Original Research Article

Antioxidant, antimicrobial and phytochemical investigations of cone of *Thuja orientalis*

ABSTRACT

Background of the study~~Aims~~: *Thuja orientalis* L. is an indigenous medicinal plant belonging to family Cupressaceae found in Darjeeling Himalayan region, Dooars and Tarai region of India. The plant cone has been used traditionally as medicine to treat various diseases, like bronchitis, bacterial skin infection, osteoarthritis, trigeminal neuralgia.

Aim: The aim of the present study is to evaluate the plant cone for phytochemical constinuents, and in vitro antioxidant, and antimicrobial properties.

Place and Duration of Study: All the experiments were done in the Department of Biotechnology, University of North Bengal, Darjeeling, West Bengal, 734013, India.

Methodology: Methanolic extract of *T. orientalis* cone was analyzed for phytochemicals by various biochemical methods. Antioxidant properties were analyzed by in vitro assays of DPPH, ABTS, NO and H_2O_2 scavenging. Antibacterial property was analyzed by agar well diffusion method and antifungal assay was monitored by radial growth bioassay.

Results: Methanolic extract of *T. orientalis* cone contained flavonoid, phenol, saponin, tannin, terpenoid and alkaloid. The extract showed significant in-vitro antioxidant and antimicrobial activity.

Conclusions: The study revealed that *T. orientalis* cone has potential as source of potent antioxidant, antibacterial and antifungal agents. Our further study is directed towards the isolation, and characterization of active compound from methanolic extract and evaluation of it's antidiabetic potential.

Comment [u1]:

Keywords: Thuja orientalis; 2, 2-diphenyl-1-picrylhydrazyl; 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

1. INTRODUCTION

Natural products (NPs) withhigh chemical diversity and biochemical specificity represent the best enduring approach to drug discovery. Plant based NPs have been the basis of traditional medicine system and World Health Organization (WHO) estimated that about 80-85% of population relies on traditional medicine for their primary health care and the major part of traditional therapy involves the use of plant extracts or their active principles [1]. Traditional medicine and ethnobotanical information have played an important role in scientific research on drug development [2]. The underlying reason for NPs as sources of such a large proportion of existing drugs might be the similar interaction of natural products with biosynthetic enzymes and therapeutic targets [3].

T. orientalis (synonym *Platycladus orientalis*), an evergreen coniferous tree belonging to the family cupressaceae is native to northeastern parts of East and North Asia. It is a traditional medicinal plant used by ethnic people of Eastern Himalayan region. It has been used for different properties like antipyretic, antiussive, astringent, diuretic, refrigerant and stomachic [4]. Flavonoids and terpenoids of this plant showed various biological activities [5]. Most of the previous research on *T. Orientalis* is based on therapeutic efficacy of its leaves; however, less information is available about its cone. Present work describes the phytochemical screening, and antioxidant, antibacterial and antifungal properties of *T. orientalis*.

2. MATERIALS AND METHODS

2.1. Plant material and chemical

T. orientalis cones were collected from University of North Bengal campus, Darjeeling district and a specimen copy submitted in the Department of Botany, University of North Bengal (Accession number: NBU-11836). All the chemicals of analytical grade were purchased from Sigma-Aldrich India Limited, Hi Media, India and E. Merck, India.

2.2. Preparation of plant extract

The cones were washed, dried properly in shade area and then crushed into fine powder. Methanolic extract of the cone was prepared by using soxhlet extraction method. 30g of sample was packed and extracted with 250 ml of methanol for 8 h. The extract was concentrated in a rotary evaporator under reduced pressure. It gave total yield of 30.26% which was calculated by the following formula: Yield percentage = (Weight of extract obtained)/ (Total weight of sample loaded) × 100.

2.3. Qualitative Phytochemical analysis

The methanolic extract of *T. orientalis* cone (THU-C) was screened for various phytochemical like flavonoids, phenols, saponins, tannins, cardiac glycosides, terpenoids, and alkaloids. For screening of flavanoid, mixture of 1g extract and 5ml of acetone was placed in hot water bath. The precipitate obtained was then mixed with 5ml warm D/W, filtered and kept at room temperature (RT).Thereafter 1ml of filtrate was mixed with 1ml of 20% NaOH and the presence of flavonoid was confirmed by the appearance of yellow colour [6]. For screening of phenol, mixture of 100mg of extract and 5 ml D/W was filtered through Whatman No.1 filter paper. Then 1ml filtrate mixed with 1ml of 1% $FeCl_3$. Presence of phenol was confirmed by appearance of blue or green colour [7]. For screening of saponin, 5ml D/W was added to 100mg of powdered extract and the mixture was filtered.To 0.5 ml of filtrate 5ml D/W was added followed by vigorous shaking for half minute. Persistent froth formation indicated the presence of saponins [8]. For screening of tannin, 2ml of previous filtrate was mixed with 1ml of 5% $FeCl_3$. The yellow brown coloured precipitate formation indicated the presence of tannin [9].For screening of cardiac glycoside, a mixture of 1ml methanolic filtrate of extract, 0.5ml glacial acetic acid, few drops of 5% $FeCl_3$ and 0.5ml of conc. H_2SO_4 was prepared; appearance of brown ring proved the presence of cardiac glycosides [10].For terpenoid screening a mixture of 1ml chloroform, 1ml methanolic extract filtrate, 1 ml of acetic anhydride and 0.5ml conc. H_2SO_4were mixed. The presence of terpenoid was confirmed if the mixture produced reddish brown color [11]. For screening of alkaloid, 2 ml of 1% HCl added to 2ml of methanolic filtrate of extract, kept on steam for few minutes. Then 1ml of mixture reacted with few drops of Mayer's reagent, appearance of red or orange precipitate confirmed the presence of alkaloid [12].

2.4. Antioxidant activity

2.4.1. DPPH scavenging activity

Free radical scavenging activity of THU-C extract was determined by its ability to reduce stable DPPH (2, 2- diphenyl-1-picrylhydrazyl). 0.1 ml of plant extract of different concentrations was mixed with 3ml of methanolic solution of DPPH and after 30 min absorbance was monitored at 517 nm. Percentage inhibition of activity was calculated as follows: Inhibition (%) = $[(A_0 – A_1)/ A_0] \times 100$; where A_0 = Absorbance of control, A_1 = Absorbance of standard/extract. L-Ascorbic acid was taken as the reference standard [13].

2.4.2. H_2O_2 scavenging activity

H_2O_2 scavenging activity was determined by the method of Keser *et al*. (2012). 2mM solution of H_2O_2 was prepared in phosphate buffer (pH 7.4). Plant extract at various concentrations was mixed with 0.6 ml of H_20_2 solution. After 10 min absorbance was determined at 230 nm against blank phosphate buffer without H_2O_2. Percentage inhibition of activity was calculated as follows: Inhibition (%) = $[(A_0 – A_1)/ A_0] \times 100$; where A_0 = Absorbance of control, A_1 = Absorbance of standard/extract. L-Ascorbic acid was taken as the reference standard [14].

2.4.3. Nitric oxide (NO) scavenging activity

NO generated by sodium nitroprusside reacts with oxygen to produce nitrite ions which is estimated by Greiss reagent [15]. Nitric oxide scavengers compete with oxygen. For determining the NO scavenging 10 mM sodium nitroprusside in PBS was mixed with varying concentrations of THU-C extract and the reaction mixture was incubated at room temperature for 150 min. Thereafter, 0.5 ml of Griess reagent was added and optical absorbance was recorded at 546 nm. Percentage inhibition of activity was calculated by using the formula, Inhibition (%) = $[(A_0 – A_1)/ A_0] \times 100$; where A_0 = Absorbance of control, A_1 = Absorbance of standard/extract. L- Ascorbic acid served as the reference standard.

2.3.4. ABTS scavenging activity

For this assay the stock solution of ABTS prepared by mixing 7mM ABTS **(**2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)and 2.45 mM potassium per sulfate in equal ratio followed by incubation at RT for 12-16 h under dark condition. Stock solution was mixed with 80% methanol to prepare working solution. Now THU-C extract in different concentrations in methanol was mixed with I ml of ABTS working solution and the reaction was incubated at RT for 7 min. in dark condition and absorbance was measured at 734 nm. ABTS working solution without plant extract served as blank. Percentage inhibition of activity was calculated by the formula, Inhibition (%) = $[(A_0 – A_1)/ A_0] \times 100$; where A_0 = Absorbance of control, A_1 = Absorbance of standard/extract. Gallic acid and L- Ascorbic acid was taken as the reference standard [16].

2.5. Determination of antibacterial activity

The antibacterial property of THU-C extract was determined by agar well diffusion method using nutrient agar (NA) plate [17]. Each bacterial culture was grown overnight in Nutrient Broth at 37°C in

a shaking incubator. After that 100µl of bacterial culture was swabbed into NA plates and wells were prepared by the help of sterile 6mm cork borer. Then 20µl plant extract of different concentrations (10 – 80 mg/ml) was added into each well and incubated the plates for 24 h at 37°C. Thereafter, zone of inhibition was measured. Ampicillin (2µg/ml), tetracycline (30 µg/ml), penicillin (2 µg/ml) were used as positive controls. The activity of extract was determined by Activity Index (AI) which is zone of inhibition obtained for extract/ zone of inhibition obtained for standard antibiotic.

2.6. Determination of antifungal activity by radial growth bioassay

In this method the effect of antifungal substances on the radial growth of fungus was monitored in the specific media [18]. The THU-C extract was mixed with sterile potato dextrose agar (PDA) to afinal concentration of 100 mg/ml and the mixture was poured into sterile petriplates. After solidifying the PDA medium, wells were prepared with the help of sterile 6mm cork borer and then 7 days oldfungal culture was inoculated aseptically into the wells. The plates were incubated at 25°C for 7 days and radial growth of the fungus was measured. Methanol (50%) and griseofulvin (1mg/ml) were used as negative and positive control, respectively. Radial growth inhibition was calculated by the following formula: (C – T/C) × 100; where C and T are the growth diameter (mm) in both positive; negative control, and treatment, respectively.

3. RESULTS AND DISCUSSIONS

T. *orientalis* is used to treat bronchial catarrh, enuresis, cystitis, psoriasis, uterine carcinomas, amenorrhea, rheumatism, asthma, skin infections, mumps, bacterial dysentery, arthritic pains, and premature blandness, among other conditions. Biological activity such as hair growth promotion, antiviral, antiallergic, anti-epileptic, anti-inflammatory, antibacterial, antioxidant, and antifungal effects have been observed in various portions of the plant. It can also be employed as a nematicidal, insecticidal, and molluscicidal activity against a variety of pests. [19]. Most of the studies on therapeutic efficiency of *T. orientalis* have been carried out using the plant leaf extract [21]. However, the plant's cone has received little attention in terms of phytoconstituent identification and biological activities. Hence in the present study, the phytoconstituents, antibacterial, and antioxidant properties of a methanolic extract of *T. orientalis* cone were investigated. The methanol extract of cone revealed the presence of terpenoids, alkaloids, tannins, flavanoids, saponin, and phenols, which are known to have antioxidant, antibacterial, and antidiabetic properties.

3.1. Qualitative phytochemical analysis:

In a preliminary experiment the methanolic extract of *T. orientalis* cone (THU-C extract) was analyzed for the presence of various classes of phytochemicals. From the result in Table 1 it is evident that the extract contained the phytochemicals, like flavonoids, tannins, phenols, saponins, terpenoids and alkaloids, whereas cardiac glycoside was altogether absent. The research work by Jasuja *et al.* [21] reported the presence of alkaloids, flavonoids, carbohydrates, glycoside, phenolic compound and tannins, saponins fixed oils and fat in the plant leaf extract.

Table 1: Qualitative phytochemical screening of

***T. orientalis* cone extract**

Phytochemical	Results
Flavonoid	+
Phenol	+
Saponin	+
Tannin	+
Cardiac Glycoside	-
Terpenoid	+
Alkaloid	+

'+' denotes presence of phytochicals, '-' denotes absence of phytochemical

3.2. Antioxidant activities:

Phytochemicals are thought to provide many health benefits by neutralizing free radicals linked to a variety of ailments. They either donate hydrogen or quench singlet oxygen to neutralize the free radical. *Thuja orientalis* cone extract demonstrated antioxidant activity, scavenging DPPH more effectively than H_2O_2, NO, and ABTS scavenging activity.

THU-C extract was assessed for antioxidant activities by methods based on various working principles. It showed concentration dependent increase in DPPH free radical scavenging activity, however, its capacity to scavenge was lower than ascorbic acid, the standard antioxidant. The IC50 value of ascorbic acid and THU-C extract for DPPH scavenging was 6 and 20 µg/ml, respectively. The THU-C extract exhibited H_2O_2 scavenging capacity with IC50 value of 77 µg/ml, which was significantly higher than that of ascorbic acid (43 µg/ml). The IC50 value of extract and ascorbic acid for scavenging of NO was 115 and 61 µg/ml, respectively. The IC50 of THU-C extract for ABTS free radical scavenging was 144 µg/ml, which was comparable to that of ascorbic acid (IC50 131µg/ml)and was more than threefold higher than that of galic acid standard (41 µg/ml) **(Figure 1).**In an earlier report, the 70% methanolic leaf extract of *T. orientalis* displayed 85.25% DPPH scavenging activity at a dose of 100µg ml^{-1} in a previous study on [21].Using the same dose of cone extract we obtained approximately same DPPH scavenging activity (82%) at 100µg ml^{-1} of methanolic cone extract. The data thus show that cones and leaves have equivalent antioxidant potential. A relatively lower antioxidant potential of the THU-C extract in comparison to the standards (gallic acid and ascorbic acid) could be due the lower antioxidant content of the extract.

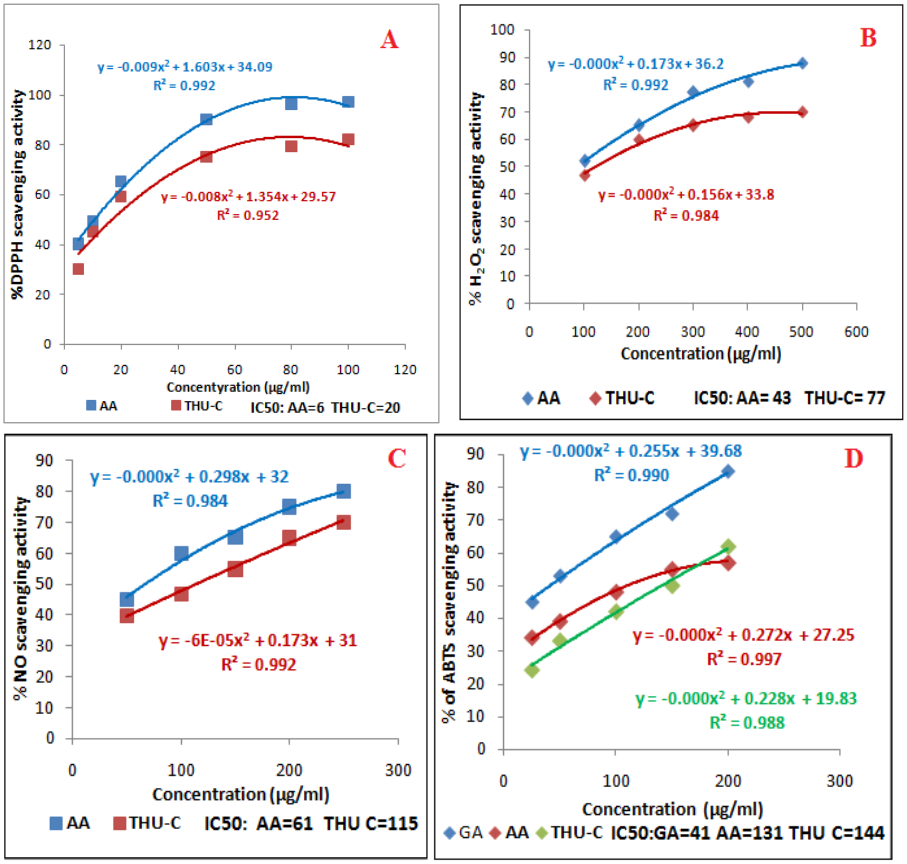

Figure 1: Antioxidant activity of methanolic extract of cone of *T.orientalis* cone. (A) DPPH Scanenging activity, (B)H_2O_2 scavenging activity, (C)NO scavenging activity, (D) ABTS scavenging activity. AA: Ascorbic acid (standard), GA: Galic acid (standard), THU-C: methanolic extract of *Thuja Orientalis* cone. Data represent the mean of three replicates.

3.3. Antibacterial effect:

The occurrence of medication resistance against bacteria is a primary cause of antimicrobial agent ineffectiveness. Even against some resistant strains of microorganisms, medicinal plants could be a source of new antibacterial agents. Because of the presence of an impermeable lipopolysacharide layer, numerous plant extracts have been reported to be more efficient against gram positive bacteria than gram negative bacteria in previous studies [21].The antibacterial property of THU-C extract was examined against *Bacillus amyloliquifaciens*, *Bacillus subtilis*, *Aeromonas liquifaciens*, *Flexibactor sp.* **(Figure 2).** Results in Table-2 show that **the** extract had antibacterial effect against both gram (+)ve and gram (–)ve bacteria with maximum Zone of Inhibition (ZOI) against *Aeromonas*

liquifaciens. The antibacterial effect of THU-C was compared with the standard antibiotics, like ampicillin, tetracycline and penicillin, by measuring the AI value (**Figure 3**). For *Bacillus amyloliquifaciens* the AI value of extract was 0.74, 0.57 and 0.90mm for ampicillin, tetracycline and penicillin, respectively. For *Bacillus subtilis* AI of the extract was 0.92, 0.65, 0.95mm, respectively, for the above three antibiotics. The AI value of the extract was 1.3, 0.7, and 2mm for *Aeromonas liquifaciens* and 0.88, 0.44, and 1.06mm for *Flexibactor sp.* for ampicillin, tetracycline and penicillin, respectively. In previous study *T. orientalis* seed coat essential oil was found to have antibacterial activity against six bacterial and five fungal pathogens [20].

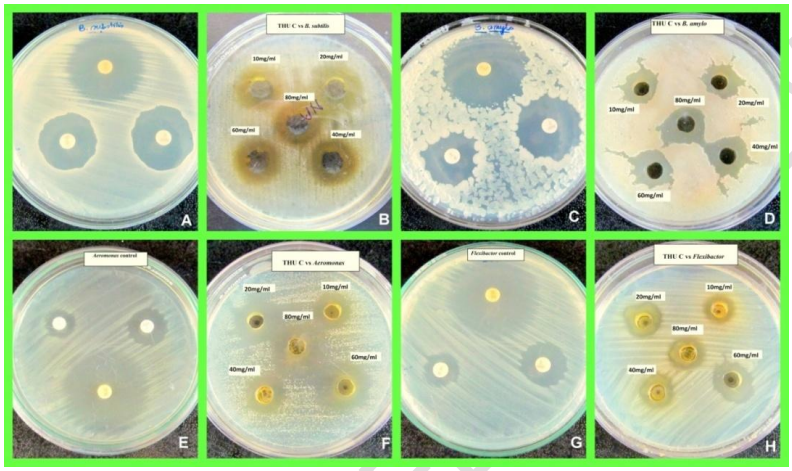

Figure 2: Antibacterial activity of methanolic extract of *T. orientalis* cone (THU-C). (A) 3 Stand. Anti. against *B. subtilis*, (B) THU-C against *B. subtilis,* (C) 3 Stnd. Anti. against *B. amyloliquifaciens,* D) THU-C against *B. amyloliquifaciens.* E) 3 Stnd. Anti. against *Aeromonas liquifaciens,* F) THU-C against *Aeromonas liquifaciens.* G) 3 Stnd. against Vs *Flexibactor*, H) THU-C against *Flexibactor*.

3 Stnd. Anti. : Standard Antibiotic (Ampicilin, Tetracyclin, Penicilin).

Table 2: Antibacterial activity show ZOI of methanolic extract of *Thuja orientalis* cone (THU-C).

Bacterial strain	Zone of inhibition (mm)							
	Conc. Of THU-C extract (mg/ml)					Conc. of standards (µg/ml)		
	10	20	40	60	80	Amp	Tet	P
Bacillus amyloliquifaciens	17	18	20	20	20	27	35	22

Bacillus subtilis	20	23	20	20	23	25	35	24
Aeromonas liquifaciens	13	19	19	23	26	20	34	13
Flexibactor sp.	13	16	13	18	16	18	36	15

Amp: Ampicilin, Tet: Tetracycline, P: Penicillin

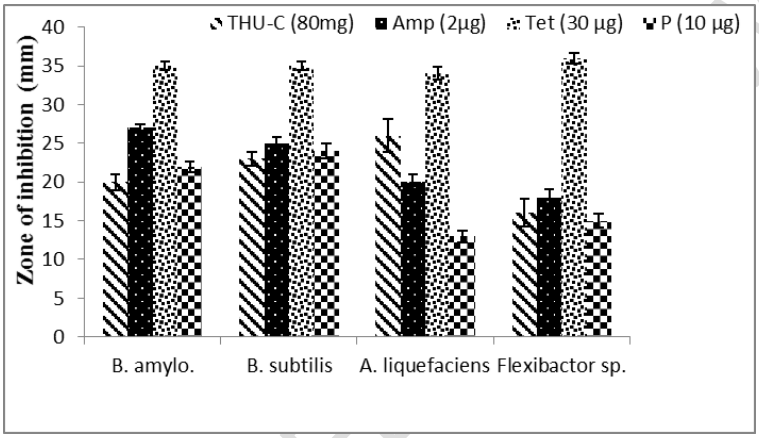

Figure 3: Comparison of antibacterial activity of methanolic extract of *Thuja orientalis* cone (80mg/ml) and standard antibiotics based on the Zone of Inhibition (ZOI). Amp: Ampicillin, Tet: Tetracycline, P: Penicillin. Data are mean± standard deviation, n=3.

3.4. Antifungal effect:

THU-C extract restricted the radial growth of three fungal genera: *Aspergillus*, *Rhizopus*, *Fusarium*. It had greater inhibitory effect on growth of *Aspergillus*, *Rhizopus.* Moreover, the effect of the extract on *Aspergillus* was greater than that of griseofulvin, the standard antifungal agent (**Figure 4 &Table 3**).

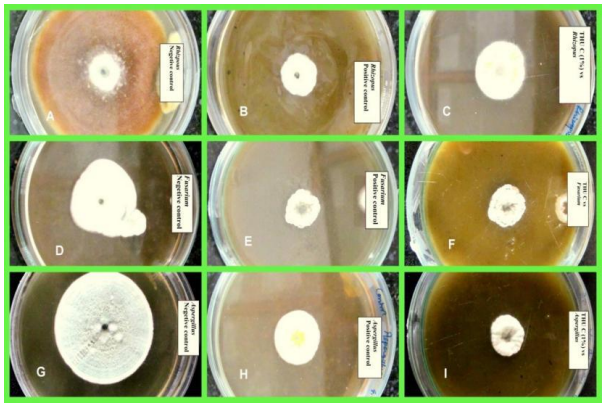

Figure 4: Antifungal activity of methanolic extract of *T. orientalis* cone (THU-C) against three fungal genera. (A) Negative control (methanol) against *Rhizopus*, (B) Positive control (Griseofulvin) against *Rhizopus*, (C) THU-C extract against *Rhizopus, (*D) methanol against *Fusarium*, (E) Griseofulvin against *Fusarium*, (F) THU-C extract against *Fusarium*.(G) methanol against *Aspergillus*, (H) Griseofulvinagainst *Aspergillus*, (I) THU-C extract against *Aspergillus*.

Table 3: The effect of methanolic extract of *Thuja orientalis* cone (THU-C) on growth of fungi

Fungal strain	Zone of radial growth (mm)		
	Negetive control*	Positive control**	THU-C
Rhizopus	60	15	21
Fusarium	30	12	17
Aspergillus	36	16	13

*Methanol, **Griseofulvin

5. Conclusion

In conclusion, methanolic extract of *Thuja orientalis* cone can be considered as good sources of natural compounds having antioxidant, antibacterial and antifungal activity. It consist various phytochemical substances like terpenoid, flavonoid, phenol, tannin. It is effective antioxidant against DPPH, H_2O_2, NO, ABTS. It also revealed significant antibacterial activity against both gram positive and negative bacteria and antigungal activity against three fungus. Our future study will based on

isolation and characterization the active compound from methanolic extract of *Thuja orientalis* cone and evaluation its different biological activities and action of mechanism.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Ignacimuthu S, Ayyanar M, Sankara SK. Ethnobotanical investigations among tribes in Madurai District of Tamil Nadu (India). J. Ethnobiol. Ethnomed. 2006;2: 25.

2. Awadhi A, Ali N, Al-rewhip IK, Lindquist U. Some medicinal plants used in Yemeni herbal medicine to treat malaria. Afr. J. Trad. Complementary Alter. Med. 2004;1: 72-76.

3. Kellenberger E, Hofmann A, Quinn RJ. Similar interactions of natural products with biosynthetic enzymes andtherapeutic targets could explain why nature produces such a large proportionof existing drugs. Nat. Prod. Rep. 2011;28: 1483-1492.

4. Yeung HM. Handbook of Chinese herbs and formulas. Institute of Chinese Medicine. Los Angeles, C. A;1985.

5. Hassanzadeh MK, Rahimizadeh M, Fazly Bazzaz BS, Emami SA, Asili J. Chemical and antimicrobial studies of *Platycladus orientalis* essential oils. Pharm. Biol. 2001;5: 388-390.

6. Evans WC. Trease and Evans Pharmacognosy. (15th Ed.), 15th edition: Sanders Co. Ltd. Singapore;2002.

7. Nabavi S, Ebrahimzadeh M, nabavi S, Hamidinia A, Bekhradnia A. Determination of antioxidant activity, phenol and flavonoids contents of *Parrotia persica Mey.* Pharmacol online. 2008;2: 560-567.

8. Kokate A. Phytochemical Methods. Phytotherapy, 2nd edition. 1999;78:126-129.

9. Harborne JB. Phytochemical methods: A guide to modern techniques of plant analysis 3rd edition. Springer, Germany;1998.

10. Trease GE, Evans WC. Isolation, purification and partial characterization of prunellin an anti – HIV compound from aqueous extract of *Prunella vulgaris*, ant. Res. 1989;11:263–73.

11. Harborne JB. Phytochemical methods. London: Chapman and Hall;1973.

12. Brain KR, Turner TD. The practical evaluation of phytopharmaceuticals, Wright-science technical 1st edition, Bristol Britain. 144;1975.

13. Braca A, Tommasi N de, Bari L Di, Pizza C, Politi M, Morelli I. Antioxidant principles from Bauhinia tarapotensis. J Nat Prod. 2001;64(7): 892-5.

14. Keser S, Celik S, Turkoglu S, Yilmaz O, Turkoglu I. Hydrogen peroxide scavenging and total antioxidant of hawthorn. Chem J. 2012;1: 9-12.

15. Sreejayan, Rao MN. Nitric oxide scavenging by curcuminoids. J Pharma and pharmacol. 1997;49: 105-107.

16. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-evans C. Antioxidant activity applying an improved ABTS radical cation decolarization assay. Free Radic Biol Med. 1999;26(9-10):1231-1237.

17. Salie F, Eagles PFK, Leng HMJ. Preliminary antimicrobial screening of four South African Asteraceae species. J. Ethanopharmacol. 1996;52: 27-33.

18. Kokubun T, Veitch NC, Bridge PD, Simmonds MSJ. Dihydroisocoumarins and a tetralone from *Cytospora eucalypticola.* Phytochemistry. 2003;62: 779-782.

19. Jain N, Sharma M. Ethanobotany, Phytochemical and Pharmacological Aspects of *Thuja orientalis* : A Review. Int. J. Pure and App. Biosci. 2017;5 (3):73-83.

20. Jain RK, Garg SC. Antimicrobial activity of the essential oil of *Thuja orientalis* L. *Anc. Sci. Life.* 1997; **3:** 186 – 189.

21.Jasuja ND, Sharma SK, Saxena R, Choudhary J, Sharma R, Joshi SC. Antibacterial, antioxidant and phytochemical investigation of *Thuja orientalis* leaves. *J. Med. Plant Res*. 2013; 7(25): 1886-1893.