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DETERMINATION OF PHYTOCHEMICALS AND *IN VITRO* ANTIOXIDANT OF DIFFERENT EXTRACTS OF HIMALYAN CYPRESS (*CUPRESSUS TORULOSA* D.DON) NEEDLES

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ABSTRACT
The use of antioxidant in treatment of oxidative stress related pathologies is a possible therapeutic strategy for future. Natural product with antioxidant properties could trigger this goal. The aim of this *in vitro* study was to assess the antioxidant activity of four (hexane, chloroform, ethanol and aqueous) extracts of *Cupressus torulosa* D.Don. The phytochemical analysis of the extracts of the plant was determined using standard assay methods and the antioxidant activity was assessed using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), 2, 2′ [azinobis (3 ethyl benothiazoline- 6 sulphonic acid) di-ammonium salt] (ABTS) and superoxide scavenging activity assay (SSA). The polar extracts (ethanol and Aqueous) had more phytochemicals than non-polar extracts (hexane and chloroform). The scavenging activity was found dose dependent. Polar extracts has been found to have higher total phenolic content (for ethanol- 467.5±6.81 and for aqueous- 379.76±7.29 mg GAE/gm dry extract weight extract), total flavonoid content (for ethanol- 248.51±3.81 and for aqueous- 137.47±2.79 mg QE/gm dry extract weight of extract) as well as high antioxidant activity with IC_{50} value ranging from 16.61±2.18 µg/ml for aqueous extract in ABTS assay to 47.40±3.06µg/ml for aqueous extract for DPPH assay. Hexane and chloroform extracts showed weak antioxidant capacity. Results of this study showed that, polar extracts has maximum antioxidant property and may be utilized as a promising source of therapeutics.

KEYWORDS: antioxidant, phenolic and flavonoid content, radical scavenging activity, *Cupressus torulosa*.

INTRODUCTION

The Indian Himalaya has one of the richest biodiversity in the world and abounds in the plants of economic and medicinal importance which have great potential in global drug development but many Himalayan plant species are under-explored because of inadequate scientific knowledge of their medicinal potentials. Antioxidant property is major attribute for medicinal value of the plant and antioxidant compounds in food play an important role as a health protecting factor as these substances are responsible for the protection against unstable molecules known as free radicals by interacting and stabilize them. Compounds like phenolic acids, polyphenols and flavonoid scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Hamid *et al*., 2010). Plants are the rich source of natural antioxidant but many plant species are still underexplored for their antioxidant potential.

The Cypress family (Cupressaceae) is a large and diverse group including approximately 30 genera and 135 species. The genus *Cupressus* consists approximately 15-17 species distributed worldwide especially in the northern hemisphere, including western North America, Central America, Northwest Africa, Asia and Mexico (Allemand, 1979; Pierre-Leandri, 2003; Wang and Ran, 2014). *Cupressus torulosa* D. Don (Bhutan cypress or Himalayan cypress) is widely distributed throughout India, Nepal, Tibet, Pakistan and Bhutan at elevation of 1800–3300 m (Gupta, 1968; Polunin and Stainton, 1984). It is an evergreen tree that grows up to 30 to 40 m tall. The wood is useful as timber for buildings and the plant is burnt in temple for incense (Gupta, 1968). The essential oil of the needles is used to cure from whooping cough, rheumatism and used as an astringent (Sellappan *et al*., 2007). The needles are also rich in Biflavones, viz. amentoflavone, cupressuflavone, hinokiflavone, and apigenin, α-pinene, δ-3-carene, limonene and sabinene (Natarajan *et al*., 1970, Lohani *et al*., 2012; Padalia *et al*., 2013). Mono-, sesqui- and di-terpenes are found to be present in the foliage of C. torulosa (Cool *et al.*, 1998). Apart from chemical constituents, biological screening have shown antimicrobial activity of different extracts (hexane, chloroform, ethanol and methanol) and antioxidant activity of volatile oil of *C. torulosa* (Dhanabal *et al*., 2000; Joshi *et al*., 2014).

Keeping in view the importance of this Himalayan plant, the present study was designed to screen its phytochemical constituents, to evaluate the total phenoilc component (TPC), total flavonoid components (TFC) and also free radical scavenging activities (RSA) as there is no record available on antioxidant activity as per our broad literature survey. To the best of our knowledge, this is the first attempt presenting comprehensive data in antioxidant compound, antioxidant activity and phytochemical constituents for the *C. torulosa* needles.

MATERIALS & METHODS

Sample collection

Leaves (needles) of *C. torulosa* were collected during the month of March 2015, from Nainital, India and authenticated by Dr. Y. P. S. Pangtey, Department of

Botany of the university. A voucher specimen was deposited in the departmental herbarium.

Solvent Extraction of Plant Material

Plant material was thoroughly washed off under running tap water and then shade dried at room temperature for three-four weeks. The air-dried plant material was finely grinded and packed in self seal air tight polythene bags for further use. Exposure to sunlight was avoided to prevent the loss of active components (Thakare, 2004).

Fine powdered plant material was soaked in four different solvents (1:10 w/v) separately *i.e.* hexane, chloroform, ethanol and double distilled water for 48 hours in closed electrical shaker (120 rpm at 25ºC) and then filtered with Whatman's filter paper no.1. Only supernatant was taken and solvent was evaporated using vacuum evaporator at 40^0C and stored at 4ºC for further studies.

Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), quercetin, sodium nitrite ($NaNO_2$), ascorbic acid, Ferric chloride ($FeCl_3$), gallic acid, Potassium di-hydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), Potassium acetate (CH_3COOK), potassium per-sulfate ($K_2S_2O_8$), Ethylene diamine tetra acetic acid (EDTA), Riboflavin and Nitro-blue tetrazolium (NBT) were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Folin-Ciocalteu's reagent, Molisch's reagent, conc. H_2SO_4, Fehling's reagent, glacial acetic acid, conc. HCl, NH_4OH, Meyer's reagent (potassiomercuric iodide solution), 2,2 [azinobis (3ethyl benothiazoline- 6sulphonic acid) diammonium salt] (ABTS), hexane, chloroform, ethanol and. methanol were obtained from Merck, Mumbai, India. All chemicals used were of analytical grade.

PRELIMINARY PHYTOCHEMICAL SCREENING

To assess the chemical composition of the plant qualitatively, phytochemical analysis was conducted using hexane (CTH), chloroform (CTC), ethanol (CTE) and aqueous (CTA) extracts following Harborne, 1998.

QUANTITATIVE PHYTOCHEMICAL ASSAYS

Determination of Total Phenolic Content (TPC)

The total phenolic content of the sample extract was determined by Folin-Ciocalteu's colorimetric method given by Singleton and Rossi (1965) with certain modifications. 10µl of sample extract was taken and make it up to 100µl with solvent. 450µl of distilled water and 50µl of 10% Folin- Ciocalteu's reagent were added further and allowed to stand for 5min. This mixture was then neutralized by adding 500µl of 7 % (w/v) sodium carbonate and kept at room temperature in dark for 90 minutes. The resulting blue colored solution was measured spectrophotometrically (UV-VIS) at 765nm. Quantification of total phenolic content was based on standard curve of Gallic acid. The results were expressed in mg gallic acid equivalent (GAE)/gm dry extract weight of the sample.

Determination of Total Flavonoid Content (TFC)

Content of flavonoids of the sample extract were determined by $AlCl_3$ colorimetric method given by Chang *et al*, 2002 with certain modifications. 10µl of sample extract was taken and make it up to 100µl with solvent. 200µl distilled water was added further; 100µl of 10 % (w/v) $AlCl_3$ was added followed by the addition of (20µl) 1M potassium acetate and 500µl of distilled water. Then the reaction mixture was incubated at room temperature for 30 minutes. Thereafter the absorbance was recorded at 415 nm using UV-VIS spectrophotometer. Quantification of total flavonoid content was done on the basis of standard curve of Quercetin. Results were expressed in mg quercetin equivalent (QE)/gm dry extract weight of the sample.

DETERMINATION OF ANTIOXIDANT ACTIVITY

The antioxidant potential of *C. torulosa* was evaluated by three different methods, DPPH (1,1-diphenyl 2-picrylhydrazyl), ABTS (2,2' [azinobis (3 ethyl benothiazoline- 6 sulphonic acid), and SSA (Superoxide scavenging activity).

DPPH Antioxidant Activity Assay

The DPPH assay was done according to the method of Brand-Williams *et al*. (1995) with certain modifications.100 µM DPPH (0.002gm) was added to absolute methanol (50ml) to generate DPPH cations (DPPH*). To different concentration of samples (1 to 50µg/ml), 400µl DPPH* was added and final volume maintained to 1 ml with double distilled water. Then the content was shacked for few seconds and kept in dark for 20 minutes at room temperature. The reduction in absorbance was recorded at 517 nm in UV-VIS spectrophotometer. Ascorbic acid (AA), Butylated hydroxyl anisole (BHA) and Butylated hydroxyl toluene (BHT) was used as standard and for control absorbance of DPPH cations was taken without adding sample extract.

ABTS Antioxidant Activity Assay

The ABTS (2,2' -Azinobis-3-ethylbenzotiazoline-6-sulphonic acid) assay was conducted according to Miller *et al*. (1993) and Re *et al*. (1999) with minor modifications. $ABTS^+$ cation was generated through the interaction of 19.2 mg of 7 mM ABTS dissolved in 5 ml of double distilled water and 88 µL of 0.0378 g/ml, 2.45 mM potassium per-sulfate ($K_2S_2O_8$) and again add 5 ml double distilled water. The ABTS* was incubated in the dark at room temperature for 16 h. The ABTS activated radical was diluted with ethanol to an absorbance of 0.70 ± 0.025 at 734 nm. Because ABTS and potassium per-sulfate react stoichiometrically at a ratio of 1**:**0.5, this will result in incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical was stable in this form for more than two days when stored in the dark at room temperature. The photometric assay was conducted by adding 900 µl $ABTS^+$ solution to different concentration (1µl to 50 µg/ml) of tested samples and mixed for 30 seconds; measurements were taken immediately after 15 minutes incubation in dark. The antioxidant activity of tested sample was calculated by determining the decrease in the absorbance at different concentrations.

Superoxide scavenging assay (SSA)

Superoxide scavenging assay was performed by method given by Gülçin (2009) with some modifications**.** Different concentrations of sample extract (1 to 50µg/ml) were diluted with phosphate buffer saline to make up the volume to 100µl, to this 100µl of each riboflavin, EDTA, and absolute methanol were added by keeping the reaction

mixture in dark. This step was followed by vigorous shaking and addition of NBT (50µl) and 500µl of phosphate buffer saline. The reaction mixture was allowed to stand for 20 min in fluorescent light. Absorbance was recorded at 590 nm. Ascorbic acid, BHA & BHT was used as standard and phosphate buffer saline as blank. For control absorbance of reagent was taken without adding sample extract.

% scavenging /Inhibition for DPPH, ABTS and SSA were calculated as:

$$\% \, scavenging = \frac{absorbance \ of \ control - absorbance \ of \ test \ sample}{absorbance \ of \ control} X100$$

Statistical Analysis

All the measurements were taken in triplicates and the results obtained were expressed as mean ± standard error (SE). The results were further analyzed by ANOVA (analysis of variance) and Duncan test using SPSS 20.0 software. At $P<0.05$ the values were considered to be significant. To calculate the IC_{50} values probit analysis was used. IC_{50} concentrations for all three assays were calculated by probit analysis using SPSS version 20 in order to obtain linearity between log concentration and percentage inhibition of free radical. Correlations were obtained by Pearson correlation coefficient in bivariate correlations.

RESULTS

Phytochemical screening of plant material

The preliminary phytochemical investigation of *C. torulosa* was conducted using four different extracts i.e. hexane, chloroform, ethanol and aqueous. Results showed the presence of alkaloids, phenols, flavonoids, glycosides, tannins, saponin, resin, volatile oils, quinones, glycosides, proteins and carbohydrates in different extracts. Resin, saponin and terpenoids were present in all four extracts used whereas positive tests for alkaloids and gallotannins were obtained only in ethanolic extract (Table 1).

TABLE 1: Phytochemical evaluation of *C. torulosa* needles extracts

S. No	Tests Conducted for Phytoconstituents	Solvent extracts			
		Hexane	Chloroform	Ethanol	Aqueous
1.	Alkaloids (Mayer and Wagner test)	+	-	+	-
2.	Carbohydrates	-	+	+	+
3.	Proteins	-	-	+	+
4.	Flavonoids	-	-	+	+
5.	Phenols (Ferric chloride test)	-	+	+	+
6.	Tannin (Ferric chloride test)	-	+	+	+
7.	Gallo-tannin (Ferric chloride test)	-	-	+	-
8.	Resin (Tubidity test)	+	+	+	+
9.	Saponin (Foam test)	+	+	+	+
10.	Quinones	-	-	+	+
11.	Volatile oils	+	-	+	-
12.	Glycosides (Keller-Kiliani test)	+	+	-	+
13.	Terpenoids (Salkowski's test)	+	+	+	+
	Total	6	7	12	10

+ = present; - = absent

QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Determination of Total Phenolic Content

Total phenolic content of different samples were calculated from the regression equation of calibration curve for Gallic acid (y=0.028x-0.044, R^2 = 0.995) expressed as mg Gallic acid equivalents (GAE) per gram of sample in dry extract weight. Results revealed that the phenolic content was highest in the ethanolic extract (467.5±6.81 mg GAE/gm dry extract weight) followed by aqueous extract (379.76.50±7.29 mg GAE /gm of dry extract weight). Lowest value of phenolic content was observed in hexane extract i.e. 227.86±10.39mg GAE / gm of dry extract weight (Table 2).

Determination of Total Flavonoids Contents

Total flavonoids content of the sample extracts was calculated from the regression equation of calibration curve of Quercetin (y = 0.029x + 0.003; R^2 = 0.989) and expressed as mg Quercetin equivalents (QE) / gm dry extract weight. Total flavonoid in needles of *C. torulosa* ranged from 51.82±7.40 mg in hexane to 248.50±3.81 mg in ethanol QE/gm of dry extract weight in different extracts (Table 2).

TABLE 2 - Total phenolic and total flavonoid content in different extracts of *C. torulosa* needles

S.N.	Extracts	TPC (mg GAE/gm dry extract weight)	TFC (mg QE/gm of dry weight)
1	Hexane	227.86±10.39	51.82±7.40
2	Chloroform	278.93±4.54	96.23±6.43
3	Ethanol	467.5±6.81	248.51±3.81
4	Aqueous	379.76±7.29	137.47±2.79

Note- Values are mean±SE of three independent observations, each in triplicate.

DETERMINATION OF ANTIOXIDANT ACTIVITY

DPPH free radical scavenging activity

DPPH free radical scavenging activity of plant extracts was analyzed using different concentrations (1-50 µg/ml).At higher concentration (50 µg/ml), ethanol extract showed maximum inhibition (67%; IC_{50}- 37.86±3.82 µg/ml) whereas hexane extract showed minimum scavenging (34%, IC_{50}- 78.24±6.23 µg/ml). At the same concentration used. At the same concentration standard antioxidant used (AA, BHA, BHT) showed 72.98%, 79.05% and 68.57% inhibition respectively (Fig. 1 and Table 3).

TABLE 3: Free radical scavenging activity- Comparison of % inhibition and IC_{50} of DPPH, ABTS & SSA

S.N.	Extract	DPPH		ABTS		SSA	
		% inhibition at 50 µg/ml	IC_{50} (µg/ml)	% inhibition at 50 µg/ml	IC_{50} (µg/ml)	% inhibition at 50 µg/ml	IC_{50} (µg/ml)
1.	AA	72.98	6.42±0.61	99.12	9.08±2.12	93.10	12.38±3.01
2.	BHA	79.05	3.48±0.74	96.64	12.77±0.83	96.30	5.87±0.96
3.	BHT	68.57	8.40±1.31	98.94	6.05±0.92	83.00	11.01±2.17
4.	CTH	34.93	78.24±6.23	61.82	35.52±1.09	51.68	53.59±3.49
5.	CTC	45.60	51.44±4.06	53.14	63.63±2.78	60.06	38.07±1.39
6.	CTE	57.10	37.86±3.82	77.45	19.79±2.10	80.91	19.16±2.15
7.	CTA	51.43	47.40±3.06	83.83	16.61±2.18	76.88	25.85±1.65

AA- Ascorbic acid, BHA- Butylated hydroxyl anisole, BHT- Butylated hydroxyl toluene, CTH- *C. torulosa* Hexane extract, CTC- *C. torulosa* chloroform, CTE- *C. torulosa* ethanol extract, CTA- *C. torulosa* aqueous extract.

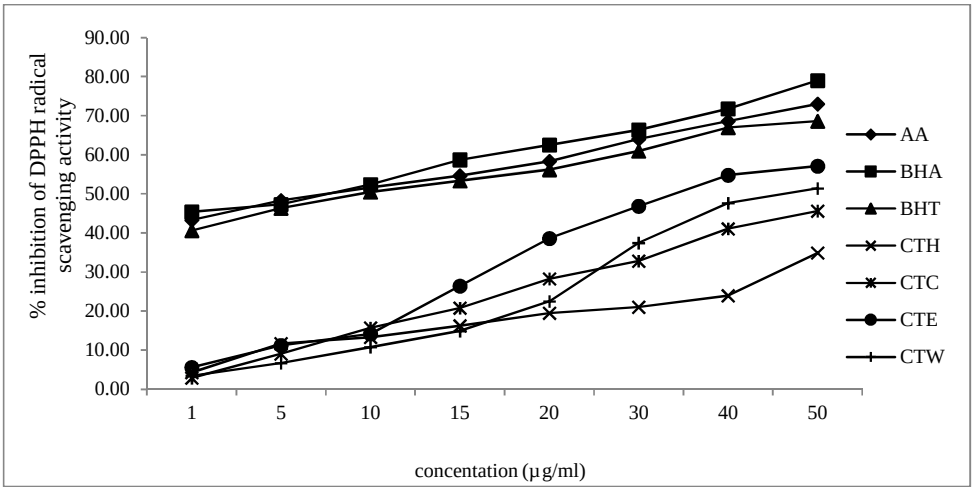

FIGURE1: DPPH radical scavenging potential of different extracts of *C. torulosa*

ABTS free radical scavenging assay

The ABTS scavenging activity of different extracts was measured using three different standards (AA, BHA, and BHT). Highest ABTS scavenging was recorded in aqueous extract (83% inhibition; IC_{50}-16.61±2.18 µg/ml) followed by ethanol (77 % inhibition; IC_{50}-19.79±2.10 µg/ml), hexane (61 % inhibition; IC_{50}-35.52±1.09 µg/ml) and chloroform (53 % inhibition, IC_{50}- 63.63±2.78 µg/ml) . Low IC_{50} value of aqueous extract was found comparable to standards used (Fig. 2 and Table 3).

Superoxide scavenging activity (SSA)

Results for %inhibition and IC_{50} value of the superoxide scavenging activity for different extracts were found in the order of 19.16±2.15 (CTE)< 25.85±1.65 (CTW)< 38.07±1.39 (CTC)< 53.59±3.49 (CTH) µg/ml whereas among different standards used, BHA stands as best superoxide scavenger (Fig. 3 and Table 3).

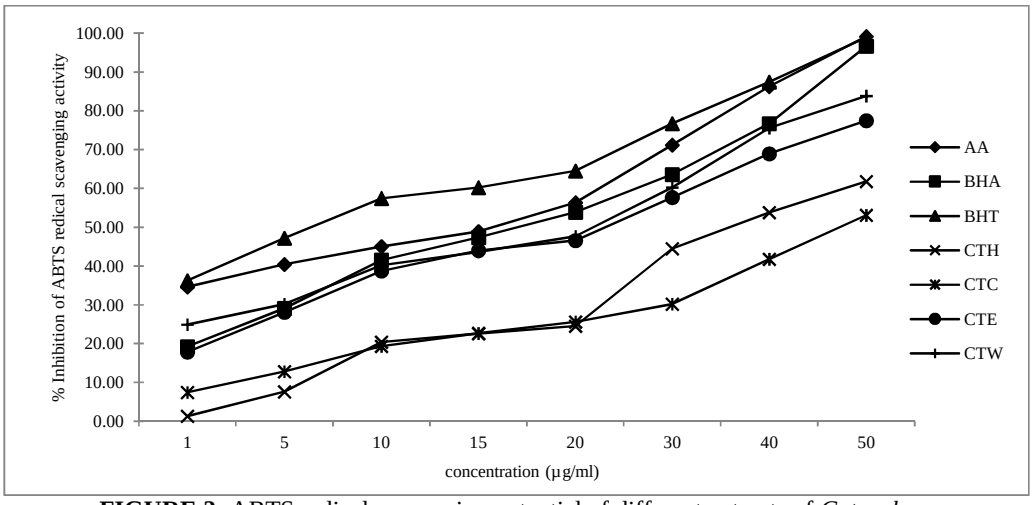

FIGURE 2- ABTS radical scavenging potential of different extracts of *C. torulosa*

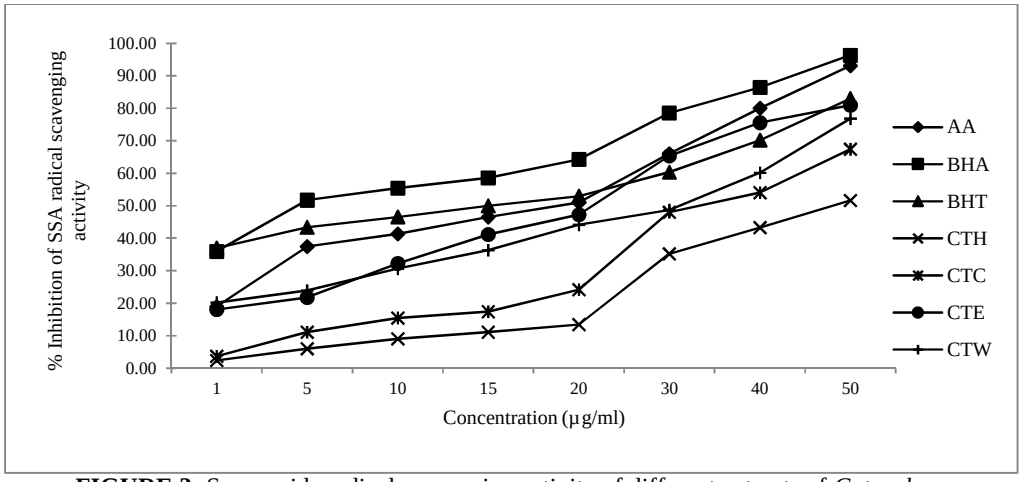

FIGURE 3: Superoxide radical scavenging activity of different extracts of *C. torulosa*

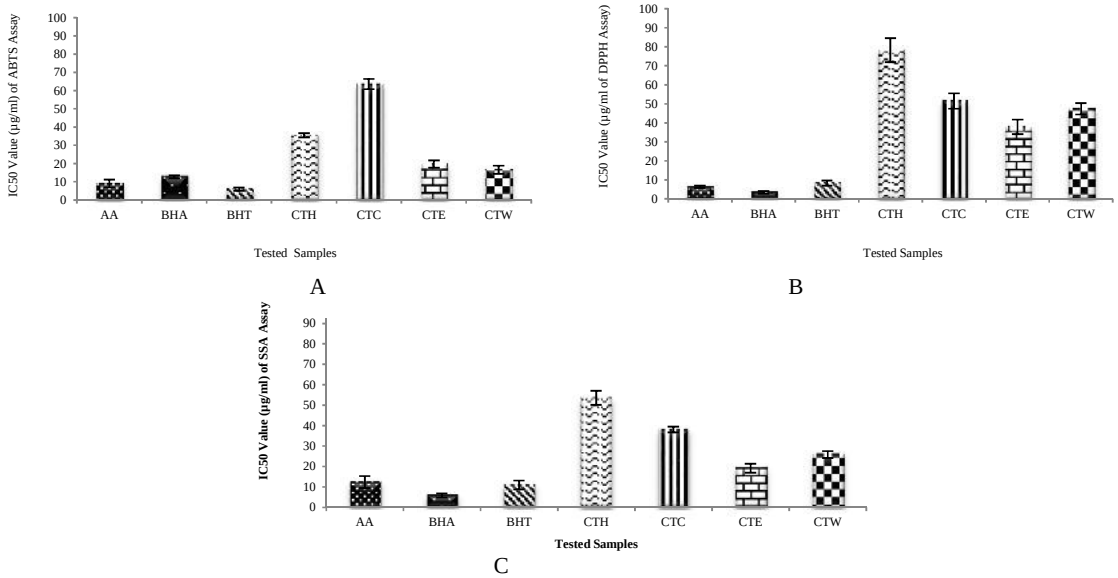

FIGURE 4- Comparison of IC_{50} values for DPPH, ABTS and SSA radical scavenging potential of different extracts of *C. torulosa* with standards.

DISCUSSION

In addition to primary metabolites, plant utilizes different pathways synthesizing various secondary metabolites such as phenols, flavonoids, alkaloids, tannins, glycosides *etc*. which are responsible for various antioxidant properties. The present study is the first attempt to report antioxidant potential of the studied plant by determining the total phenolic content, total flavonoid content of *C. torulosa* as well as its free radical scavenging potential using ABTS, DPPH and SSA assays. To evaluate the various phytochemical and antioxidant potential of the plant, sequential extraction using four different solvents was done. Antioxidant compounds may be soluble in polar solvents or non-polar solvent as well, that's why for the quantification of broader antioxidant activity proper extraction procedure plays an important role as noticed in this study. This observation has also been reported earlier by Arabshahi-Delouee and Urooj (2007). Out of 52 tests performed 67% tests showed positive results for different phytochemicals in which resin, saponin and terpenoids were present in all the extracts suggesting their high content in plant samples, signifying the importance of polar solvents as better extractants. While working on other species of *Cupressus* i.e. *C. macrocarpa*, alkaloids were reported absent by Thukral *et al*. (2014), but in present investigation alkaloids were present in hexane and ethanol extracts.

Various phyto-constituents have been reported to have bioactive properties through *in vitro* studies (Sodipo *et al.*, 2000; Aguinaldo *et al*., 2005; Doughari, 2009). The phenolic compounds have attracted much interest due to their potential as antioxidant as they protect plants and humans from oxidative damage (Duthie *et al*., 1997; Skaper *et al*., 1997). Other important groups of compounds are flavonoids which are a widely distributed group of polyphenolic compounds with health protecting prospects, mainly based on their antioxidant activity (Benavente-Garcia *et al*., 1997). These are referred as a glycones in plants and are extracted with solvents based on polarity.

A mixture of different antioxidant can attribute to the antioxidant capacity of plant; therefore, the antioxidant capacity of plant must be evaluated using various methods which can address the different mechanisms. To this effect, three antioxidant systems have been selected in the present work, which are based on measurement of color degradation. The ABTS method generally indicative for the antioxidant activity of hydrophilic compound (Rufino *et al*., 2010), DPPH method is commonly used for aqueous/ organic extracts with hydrophilic compounds while SSA method analyses the protective capacity of chemical agent against highly reactive superoxide radical (Janknegt *et al*., 2007). When the DPPH assay was used, higher antioxidant activity corresponded to ethanol extract (IC_{50}- 37.86±3.82 µg/ml) and aqueous extract (IC_{50}- 47.40±3.06 µg/ml) in comparison with hexane and chloroform extract. Similar trend for SSA and ABTS was observed with no significant difference (Fig. 4 A,B and C). These results are superior to those reported for other gymnosperms by many workers (Gupta *et* al., 2011; Souza *et al*., 2014; Kumar *et al*., 2014; Gautam *et al*., 2014).

Statistically ethanol extract showed a strong correlation between DPPH and ABTS free radical scavenging activity and total phenolic content which was found highest (57 %, 77% inhibition and 467.5±6.81 mg GAE/gm dry extract weight respectively) among all extracts used. Phenolic compounds possess a high potential to scavenge free radicals can be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups (Sawa *et al*., 1999). The extracts could have reducing power to donate electron and according to Yen and Wu (1993) this ability serves as a significant indicator of potential antioxidant activity. Some authors demonstrated that antioxidant activity was not solely due to the phenolic content but due to the combined effect of different phytochemical (Ho *et al.*, 2012; Wong *et al.*, 2006). In present study solvent with different polarity had significant effect on the number of phytochemicals present, total phenolic as well as total flavonoid content and the trend indicated relatively higher number of phytochemicals and greater yield of total phenolic and total flavonoid in polar solvents. Further, positive correlation observed between antioxidant activity and phenolic and flavonoid content is might be due to the scavenging activity of different compounds such as flavonoids, phenols and other phytochemicals acting singly or synergistically. The high antioxidant acivity of polar extracts in the present investigation supports a liner correlation between total phenolic and flavonoid content and their antioxidant capacity as observed in earlier studies (Wu *et al*., 2004; Cai *et al*., 2004; Katsube,2004; Djeridane *et al*., 2006; Katalinic *et al*., 2006; Wojdylo *et al*., 2007; Asadi *et al*., 2010). These finding may be explained on the basis of possible synergism between phenolic compound and other phytochemical compounds released in the polar solvents.

CONCLUSION

In conclusion, the results of these screening investigations confirms the great potential of the tested plant for the production of bioactive compounds and is useful for rationalizing the use of medicinal plants in primary health care. Further study will be aimed isolating and identifying the substances responsible for the biological activity of these plant extracts, which may be further exploited in herbal formulations.

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Conflicts of Interest

We declared that we have no conflict of interest.

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