JoP Journal of Pharmacopuncture
Journal of Pharmacopuncture 2022;25(4):364-368
pISSN: 2093-6966 • eISSN: 2234-6856
https://doi.org/10.3831/KPI.2022.25.4.364
Original Article
Check for updates

Bacterial Reverse Mutation Test of Verbenalin

Hye Jeong Shin[1], Yi Gun Lim[1], Ji Su Ha[2], Gabsik Yang[1], Tae Han Yook[1]*

[1]Department of Korean Medicine, College of Korean Medicine, Woosuk University, Jeonju, Republic of Korea
[2]Department of Acupuncture & Moxibustion Medicine, National Medical Center, Seoul, Republic of Korea

Received August 18, 2022
Reviewed August 24, 2022
Accepted September 6, 2022

***Corresponding Author**
Tae Han Yook
Department of Korean Medicine, College of Korean Medicine, Woosuk University, 61 Seonneomeo 3-gil, Wansan-gu, Jeonju 54986, Republic of Korea
Tel: +82-63-220-8625
E-mail: nasiss@naver.com

Objectives: Verbenalin is a compound found in herbs such as *Cornus officinalis* and *Verbena officinalis*. This study investigated whether verbenalin is safe by analyzing its mutagenicity.
Methods: To examine the mutagenic potential of verbenalin, a bacterial reverse mutation test (Ames test) was conducted with *Salmonella typhimurium* and *Escherichia coli* strains. Experiments with and without metabolic activity were performed.
Results: The mean colony number was less than double that of the control. Growth inhibition and precipitation of verbenalin were not apparent in all strains at different concentrations regardless of metabolic activity.
Conclusion: Verbenalin did not show any signs of mutagenicity in this study. Additional toxicity studies including repeated oral toxicity, reproductive toxicity, and carcinogenicity tests are needed.

Keywords: dementia, mutagenicity test, verbenalin

INTRODUCTION

Dementia causes behavioral abnormalities and personality changes including cognitive decline due to biological aging [1]. It is estimated that there are around 55 million people with dementia, which is predicted to reach 78 million in 2030 [2]. In Korean medicine, the recognized causes of dementia include aging, poor movement of qi and blood due to stress, damage to qi and blood due to inability to control food intake, trauma to the head, and chronic consumptive disease [3].

Various studies have reported the therapeutic efficacy of herbal extracts for dementia. Standard extracts of *Ginkgo biloba* leaves [4], an alkaloid compound constituting the medicinal herb *Huperzia serrata* (huperzine A) [5], standard ethanolic extracts of *Angelica gigas* Nakai (INM-176) [6], dehydroevodiamine hydrochloride (DHED), and the active ingredients of *Evodia officinalis* [7], *Perilla frutescens* [8], and *Gastrodiae Rhizoma* [9] were demonstrated to have potential therapeutic efficacy against dementia.

Verbenalin is an alkaloid present in herbs such as *Verbena officinalis*, *Cornus officinalis*, and *Symplocos glauca* [10-12]. Verbenalin was found to improve brain microcirculation and protect brain tissue and neurons in a study using a cerebral ischemia rat model. Other studies reported that it exhibited sleep-promoting and antioxidant effects [13, 14] and the potential to reduce apoptosis [15].

To confirm the therapeutic effect of verbenalin on dementia, studies have been conducted to determine whether verbenalin inhibits the production of amyloid-β (Aβ) peptides. Various effects of verbenalin have been identified; however, its toxicity has not been sufficiently investigated. To use verbenalin for dementia treatment, it is important to identify any toxic and mutagenic properties. Therefore, an oral dose toxicity study using ICR mice is currently ongoing to evaluate the toxicity of verbenalin. In this study, the Ames test was performed to determine whether verbenalin is mutagenic. The Ames test can sensitively, quickly, and accurately identify mutant activity [16]. This study was based on the Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals, No. 471 'Bacterial Reverse Mutation Test' (1997).

MATERIALS AND METHODS

Verbenalin (purity > 99.3%, molecular formula: $C_{17}H_{24}O_{10}$, molecular weight: 388.37 g/mol) was obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Dimethylsulfoxide (DMSO) and other solvents and chemicals were purchased from Merck (Darmstadt, Germany). *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2uvrA were obtained from Molecular Toxicology Inc. (Boone, NC, USA). DMSO was used as a solvent for verbenalin as well as a negative control.

The Ames test was performed with 4 *Salmonella typhimurium* strains and 1 *Escherichia coli* strain, both with and without metabolic activity [17].

Verbenalin was dissolved in DMSO and mixed with a vortex mixer. Solvents were added to achieve the desired concentration level. The maximum concentration of verbenalin was set to 5,000 μg/plate and diluted to obtain 2,500, 1,000, 500, 100, 50, 10, and 5 μg/plate concentrations. A negative control group was also established. The test was performed using the pre-incubation method, and a total of 2 plates were used for all concentration levels with and without metabolic activity.

In experiments without metabolic activity, 100 μL of verbenalin and the negative control were placed in separate tubes. Then, a mixture of 500 μL of 0.1 M sodium phosphate buffer and 100 μL of strain suspension was incubated in a shaking incubator (90 rpm, 37℃). Heated agar (2 mL) was added and mixed in a vortex mixer. The mixtures were solidified on glucose agar plates at room temperature.

In experiments with metabolic activity, instead of sodium phosphate buffer, 500 μL of S9 mixture was added. After solidification, the inverted plates were cultured at 37℃ for 48 h in an incubator (DK-LI020-P; Daiki Scientific Co., Ltd., Seoul, Korea).

Precipitation was observed visually and recorded during the treatment with verbenalin. The number of revertant colonies was automatically calculated by a colony counter (ProtoCOL3; Synbiosis, Cambridge, UK) after incubation. When the number obtained by automatic counting was incorrect, the colonies were counted manually.

To detect growth inhibition, the background lawn was examined with a stereoscopic microscope (45-fold magnification, SZ61; Olympus, Tokyo, Japan). Growth inhibition was judged based on the extent to which the number of revertant colonies was decreased or the background lawn was reduced compared with those of the control group.

RESULTS

If a dose-related increase was observed over the tested range and/or the increase reflected the average number of revertant colonies for at least one strain regardless of metabolic activity, the result was considered positive. Cytotoxicity was defined as the reduction of the background lawn or colony number by more than 50% compared with those of the vehicle control.

Overall, the number of revertant colonies was less than double that of the control at all concentrations for all strains regardless of metabolic activity. Growth inhibition and precipitation of verbenalin were not observed at all concentrations in all strains regardless of metabolic activity (Table 1).

DISCUSSION

Dementia is a syndrome with behavioral and psychological symptoms such as cognitive and memory deterioration, emotional abnormalities, and hallucinations [18].

Recently, herbal extracts have attracted attention due to their medicinal properties including rapid action, low frequency of side effects, and potential synergistic effects [19, 20].

The active ingredients of herbal extracts may be used for the treatment of dementia. For example, galantamine and huperzine A, which are derived from herbal sources, have been developed clinically to treat mild-to-moderate dementia [21, 22]. Extracts of *Ginkgo biloba* leaves were reported to protect brain tissue against hypoxic damage [4] and partially prevent oxidative damage in the brain of aged animals [23]. Huperzine A from the medicinal herb *Huperzia serrata* was demonstrated to treat cognitive deficits [5] and reduce glutamate-induced impairment in the brain [24]. INM-176, an extract of *Angelica gigas* Nakai, showed memory-improving effects against scopolamine-induced memory damage [6]. DHED, the active ingredient of *Evodia officinalis*, demonstrated memory-improving and neuroprotective effects in experiments with various models [7]. The positive effects of *Gastrodiae Rhizoma* on learning and memory were confirmed in experiments using APP/PS1 mice [9]. The effects of *Perilla frutescens* extract on a mouse model with memory disorder were demonstrated through Y-maze and other types of tests [8]. In addition, studies using various herbal extracts are being conducted to develop new drugs that can treat the cause of dementia.

Table 1. The number of revertant colonies

Strain	Test substance	Dose (μg/plate)	Absence of metabolic activation			Presence of metabolic activation		
			Individual revertant colony counts		Mean	Individual revertant colony counts		Mean
TA98	VC	0	14	10	12	27	33	30
	Verbenalin	5	11	11	11	25	24	25
		10	15	16	16	29	34	32
		50	10	12	11	27	32	30
		100	14	10	12	24	30	27
		500	13	10	12	30	27	29
		1,000	13	9	11	27	29	28
		2,500	13	11	12	25	27	26
		5,000	13	12	13	33	27	30
TA100	VC	0	99	110	105	111	123	117
	Verbenalin	5	101	111	106	119	120	120
		10	100	110	105	120	110	115
		50	97	109	103	122	121	122
		100	112	123	118	112	121	117
		500	119	110	115	116	120	118
		1,000	116	115	116	117	133	125
		2,500	94	113	104	118	128	123
		5,000	98	83	91	119	125	122
TA1535	VC	0	5	8	7	6	9	8
	Verbenalin	5	10	7	9	9	7	8
		10	8	5	7	4	8	6
		50	6	10	8	10	8	9
		100	5	8	7	8	5	7
		500	5	8	7	7	4	6
		1,000	5	5	5	7	7	7
		2,500	5	5	5	5	8	7
		5,000	7	6	7	11	8	10
TA1537	VC	0	5	7	6	10	15	13
	Verbenalin	5	5	6	6	7	11	9
		10	5	5	5	11	10	11
		50	6	10	8	15	15	15
		100	5	9	7	12	9	11
		500	5	7	6	9	10	10
		1,000	5	7	6	11	9	10
		2,500	8	8	8	9	6	8
		5,000	8	7	8	9	10	10
WP2uvrA	VC	0	28	32	30	35	31	33
	Verbenalin	5	31	26	29	29	32	31
		10	30	35	33	35	37	36
		50	24	31	28	33	39	36
		100	31	35	33	32	37	35
		500	32	38	35	31	36	34
		1,000	27	29	28	32	34	33
		2,500	30	35	33	40	32	36
		5,000	26	32	29	37	32	35

VC, vehicle control (dimethyl sulfoxide).

 https://doi.org/10.3831/KPI.2022.25.4.364

Verbenalin, also known as cornin, is an iridoid glucoside component of the herbs *Cornus officinalis* and *Verbena officinalis* (a plant of the Verbenaceae family) [10-12]. Verbenalin protects brain tissue, improves brain microcirculation, and reduces cerebral ischemic damage. It has also been reported to have antioxidant activity and promote sleep [13, 14]. Previous studies showed that human amniotic epithelial cells treated with verbenalin might exert therapeutic activity against Alzheimer's disease by regulating gene expression associated with neurometabolic aging, lysosomal dysfunction, pathological angiogenesis, and 24 h periodic rhythm. Furthermore, verbenalin has been reported to significantly reduce cell death [15].

Studies are currently being conducted to investigate the efficacy of verbenalin in inhibiting Aβ peptide production, the effect of verbenalin on the behavioral characteristics of APPswe mice with dementia, and the anti-inflammatory effect of verbenalin on lipopolysaccharide-activated BV2 microglial cells. To evaluate the safety of verbenalin, a single oral dose toxicity study using ICR mice is also ongoing. This study was conducted to investigate the genetic toxicity of verbenalin.

To investigate the potential mutagenicity of verbenalin, the Ames test was performed with *Escherichia coli* and *Salmonella typhimurium* strains that required specific amino acids. The study attempted to determine whether specific chemicals could mutate the DNA of the test organisms [25]. Based on the number of revertant colonies, there was no mutagenic activation by verbenalin in all strains compared with the control group regardless of metabolic activity.

CONCLUSION

All bacterial strains (TA98, TA100, TA 1535, TA1537, and WP2uvrA) showed a negative result over the tested dose range. Therefore, verbenalin is a non-genotoxic substance.

A bacterial reverse mutation test for verbenalin was conducted in this study. Furthermore, the findings of an ongoing single oral toxicity study will be reported in the future. Nevertheless, safety tests including carcinogenicity, repeated oral toxicity, reproductive toxicity, and local toxicity tests should be conducted. Additional toxicity studies with different doses of verbenalin are needed to confirm its safety.

ACKNOWLEDGMENT

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP; Ministry of Science, ICT & Future Planning) (No. NRF-2018R1D1A1B07044595).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Hye Jeong Shin, https://orcid.org/0000-0002-5434-7240
Yi Gun Lim, https://orcid.org/0000-0002-7859-4078
Ji Su Ha, https://orcid.org/0000-0001-9486-8924
Gabsik Yang, https://orcid.org/0000-0002-9158-6531
Tae Han Yook, https://orcid.org/0000-0001-6379-7596

REFERENCES

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders: DSM-5. 5th ed. Washington, D.C.: American Psychiatric Association; 2013.
2. World Health Organization. Fact Sheets of Dementia [Internet]. Geneva: WHO; 2021 [cited 2022 Jul 7]. Available from: https://www.who.int/health-topics/dementia#tab=tab_1.
3. Oriental Neuropsychiatry Editorial Committee of Korean Medicine schools. Oriental neuropsychiatry: revised ed. Gyeonggi: Jipmoondang; 2011. 332-41 p.
4. Loew D. [Value of Ginkgo biloba in treatment of Alzheimer dementia]. Wien Med Wochenschr. 2002;152(15-16):418-22. German.
5. Xu SS, Gao ZX, Weng Z, Du ZM, Xu WA, Yang JS, et al. Efficacy of tablet huperzine-A on memory, cognition, and behavior in Alzheimer's disease. Acta Pharmacol Sin. 1995;16(5):391-5.
6. Park SJ, Jung JM, Lee HE, Lee YW, Kim DH, Kim JM, et al. The memory ameliorating effects of INM-176, an ethanolic extract of Angelica gigas, against scopolamine- or Aβ(1-42)-induced cognitive dysfunction in mice. J Ethnopharmacol. 2012;143(2):611-20.
7. Choi SH, Park CH, Seo JH, Suh YH. Dehydroevodiamine.HCl protects neuron from cytotoxicity induced by Aβ or oxidative stress: its antioxidant. J Neurochem. 2002;81(s1):101-5.
8. Lee JH, Lee EH, Jung EM, Kim DH, Kim SK, Park MH, et al. Perilla Frutescens extract protects against Scopolamine-induced memory deficits in mice. J Physiol Pathol Korean Med. 2021;35(3):97-103.
9. Gao YM, Wang XL, Deng YX, Reng JM, Shen XC, Guan ZZ, et al. Effect of Gastrodiae Rhizoma powder on prevention of de-

mentia and oxidation resistance in mice. Chin J Exp Tradit Med Formulae. 2020;24:52-8.
10. Bahramsoltani R, Rostamiasrabadi P, Shahpiri Z, Marques AM, Rahimi R, Farzaei MH. Aloysia citrodora Paláu (Lemon verbena): a review of phytochemistry and pharmacology. J Ethnopharmacol. 2018;222:34-51.
11. Bilia AR, Giomi M, Innocenti M, Gallori S, Vincieri FF. HPLC-DAD-ESI-MS analysis of the constituents of aqueous preparations of verbena and lemon verbena and evaluation of the antioxidant activity. J Pharm Biomed Anal. 2008;46(3):463-70.
12. Schönbichler SA, Bittner LK, Pallua JD, Popp M, Abel G, Bonn GK, et al. Simultaneous quantification of verbenalin and verbascoside in Verbena officinalis by ATR-IR and NIR spectroscopy. J Pharm Biomed Anal. 2013;84:97-102.
13. Cao L, Miao M, Qiao J, Bai M, Li R. The protective role of verbenalin in rat model of focal cerebral ischemia reperfusion. Saudi J Biol Sci. 2018;25(6):1170-7.
14. Makino Y, Kondo S, Nishimura Y, Tsukamoto Y, Huang ZL, Urade Y. Hastatoside and verbenalin are sleep-promoting components in Verbena officinalis. Sleep Biol Rhythms. 2009;7(3):211-7.
15. Ferdousi F, Kondo S, Sasaki K, Uchida Y, Ohkohchi N, Zheng YW, et al. Microarray analysis of verbenalin-treated human amniotic epithelial cells reveals therapeutic potential for Alzheimer's disease. Aging (Albany NY). 2020;12(6):5516-38.
16. Diehl MS, Willaby SL, Snyder RD. Comparison of the results of a modified miniscreen and the standard bacterial reverse mutation assays. Environ Mol Mutagen. 2000;36(1):72-7.
17. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res. 1983;113(3-4):173-215.
18. Korean Neuropsychiatric Association. Textbook of neuropsychiatry. 3rd ed. Seoul: iMiS Company; 2019. 579-89 p.
19. Kumar A, Singh A, Ekavali. A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacol Rep. 2015;67(2):195-203.
20. Lane RF, Dacks PA, Shineman DW, Fillit HM. Diverse therapeutic targets and biomarkers for Alzheimer's disease and related dementias: report on the Alzheimer's Drug Discovery Foundation 2012 International Conference on Alzheimer's Drug Discovery. Alzheimers Res Ther. 2013;5(1):5.
21. Wang R, Yan H, Tang XC. Progress in studies of huperzine A, a natural cholinesterase inhibitor from Chinese herbal medicine. Acta Pharmacol Sin. 2006;27(1):1-26.
22. Takata K, Kitamura Y, Saeki M, Terada M, Kagitani S, Kitamura R, et al. Galantamine-induced amyloid-{beta} clearance mediated via stimulation of microglial nicotinic acetylcholine receptors. J Biol Chem. 2010;285(51):40180-91.
23. Sastre J, Millán A, García de la Asunción J, Plá R, Juan G, Pallardó FV, et al. A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. Free Radic Biol Med. 1998;24(2):298-304.
24. Wang BS, Wang H, Wei ZH, Song YY, Zhang L, Chen HZ. Efficacy and safety of natural acetylcholinesterase inhibitor huperzine A in the treatment of Alzheimer's disease: an updated meta-analysis. J Neural Transm (Vienna). 2009;116(4):457-65.
25. OECD. Test No. 471: bacterial reverse mutation test. Paris: OECD; 1997.