

Antioxidant, antibacterial and antiacetylcholinesterase activities of abietic acid from *Isodon wightii* (Bentham) H. Hara

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ABSTRACT

Objective: *Isodon* is an important genus of the family Lamiaceae which contains diterpenoids of diverse functionalities. The present study attempted with isolation, structure elucidation and bioactivity evaluation of isolated compound from the leaves of *Isodon wightii* (Bentham) H. Hara. **Methods:** Petroleum ether extract of powdered leaves was obtained and concentrated in vacuo at 45°C. Compound was isolated using silica gel column chromatography (60-120 mesh). Antioxidant activity of isolated compound was tested for DPPH free radical and hydroxyl radical scavenging, inhibition of linoleic acid peroxidation and metal ion chelation activities. Antibacterial activity was tested against six pathogenic bacteria by micro broth dilution method and *in vitro* antiacetyl cholinesterase activity of isolated compound was also studied. **Results:** Abietane diterpenoid, abietic acid was isolated from the leaves of *I. wightii* and the structure has been elucidated on the basis of spectroscopic analysis. Abietic acid isolated from petroleum ether extract of leaves showed moderate DPPH free radical, hydroxyl radical scavenging and less inhibition of linoleic acid peroxidation and metal ion chelating activities. Antibacterial activity of abietic acid against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris* and *Staphylococcus aureus* was found in the range of 340-980 µg/mL. Antiacetylcholinesterase activity of abietic acid was less when compared with positive control. **Conclusion:** Abietic acid showed concentration dependent antioxidant, antibacterial and antiacetylcholinesterase activities.

Keywords: Abietic acid, Antioxidant, Antibacterial, Antiacetylcholinesterase, Column Chromatography, Silica gel G-60, NMR.

INTRODUCTION

Nature is a rich source of medicinal agents for more than thousand years and an impressive number of drugs have been isolated from natural resources.¹ *Isodon* is a widely distributed and well represented genus of the family Lamiaceae. *Isodon* species are known for its ethnomedicinal

and phytochemical values. Diterpenoids like labdanes, clerodanes, abietanes and kauranes of *Isodon* with diverse structures had shown antibacterial, antiinflammatory and antitumor activities.² *Isodon wightii* (Bentham) H. Hara is a perennial herb commonly distributed in Western Ghats, South India up to 8000 feet. *Ent*-kaurene diterpenoid, melissoidesin isolated from the leaves showed antioxidant, antiacetylcholinesterase, cytotoxic and anticarcinogenic activities.³⁻⁴ *in vitro* mass multiplication protocol for this important species has also been developed.⁵ Based on the biological activities of diterpenoids, the present study was aimed to evaluate the antioxidant, antibacterial and antiacetylcholinesterase activities of diterpenoid isolated from the leaves of *I. wightii*.

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MATERIALS AND METHODS

General

¹H (400MHz), ¹³C (100MHz) (Bruker-400) and 2D NMR (500MHz) spectra including ¹H-¹H correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) were recorded in Bruker-500 spectrometer with the residual solvent signals as internal reference. IR spectrum was recorded on a Shimadzu FT-IR -820 ipc using KBr pellets. EI-MS was recorded on Shimadzu LC-MS. Silica gel 60-120 (Hi media Laboratories Pvt Ltd, Mumbai, India) mesh was used for column chromatography and eluted with petroleum ether (100%). Fractions were monitored by silica gel G-60.

Plant material

The leaves of *Isodon wightii* (Bentham) H. Hara, a perennial herb was collected from Coonoor, Tamil Nadu, India during January 2014.

Extraction and isolation

The dried and powdered leaves (715 g) were extracted with petroleum ether in Soxhlet apparatus at room temperature to yield crude extract (15 g). After evaporating the solvent in vacuo at 45°C the extract was subjected to silica gel column chromatography (60-120 mesh size, Ranbaxy Fine Chemicals Limited, New Delhi, India) and eluted with petroleum ether (100%) (Ranbaxy Fine Chemicals Limited, New Delhi, India). Further the fractions were collected, combined and monitored by TLC coated with silica gel G-60 (Hi media Laboratories Pvt Ltd, Mumbai, India). Yellowish amorphous powder (82 mg) was obtained after 70th h.

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), tris-HCl, trichloroacetic acid (TCA), phosphate buffer, ferrous chloride, ferrous sulphate, thiobarbituric acid (TBA), ferrozine, 1,10-phenanthroline, sodium hydroxide, deoxyribose, linoleic acid, physostigmine and butylated hydroxyl toluene (BHT) were purchased from Hi Media Laboratories Pvt. Ltd, Mumbai, India. H₂O₂ was purchased from S.D Fine Chemicals Limited, Mumbai, India. Acetylcholinesterase (AChE) type VI-S, from electric eel 349Umg⁻¹ solid, 411Umg⁻¹ protein, 5,5-dithiobis [2-nitrobenzoic acid] (DTNB) and acetylthiocholine iodide (AChI) were purchased from Sigma- Aldrich, Inc., 3050 Spruce Street, St Louis, Mo, USA.

Antioxidant activities

DPPH free radical scavenging activity

Different concentrations of test sample mixed individually with 0.1 mM DPPH and 50 mM tris-HCl buffer (pH 7.4).

Reaction mixture was incubated at 37°C for 30 min and then absorbance was measured at 517 nm.^{6,7} The percentage of DPPH free radical scavenging activity was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

where A_B; absorption of blank sample,

A_A; absorption of test sample.

Hydroxyl radical scavenging activity

Reaction mixture includes 7.5 mM FeSO₄, 7.5 mM 1, 10-phenanthroline, 0.2 M phosphate buffer (pH 7.8), 30 mM H₂O₂ and test sample at different concentrations. The reaction was started by adding H₂O₂. After incubation at room temperature for 5 min, the absorbance of the mixture was read at 536 nm.⁸

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

where A_B; absorption of blank sample,

A_A; absorption of test sample.

Inhibition of linoleic acid peroxidation

Briefly, 20 mM linoleic acid, 100 mM HCl (pH 7.5), 5 mM ascorbic acid were mixed with test sample of various concentrations. Linoleic acid peroxidation was initiated by the addition of 4 mM FeSO₄·7H₂O, incubated for 60 min at 37°C and terminated by the addition of 2 mL of ice cold trichloroacetic acid (10% v/v). An amount of 1 mL of thiobarbituric acid (1% w/v in 50 mM NaOH) was added to 1 mL of the reaction mixture, followed by heating at 95°C for 60 min. The reaction sample was read at 532 nm.⁹ The percentage of linoleic acid peroxidation inhibition activity was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

where A_B; absorption of blank sample,

A_A; absorption of test sample.

Metal chelating activity

Briefly, 2 mM FeCl₂ was added to different concentrations of test sample and reaction was initiated by the addition of 5 mM ferrozine. The mixture was vigorously shaken and left to stand at room temperature for 10 min. Absorbance was measured at 562 nm after 10 min.¹⁰ The percentage inhibition was calculated using

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Table 1: NMR spectrum data of abietic acid (δ in ppm, J in Hz)^a

Position	δ H	δ C	HMBC ^b
1	1.23(m)	38.27	C-3,C-4
2	1.58(m)	18.06	
3	1.79(m)	37.19	
4	-	46.35	
5	2.07(m)	44.90	C-4,C-18,C-19,C-20
6	1.89(m)	25.63	
7	5.37(s)	120.51	
8	-	135.59	
9	1.92(m)	50.94	
10	-	34.47	
11	1.79(m)	22.49	
12	2.07(m)	27.47	
13	-	145.34	
14	5.77(s)	122.37	C-15,C-9,C-7,C-8,C-11
15	2.21(m)	34.90	
16	1.00(d,j= 2)	20.89	
17	1.00(d,j= 2)	21.44	C-17,C-15
18	-	185.0	
19	1.21(s)	16.73	C-10,C-18
20	0.82(s)	14.05	C-9,C-4,C-1

^aData were recorded in in CDCl₃ at 400MHz (¹H) and 100 MHz (¹³C)

^bHMBC correlations are from proton (s) stated to the indicated carbon

where A_B, absorption of blank sample,

A_A; absorption of test sample.

Antibacterial activity

Clinical isolates of *Aeromonas hydrophila*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris* and *Staphylococcus aureus* were procured from Microbiology Laboratory of KMCH Hospital, Coimbatore,

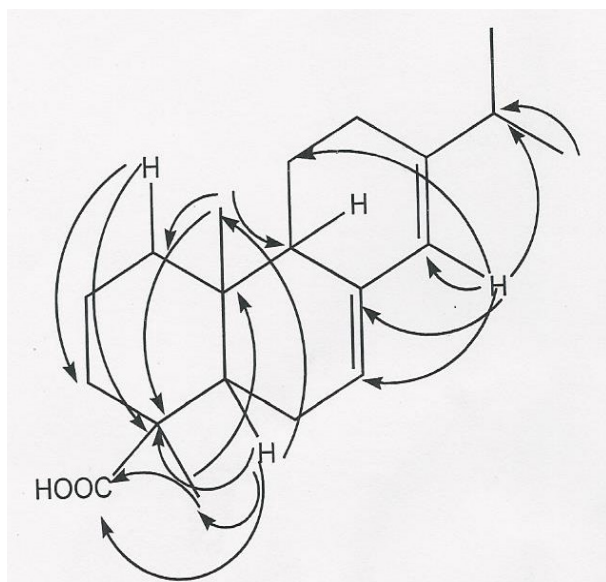


Figure 1: HMBC correlations of abietic acid

Tamil Nadu, India. The minimum inhibitory concentration of abietic acid was studied by broth micro dilution method using 96-well microtitre plates.¹¹ Test compound was dissolved in DMSO (1%) with the addition of Tween-80(0.5%) and diluted in Muller Hinton Broth to get a concentration range of 100-1000 μ g/mL. The solution was then two fold diluted in Muller Hinton Broth (100 μ L), inoculated with bacterial strains and then incubated at 37°C for 24 h. The bacterial growth was measured as turbidity with a microplate reader (Cyberlab) at 405 nm. The minimum inhibitory concentration was defined as the lowest concentration of test compound that inhibits the growth of the test bacteria. DMSO assayed as the negative control at a concentration of 1% did not inhibit any of the strains tested. All tests were assayed in triplicate in three independent experiments and median values were used for MICs calculation. Both gentamicin and ampicillin were served as positive control.

Antiacetylcholinesterase activity

The enzymatic activity was measured using the method described earlier.¹² 500 μ L of DTNB 3 mM, 100 μ L of AChI 15 mM, 275 μ L of tris-Hcl buffer 50 mM, pH 8 and different concentrations of test compound was added. In the reaction mixture, 25 μ L of buffer were replaced by the same volume of an enzyme solution containing 0.28 U/mL⁻¹. The reaction was monitored for 5 min at 405 nm. Physostigmine was used as positive control.

Statistical analysis

Data obtained from *in vitro* experiments were expressed as mean (n=3) standard error (\pm SE). IC₅₀ values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

The ¹H NMR signals at δ 5.37 (1H, s, H-7) and 5.77 (1H, s, H-14) showed the presence of ethylenic bond; whereas the ¹³C NMR spectrum showed a carboxylic carbon signal at δ 145.3,135.59,122.3 and 120.5. Based on the calculation of unsaturation degree, compound could be estimated as a tricyclic compound. The HSQC spectrum showed that the protons at δ H 5.37 (H-7) and 5.77 (H-14) were connected to the carbons at δ C 120.5 (C-7) and 122.3 (C-14) respectively. These were further verified by COSY correlations. In COSY spectrum, correlation of H-19 with C-5, H-16 with C-15 and H-17 with C-15 confirmed the position of its protons attached to the carbon as adjacent manner (Table 1). The IR spectrum showed the presence of acid group. The molecular formula of compound is established as C₂₀H₃₀O₂ by EI-MS. On the basis of spectral evidence, the structure was determined to be abieta-7,13-dien-18-oic acid (Figure 1).

Table 2: Antioxidant activity of abietic acid

Assays	IC50*(µg/mL)
DPPH free radical scavenging	660.36±0.03
Hydroxyl radical scavenging	467.43±0.03
Inhibition of linoleic acid peroxidation	1924.75±0.04
Metal ion chelation	2558.06±0.04

*IC50- inhibitory concentration values were expressed as the mean ± SD of three replicates

Table 3: Antibacterial activity of abietic acid

Name of the bacteria	MIC* (µg/mL)		
	Abietic acid	Ampicillin (10 µg)	Gentamicin (10 µg)
<i>Escherichia coli</i>	378.33±0.003	14.1±0.002	9.89±0.001
<i>Klebsiella pneumoniae</i>	343.12±0.002	13.8±0.001	12.5±0.001
<i>Pseudomonas aeruginosa</i>	523.18±0.002	14.7±0.001	11.18±0.001
<i>Proteus mirabilis</i>	983.22±0.003	16.4±0.001	13.1±0.002
<i>Proteus vulgaris</i>	750.32±0.002	15.9±0.01	12.3±0.001
<i>Staphylococcus aureus</i>	496.46±0.003	12.8±0.002	11.9±0.001

*MIC-Minimum inhibitory concentration values are expressed as the mean ± SD of three replicates

Abietic acid isolated from resina pini of *Pinus* sp. and *Pimenta racemosa* showed testosterone 5 α -reductase inhibition, antiinflammatory, antiobesity and lipoxygenase inhibitory activities.¹³⁻¹⁵ Based on the pharmacological importance, abietic acid was tested for its antioxidant, antibacterial and antiacetylcholinesterase activities. Antioxidant activity was tested using four different test systems. The concentration needed to scavenge 50% of DPPH and hydroxyl radicals were calculated as 660.36 ± 0.03 and 467.43 ± 0.03 µg/mL respectively. Meanwhile abietic acid had shown less activity against metal ion chelation and inhibition of lipid peroxidation (Table 2). When compared with BHT (IC₅₀=39.12 ± 0.02 µg/mL) antioxidant activity of abietic acid was less. Antioxidant activity of abietane diterpenoids such as carnosol, isorosmanol, carnosic acid, rosmanol, epirosmanol and galdosol from *Salvi officinalis* and inuroyleanol from *S.barrelieri* are reported in the literature.¹⁶⁻¹⁷

Antibacterial activity of abietic acid was tested against six pathogenic bacteria and most sensitive strains were *Klebsiella pneumoniae* and *Escherichia coli* followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The least activity of the compound was observed against *Proteus mirabilis* and *Proteus vulgaris* (Table 3). The bacteriolytic action of abietic acid is associated with interaction and lysis of cell membranes.¹⁸ Abietanes such as royleanone and coleon reported in the

genus *Plectranthus* possesses good antimicrobial activity.¹⁹⁻²⁰ Antiacetylcholinesterase activity of abietic acid was 750 ± 0.02 µg/mL whereas physostigmine showed IC₅₀ value of 13.12 ± 0.02 µg/mL. Similar result was reported in the literature where abietane diterpenoids isolated from *Salvia staminea* showed antiacetylcholinesterase and butrylcholinesterase activities.²¹

CONCLUSION

In conclusion, petroleum ether extract of leaves of *I.wightii* yielded abietic acid. Free radical scavenging, metal chelation, prevention of lipid peroxidation and antibacterial activities of abietic acid proves it may be useful in food products preparation and storage. Antiacetylcholinesterase activity of abietic acid will give new idea to develop natural drug to treat Alzheimers disease.

CONFLICT OF INTEREST

All authors have none to declare.

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REFERENCES

- Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of ayurveda. Phcog Rev. 2014; 8(16): 73-80.
- Sun HD, Huang SX, Han QB. Diterpenoids from *isodon* species and their biological activities. Nat Prod Rep. 2006; 23(5): 673-98.
- Thirugnanasampandan R, Jayakumar R, Narmatha Bai V, Ebenzer martin, Rajendra Prasad KJ. Antiacetyl cholinesterase and antioxidant *ent*-Kaurene diterpenoid, melissoidesin from *Isodon wightii* (Bentham) H. Hara. Nat Prod Res. 2008; 22(8): 681-8.
- Thirugnanasampandan R, Jayakumar R. Cytotoxic and anticarcinogenic activity of *ent*-kaurene diterpenoid, melissoidesin from *Isodon wightii* (Bentham) H. Hara. Nat Res Prod. 2009; 23(16): 1499-506.
- Thirugnanasampandan R, Mahendran G, Narmatha Bai V. High frequency

- in vitro* propagation of *Isodon wightii* (Bentham) H. Hara. *Acta Physiol Plant.* 2010; 32(2): 405-9.
6. Blois MS. Antioxidant determination by the use of a stable free radical. *Nature* 1958; 181(4617): 1199-200.
 7. Parasuraman S, Petchi RR, Vijaya C, Dhanaraj SA. Evaluation of free radical scavenging properties and hypoglycemic activity of ethanolic extract of *Tridax procumbens* Linn. in Wistar rats. *Drug Dev Ther.* 2014; 5(2): 164-7.
 8. Zhao GR, Xiang ZJ, Ye TX, Yuan YJ, Guo ZX. Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginseng*. *Food Chem.* 2006; 99(4): 767-74.
 9. Choi B, Kang S, Ha Y, Park G, Ackman RG. Conjugated linoleic acid as a supplemental nutrient for common carp (*Cyprinus carpio*). *Food Sci Biotechnol.* 2002; 11(5): 457-61.
 10. Dinis TCP, Madeira VMC, Almeida MLM. Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. *Arch Biochem Biophys.* 1994; 315(1): 161-9.
 11. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard. 8th ed, Wayne, PA: CLSI [CLSI document M7-A8]; 2009.
 12. Ingkaninan K, Temkitthawon P, Chuenchon K, Yuyaem T. Screening for acetylcholinesterase inhibitory activity in plants used in Thai traditional rejuvenating and neurotonic remedies. *J Ethnopharmacol.* 2003; 89(2): 261-4.
 13. Roh SM, Park Y, Kim. Abietic acid from resina pini of *pinus* species as a testosterone 5 α -reductase inhibitor. *J Health Sci.* 2010; 56(4): 451-5.
 14. Fernandez MA, Tornos MP, Garcia MD, De las Heras, Villar AM, Saenz1 MT. Anti-inflammatory activity of abietic acid, a diterpene isolated from *Pimenta racemosa* var. *grisea*. *J Pharm Pharmacol.* 2001; 53(6): 867-72.
 15. Hwang KJ, Ahn S, Kim J, Park T Ha. Abietic acid has an anti-obesity effect in mice fed a high-fat diet. *J Med Food.* 2011; 14(9): 1052-6.
 16. Miura K, Kikuzaki H, Nakatani N. Antioxidant activity of chemical compounds from sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) measured by the oil stability index method. *J Agric Food Chem.* 2002; 50(7): 1845-51.
 17. Kabouche A, Kabouche Z, Ozturk M, Kolak U, Topcu G. Antioxidant abietane diterpenoids from *Salvia barrelieri*. *Food Chem.* 2007; 102(4): 1281-7.
 18. Aranda FJ, Villalain J. The interaction of abietic acid with phospholipid membranes. *Biochem Biophys Acta.* 1997; 1327(2): 171-80.
 19. Batista O, Simoes MF, Duarte A, Valdeira ML, Torre MC, Rodriguez B. An antimicrobial abietane from the roots of *Plectranthus hereroensis*. *Phytochemistry* 1995; 38(1): 167-9.
 20. Teixeira AP, Batista O, Simoes MF, Nascimento J, Duarte A, Torre MC, *et al.* Abietane diterpenoids from *Plectranthus grandidentatus*. *Phytochemistry* 1997; 44(2): 325-7.
 21. Topcu G, Kolak U, Ozturk M, Seda DH, Fatemeh B, Burcu C, *et al.* Investigation of antiacetylcholinesterase activity of a series of *Salvia* extracts and the constituents of *Salvia staminea*. *The Nat Prod J.* 2013; 3(1): 3-9.