in the test extracts. Antibacterial and antifungal activities were determined using disc diffusion assay against human and phytopathogens. MIC was carried out using Micro-broth dilution method for pathogenic bacteria and fungi. Radical scavenging activity was also studied using DPPH and ABTS method.

Results: The study revealed the presence of carbohydrates, proteins, phytosterols, flavonoids, glycosides, tannins and phenolic compounds. Ethyl acetate and methanol extracts showed moderate activity against test phytopathogenic bacteria compared to tetracycline. Moderate activity was found against *Proteus mirabilis* and least activity against *Klebsiella pneumoniae*, *Salmonella typhi* and *Staphylococcus aureus* with ethyl acetate and methanol extracts. Ethyl acetate and methanol extracts of *Toona ciliata* exhibited lowest MIC varied from 10-2.5 mgml⁻¹ against test human pathogenic and phytopathogenic bacteria. Significant antifungal activity against *Microsporum canis* was observed in methanol extract with an MIC of 1.25 mgml⁻¹ compared to miconazole. All the test extracts showed significant DPPH and ABTS radical scavenging activity in comparison with BHT.

Conclusions: The present study concludes that the plant *Toona ciliata* could be exploited for the isolation of bioactive compounds which could be a potential source for antimicrobials and antioxidants.

Keywords: *Toona ciliata*, Antioxidant activity, Antimicrobial activity, Phytochemical analysis

Background

Phytobiology perceives medicinal plants as a source of bioactive compounds which can be traced since evolution. The diverse living systems bear a rich biodiversity in nature. Since ancient era before scientific knowledge would evolve plants performed myriad functions on the biosphere. Among which use of plants in curing illness has been well documented. But it was after the advance technology and improved scientific knowledge transformed plants as source of therapeutic agents as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Nagumanthri et al. 2012). It was estimated that current global market for plant-derived drugs is worth more than 20 billion and the market

continues growing (Lin et al. 2013). Perusal of literatures reports the medicinal properties of most of the plants bearing biological activity one such species is *Toona ciliata* (Meliaceae) which has been exploited for many traditional uses like construction purpose, dye preparation, furniture, medicines etc., (Negi et al. 2011). *Toona ciliata* along with Siderin, a compound isolated from petroleum ether extract showed significant antibacterial activity and also exhibited significant cytotoxicity (Chowdhury et al. 2003).

The plant extract also showed gastro protective activity (Malairajan et al. 2006). The inhibitive effects on formed protein non-enzymatic glycation an end product was studied from the ethanolic leaf extract (Shao-Hong 2010) Cedrelone, a tetra nortriterpenoid, isolated from *Toona ciliata* (Gopalakrishnan et al. 2000). Compounds such as 12-Deacetoxytoonacilin and 6 α -acetoxy-14 α ,15 α -epoxyazadirone were isolated from the seeds (Neto et al.

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1995), 12- α -Hydroxystigmast-4-en-3-one was isolated from the petroleum ether extract of *Toona ciliata* together with two steroids and three C-methyl coumarins (Chowdhury et al. 2002), norlimonoids and limonoids from the leaves and stems (Liao et al. 2007), three new norlimonoids (1–3), two new tirucallane-type triterpenoids (4 and 5), and a new pimaradiene-type diterpenoid (6), along with two known limonoids and eight known tirucallane-type triterpenoids, from the leaves and twigs (Chen et al. 2009), toonacilatone, methyl-3 α -acetoxy-1-oxomelic-14(15)-enate, perforin A and cholest-14-ene-3,7,24,25-tetrol-21,23-epoxy-21-methoxy-4,4,8-trimethyl-3-(3-methyl-2-butenate) from the leaves (Ning et al. 2010) and protolimonoids and norlimonoids from the stem bark of *Toona ciliata* (Wang et al. 2011). Although, several synthetic antioxidants and drugs are commercially available, natural products still substitute most of the chemical agents. In the present study, solvent extracts such as petroleum, chloroform, ethyl acetate and methanol of *Toona ciliata* were evaluated for the qualitative phyto-chemical analysis, *in vitro* antimicrobial and antioxidant activity which may lead to the finding of more effective agent for the management of diseases and effective potential source of natural antioxidant that may help in preventing various oxidative stresses.

Methods

Preparation of the extract

Plant material of *Toona ciliata* leaf and flower were washed with distilled water and shade dried. The dried leaves and flower were ground together to a fine powder using Waring blender. The coarsely powdered sample (50 g) was filled in the thimble and extracted successively with petroleum ether, chloroform, ethyl acetate and methanol using a Soxhlet extractor. The filtrate was evaporated to dryness under reduced pressure using rotary vacuum evaporator. The extracts were stored in ambient bottles until further use (Satish et al. 2007).

Preliminary phytochemical screening

The freshly prepared crude solvent extracts of *Toona ciliata* were qualitatively tested for the presence of phytochemical constituents such as alkaloids, flavones, terpenoids, phenols, tannins etc., by standard methods (Harborne 1973; Sofowara 1993; Ghani 2003).

Bacterial strains

Authenticated cultures of Gram positive bacteria such as *Bacillus subtilis* (MTCC 121), *Listeria monocytogenes* (MTCC 839), *Staphylococcus aureus* (MTCC 7443), *Staphylococcus epidermidis* (MTCC 435), Gram negative - *Escherichia coli* (MTCC 7410), *Enterobacter aerogenes* (MTCC 7325), *Klebsiella pneumoniae* (MTCC 7407), *Proteus mirabilis* (MTCC 425), *Pseudomonas aeruginosa* (MTCC 7903), *Salmonella typhimurium* (MTCC 1254),

Vibrio parahaemolyticus (MTCC 451) and *Erwinia carotovora* (MTCC 1428) were procured from MTCC, Chandigarh, India. Authentic pure cultures of phytopathogens *Xanthomonas axonopodis* pv. *malvacearum*, *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas oryzae* pv. *oryzae* were procured from DANIDA research laboratory, University of Mysore, India.

Fungal strains

Four plant fungi *Aspergillus niger*, *Aspergillus flavus*, *Drechslera* and *Fusarium verticillioides* and three human dermatophytic fungi *Candida albicans*, *Microsporum canis* and *Microsporum gypsum* were used.

Preparation of inoculum

Bacterial and fungal inoculum were prepared from 24 h old pure culture grown on nutrient agar for bacteria and a week old culture on potato dextrose agar for fungi. Bacterial colonies were pre-cultured in nutrient broth medium and kept overnight, then centrifuged at 10,000 rpm for 5 min. Pellet was suspended in sterilized distilled water and the cell turbidity was assessed spectroscopically in comparable to that of the 0.5 McFarland standards (approximately 1.5×10^8 CFU/ml) whereas, the fungal spores was scraped from the mother culture and dispensed in sterilized distilled water. Then the spore density was adjusted spectrophotometrically to obtain approximately 10^5 spores/ml final concentration. Then the inoculum were used for the antibacterial and antifungal assays (Mahesh et al. 2008).

Antibacterial and antifungal activity

Antibacterial and antifungal activity of *Toona ciliata* solvent extracts was determined using a modified Kirby Bauer disc diffusion method. Briefly, 100 μ l of the test bacteria/fungi was spread onto the nutrient agar and potato-dextrose agar plates respectively. The different test solvent extracts (petroleum ether, chloroform, ethyl acetate and methanol) were loaded to the sterilized sterile 6 mm discs, allowed to dry and then the impregnated discs with 50 μ l (100 mgml^{-1} concentration) onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation with the test microbial agents. Bacterial plates were incubated at 37°C for 24 h and at room temperature for 3–4 days for fungi. The diameters of the inhibition zones were measured in mm. All the assays were done in triplicate and the results were given in mean \pm SD. Standard antibiotics such as gentamicin and tetracycline for pathogenic bacteria, bavistin and miconazole for pathogenic fungi served as positive controls (Bauer et al. 1966).

Table 1 Qualitative chemical analysis of test solvent extracts of *Toona ciliata* leaf and flower

Sl no.	Tests	Solvent extracts of <i>Toona ciliata</i>				
		Petroleum ether	Chloroform	Ethyl acetate	Methanol	Aqueous
I	Carbohydrates test					
	a) Molisch's test	+	+	+	+	+
	b) Fehling's test	+	+	+	+	+
II	Proteins & Aminoacids					
	a) Ninhydrin test	-	-	-	-	-
	b) Biuret test	-	+	+	-	-
	c) Sodium bicarbonate test	-	-	-	-	-
	d) Tannic acid test	-	-	-	-	-
	e) Xanthoprotein test	+	+	+	+	+
III	Alkaloids					
	a) Wagner's test	+	+	+	+	+
	b) Dragendorff's test	+	+	+	+	+
	c) Mayer's test	+	+	+	+	+
IV	Saponins					
	a) Foam test	-	-	-	-	+
V	Flavonoids					
	a) Ferric chloride test	-	-	+	+	+
	b) Shinoda test	-	+	+	+	+
	c) Alkali & acid test	+	-	+	-	-
VI	Tannins & phenolic compounds					
	a) Ferric chloride test	-	-	+	+	+
	b) Heavy metals test					
	i) Copper sulphate test	-	+	+	+	+
	ii) Potassium ferricyanide test	-	+	+	-	+
	iii) Nitric acid test	+	+	+	+	+
VII	Glycosides					
	a) Modified Borntrager's test	+	-	-	-	+
VIII	Phytosterols					
	a) Libermann- Burchard's test	+	+	+	+	+
	b) Terpenoids					
	i) 2,4-DNPH test	+	+	+	+	+
	c) Anthraquinones					
	i) Borntrager's test	+	+	+	+	+
	d) Steroids					
	i) Salkowski's test	+	+	+	+	+

Note: +: Present; -: Absent.

Microbroth dilution method

Minimal inhibition concentration was determined by 2,3,5- triphenyl tetrazolium chloride (TTC) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using microtitre ELISA plate for bacteria and fungi respectively (Sette et al. 2006; Buatong et al. 2011). The 96 wells were filled with Muller–Hinton broth and Sabouraud's broth medium containing

different concentration of solvent extracts, standard reference antibiotics such as gentamycin and miconazole against bacteria and dermatophytic fungi respectively. Antibacterial activity was detected by adding 0.5% TTC (Merck) aqueous solution and antifungal activity by adding 10 µl of a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution [5 mgml⁻¹ MTT in phosphate buffered saline (PBS), pH 7.4]. MIC was

Table 2 Antibacterial activity measured as zone of inhibition at 50 µl (100 mg/ml) of solvent extracts of *Toona ciliata* (leaf and flower) and standard antibiotics

SL no.	Pathogenic bacteria	Solvent extracts (50 µl) zone of inhibition in mm (MIC in mg/ml)				Antibiotics
		Petroleum ether	Chloroform	Ethyl acetate	Methanol	Gentamicin
1	<i>Bacillus subtilis</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	34.66 ± 0.57
		(ND)	(ND)	(ND)	(ND)	(0.156)
2	<i>Escherichia coli</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	25.33 ± 0.57
		(ND)	(ND)	(ND)	(ND)	(0.625)
3	<i>Klebsiella pneumoniae</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.00 ± 0.00	21.33 ± 1.15
		(ND)	(ND)	(ND)	(5)	(1.25)
4	<i>Listeria monocytogenes</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	25.00 ± 1.00
		(ND)	(ND)	(ND)	(ND)	(0.3125)
5	<i>Pseudomonas aeruginosa</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	25.00 ± 1.00
		(ND)	(ND)	(ND)	(ND)	(0.3125)
6	<i>Proteus mirabilis</i>	0.00 ± 0.00	0.00 ± 0.00	20.66 ± 0.57	15.33 ± 0.57	30.66 ± 0.57
		(ND)	(ND)	(2.5)	(2.5)	(0.156)
7	<i>Salmonella typhi</i>	0.00 ± 0.00	0.00 ± 0.00	10.00 ± 0.00	10.00 ± 0.00	30.00 ± 0.00
		(ND)	(ND)	(5)	(5)	(0.156)
8	<i>Staphylococcus aureus</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.66 ± 0.57	24.00 ± 1.00
		(ND)	(ND)	(ND)	(5)	(0.625)
9	<i>Staphylococcus epidermidis</i>	0.00 ± 0.00	0.00 ± 0.00	13.33 ± 0.57	14.00 ± 0.00	28.66 ± 1.15
		(ND)	(ND)	(5)	(5)	(0.156)
10	<i>Vibrio parahaemolyticus</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	25.33 ± 0.57
		(ND)	(ND)	(ND)	(ND)	(0.3125)
	Plant pathogens					Tetracycline
11	<i>Erwinia carotovora</i>	0.00 ± 0.00	0.00 ± 0.00	10.66 ± 0.57	11.66 ± 0.57	34.00 ± 1.00
		(ND)	(ND)	(5)	(5)	(0.156)
12	<i>Xanthomonas axonopodis</i> pv. <i>malvacearum</i>	0.00 ± 0.00	0.00 ± 0.00	15.33 ± 0.57	13.66 ± 0.57	30.33 ± 0.57
		(ND)	(ND)	(5)	(5)	(0.156)
13	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	0.00 ± 0.00	0.00 ± 0.00	12.33 ± 0.57	8.33 ± 0.57	30.33 ± 0.57
		(ND)	(ND)	(5)	(5)	(0.156)
14	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	0.00 ± 0.00	0.00 ± 0.00	10.66 ± 0.57	12.00 ± 0.00	31.00 ± 0.00
		(ND)	(ND)	(5)	(5)	(0.156)

Values are the mean of triplicates ± SD p < 0.05.
 ND Not Done.

defined as the lowest concentration of extract that inhibited visible growth, as indicated by the TTC and MTT staining (dead cells will not be stained).

DPPH radical scavenging activity

The antioxidant activity of *Toona ciliata* test solvent extracts was determined in terms of radical scavenging ability by DPPH method. Stock solution of 0.1 mM DPPH in methanol was diluted using methanol. 1.0 ml of solvent extracts solution of differing concentrations (50–250 µgml⁻¹) was added to 1.0 ml of DPPH and made volume up to 3 ml. A negative control (reaction

mixture without test extract) was also used in this test. The absorbance was measured at 517 nm after 30 min.

Inhibition was calculated by using the following equation:

$$\% \text{ inhibition} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

IC50 values were calculated as the concentration of each sample required to give 50% DPPH radical scavenging activity with respect to absorbance of blank from the graph. The results were compared with BHT. The experiment was performed in triplicates and values are expressed in ± SD (Zhang et al. 2010).

Table 3 Antifungal activity measured as zone of inhibition at 50 μ l (100 mg/ml) of solvent extracts of *Toona ciliata* (leaf and flower) and standard antibiotics

SL no.	Pathogenic fungi	Solvent extracts (50 μ l) zone of inhibition in mm(MIC in mg/ml)				Antibiotics
		Petroleum ether	Chloroform	Ethyl acetate	Methanol	Miconazole
1	<i>Candida albicans</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	13.66 \pm 0.57
		(ND)	(ND)	(ND)	(ND)	(ND)
2	<i>Microsporium gypseum</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	18.66 \pm 0.57
		(ND)	(ND)	(ND)	(ND)	(ND)
3	<i>Microsporium canis</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	16.66 \pm 0.57	18.00 \pm 0.00
		(ND)	(ND)	(ND)	(1.25)	(1.25)
Plant pathogens						Bavistin
4	<i>Aspergillus niger</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	18.00 \pm 0.00
		(ND)	(ND)	(ND)	(ND)	(ND)
5	<i>Aspergillus flavus</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	17.66 \pm 0.57
		(ND)	(ND)	(ND)	(ND)	(ND)
6	<i>Drechslera</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	15.00 \pm 0.00
		(ND)	(ND)	(ND)	(ND)	(ND)
7	<i>Fusarium verticillioides</i>	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	14.66 \pm 0.57
		(ND)	(ND)	(ND)	(ND)	(ND)

Values are the mean of triplicates \pm SD p < 0.05.
 ND Not Done.

ABTS assay

The antioxidant activity of *Toona ciliata* test solvent extracts was determined for radical scavenging ability by ABTS method (Adedapo et al. 2009). The stock solution was prepared by using 7 mM ABTS solution and 2.4 mM potassium per sulfate solution separately. The working solution was made by mixing the two stock solutions in equal quantities and allowed them to react for 12 h. at room temperature in dark condition. 1.0 ml of *Toona ciliata* test solvent extracts of differing concentrations (50–250 μ gml⁻¹) was added to 1.0 ml of ABTS solution and diluted with methanol. The absorbance was taken at

734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as

$$\text{ABTS radical scavenging activity (\%)} = \frac{[(A_{\text{control}} - A_{\text{sample}})]}{(A_{\text{control}})} \times 100$$

Where, A_{control} is the absorbance of ABTS radical + methanol; A_{sample} is the absorbance of ABTS radical + sample extract/standard. The experiment was performed in triplicates and values are expressed in \pm SD.

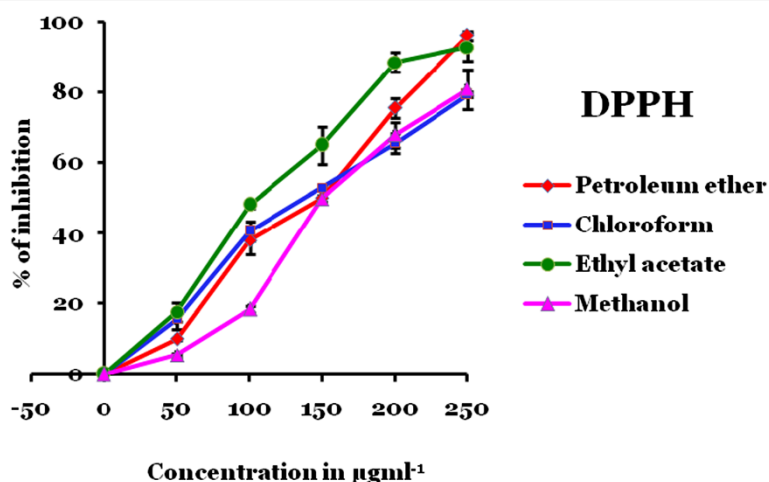


Figure 1 DPPH scavenging activity of test solvent extracts of *Toona ciliata*. Data expressed at p < 0.05.

Results and discussion

In the present study, preliminary chemical analysis of *Toona ciliata* leaf and flower extracts of different solvent extracts viz. petroleum ether, chloroform, ethyl acetate, methanol and water revealed the presence of carbohydrates, proteins, alkaloids and phytosterols (Table 1). Saponins was absent in all the extracts whereas flavonoids, tannins and phenolic compounds were found to be present in ethyl acetate and methanol extracts and glycosides was present in petroleum ether and aqueous extracts. Tables 2 and 3 shows the antibacterial and anti-fungal activities of test solvent extracts of *Toona ciliata* at 50 μl (100 mgml^{-1}) concentrations against human and phytopathogenic bacteria and fungi. Ethyl acetate extract showed moderate antibacterial activity against *Proteus mirabilis* and least activity against *Salmonella typhi* and *Staphylococcus epidermidis*. Similarly, methanol extract also showed moderate antibacterial activity against *Proteus mirabilis* and least activity against *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, whereas other test bacteria did not show any inhibition zone. Antibacterial activity against human and phytopathogenic bacteria was not observed with petroleum ether and chloroform extracts. Ethyl acetate and methanol extracts exhibited moderate activity against *Xanthomonas axonopodis* pv. *malvacearum* and least activity against *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas oryzae* pv. *oryzae* and *Erwinia carotovora* in comparison with the standard tetracycline. Petroleum ether, chloroform and ethyl acetate solvent extracts did not exhibited any zone of inhibition against *Aspergillus niger*, *Aspergillus flavus*, *Drechslera*, *Fusarium verticillioides*, *Candida albicans* and *Microsporium gypsum* whereas, methanol extract showed significant activity only against *Microsporium canis* compared to the standard miconazole. Ethyl acetate and

methanol extracts of *Toona ciliata* exhibited lowest MIC varied from 10–2.5 mgml^{-1} against *Proteus mirabilis*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Erwinia carotovora*, *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas axonopodis* pv. *malvacearum* wherein, standard antibiotics gentamicin and tetracycline showed MIC ranging between 0.625–0.156 mgml^{-1} and 0.156 mgml^{-1} against human pathogenic and phytopathogenic bacteria respectively. Methanolic extract of *Toona ciliata* showed MIC of 1.25 mgml^{-1} against *Microsporium canis*.

Antioxidant activity using DPPH and ABTS method is illustrated in the Figures 1 and 2. The test solvent extracts viz., petroleum ether, chloroform, ethyl acetate and methanol exhibited significant free radical scavenging activity wherein, as the concentration increases the percentage inhibition of free radical also increases. Petroleum ether, chloroform, ethyl acetate and methanol extracts of *Toona ciliata* showed DPPH significant activity with IC_{50} value of 150, 135.5, 105 and 92.5 μgml^{-1} whereas ABTS scavenging activity showed IC_{50} value of about 145, 120, 120.5 and 95 μgml^{-1} respectively. Standard BHT showed significant DPPH and ABTS scavenging activity with IC_{50} value of 8 μgml^{-1} and 11.5 μgml^{-1} respectively.

In the present study, methanol extract of *Toona ciliata* revealed the presence of carbohydrates, alkaloids, flavonoids, phytosterols, tannins and phenolic compounds which justifies the earlier findings of Gautam et al. (2010). Scientific literatures perused by far suggest antimicrobial activity against human pathogens whereas no reports confer the evaluation of *Toona ciliata* against phytopathogens which has been reported in the present investigation with ethyl acetate and methanol extracts of *Toona ciliata* against *Erwinia carotovora*, *Xanthomonas axonopodis* pv. *malvacearum*, *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas oryzae* pv. *oryzae* along

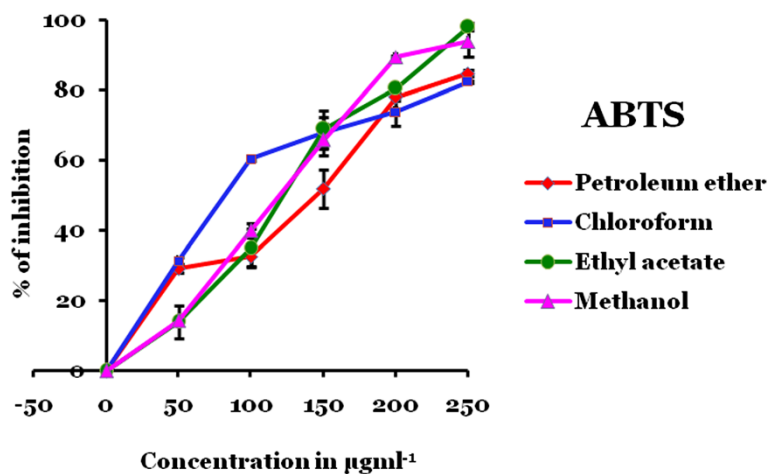


Figure 2 ABTS scavenging activity of test solvent extracts of *Toona ciliata*. Data expressed at $p < 0.05$.

human pathogen viz *Salmonella typhi*, *Staphylococcus epidermis* and *Klebsiella pneumoniae* suggests its antimicrobial potential which has also been studied previously against various pathogenic bacteria (Bibi et al. 2011; Kiladi 2012). Crude extracts upon evaluation of antioxidant activity using DPPH and ABTS method showed IC₅₀ value ranging from 100–150 µgml⁻¹ and 80–150 µgml⁻¹ respectively with different test solvent extracts. Earlier report of antioxidant compound has been well described with potent DPPH activity resulting IC₅₀ value 1.02 µgml⁻¹ (Ekaprasada et al. 2009).

Conclusions

The present investigation concludes that methanol extract of *Toona ciliata* exhibited maximum inhibition against test human and phytopathogens. Crude extracts displayed significant antioxidant activity, thus results obtained in the present investigation are promising enough for further isolation and characterization to reveal any novel metabolite of pharmaceutical importance.

Competing interests

Both authors declare that they have no competing interests.

Authors' contributions

KSK design the experiment, carried out the experiment, and contributed in framing the article. SS supervised the work. Both authors read and approved the final manuscript.

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