Bioassay-guided isolation of antimutagenic naphthoquinones from Plumbago auriculata

Daisuke Imahori
Sanyo-Onoda City University
Takuya Muraoka
Sanyo-Onoda City University
Keiko Inami
Sanyo-Onoda City University
Hiroyuki Tanaka
htanaka@rs.socu.ac.jp

Sanyo-Onoda City University

Short Report

Keywords: Plumbago auriculata, roots, naphthoquinones, Ames test, mutagenicity, chemoprevention

Posted Date: December 22nd, 2025

DOI: https://doi.org/10.21203/rs.3.rs-8331701/v1

Additional Declarations: No competing interests reported.

Abstract

Background

Mutagen-induced genotoxicity plays a central role in carcinogenesis, highlighting the need for effective chemopreventive agents. Naphthoquinones, such as plumbagin, exhibit diverse biological activities; however, their antimutagenic potency at pharmacologically relevant concentrations remains poorly characterized. In this study, the acetone extract of *Plumbago auriculata* roots was subjected to bioassay-guided fractionation, yielding two known naphthoquinone derivatives: plumbagin and *cis*-isoshinanolone.

Results

Antimutagenic activity was evaluated using the Ames test with *Salmonella typhimurium* TA1535 against *N*-methyl-*N*-nitrosourea. The chloroform-soluble fraction (PAC) exhibited stronger activity than the residual fraction (PAAR), and purified plumbagin potently inhibited mutagen-induced revertant formation at nanomolar concentrations. No toxicity was observed at any of the tested doses.

Conclusions

These findings provide the first evidence that *P. auriculata* is a source of nanomolar-active antimutagenic naphthoquinones, highlighting their chemopreventive potential and expanding the biological relevance of quinone-type natural products beyond cytotoxicity.

1. Introduction

Cancer is a leading cause of morbidity and mortality worldwide, underscoring the need for effective chemopreventive strategies [1]. Mutations are key drivers of the initiation and progression of various diseases, including atherosclerosis, heart disease, and cancer [2]. Therefore, preventing mutational events at an early stage is an important strategy for cancer chemoprevention [3]. Among the multiple factors that contribute to carcinogenesis, genotoxic insults caused by mutagens play a central role in initiating DNA damage and promoting malignant transformation [4]. Mutagens can be broadly classified into physical (ionizing radiation), chemical (alkylating agents and polycyclic aromatic hydrocarbons), and biological agents (viruses) [5]. Chemical mutagens are of particular concern because of their widespread presence in the environment, food, and pharmaceutical products [5]. One such compound, *N*-methyl-*N*-nitrosourea (MNU), is a potent direct-acting carcinogen that induces cancer in multiple organs, primarily the forestomach, brain, and nervous system [6]. MNU exerts genotoxic effects by generating reactive methyldiazonium ions that methylate the O^6 position of guanine, producing O^6-methylguanine lesions [7]. These lesions mispair with thymine during DNA replication, predominantly causing GC transition mutations [7]. Several *in vitro* and *in vivo* studies using animal models have demonstrated the antimutagenic potential of phytochemicals, including phenylpropanoids, triterpenoids, flavonoids,

isoflavones, and naphthoquinones [2, 8, 9]. However, only a limited number of natural compounds have exhibited strong antimutagenic effects, and identifying new and highly potent agents remains an important research objective [10].

Plumbago species and other members of the Plumbaginaceae family biosynthesize various naphthoquinones, among which plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is the most prominent [11]. Plumbagin exhibits a broad spectrum of biological activities, including antimicrobial, cytotoxic, anti-inflammatory, antioxidant, and antitumor effects [11, 12]. Recent studies have highlighted its potential antimutagenic properties, particularly its ability to suppress mutagenicity induced by reactive oxygen species and nitroaromatic compounds in short-term bacterial mutation assays, such as the Ames test [2]. However, most of these findings are based on crude plant extracts or high micromolar concentrations, and the antimutagenic potency of purified plumbagin and related naphthoquinones at nanomolar concentrations remains underexplored [11]. Moreover, most studies have focused on *P. zeylanica* [13] and *P. indica* [11], whereas other species such as *P. auriculata*, an ornamental plant widely cultivated in subtropical regions [14], have received limited attention. Although *P. auriculata* is taxonomically close to medicinally relevant *Plumbago* species and is known to accumulate naphthoquinone derivatives, comprehensive phytochemical and antimutagenic evaluations of this species are lacking.

In this study, we isolated and structurally characterized two known naphthoquinone derivatives, including plumbagin, from *P. auriculata* roots using bioassay-guided fractionation. Purified plumbagin exhibited potent antimutagenic activity at nanomolar concentrations, indicating that *P. auriculata* is an underrecognized source of highly active antimutagenic compounds. To the best of our knowledge, this is the first report demonstrating the nanomolar-level antimutagenic activity of plumbagin derived from this species. These findings highlight the chemopreventive potential of naphthoquinones and further support their broad biological relevance, beyond cytotoxicity.

2. Materials and methods

2.1. General experimental procedures

Specific rotations were measured using a P-2200 digital polarimeter (l = 5 cm; JASCO, Tokyo, Japan). Fourier-transform infrared (FT-IR) spectra were recorded using a JASCO FT/IR-4600 spectrometer. Ultraviolet–visible (UV–vis) spectra were acquired using a Shimadzu UV-1850 spectrophotometer (Shimadzu, Kyoto, Japan). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was performed using a JMS-T100LP AccuTOF LC-Plus 4G instrument (JEOL, Tokyo, Japan). ^{1}H (600 MHz), ^{13}C (150 MHz), and 2D nuclear magnetic resonance (NMR) spectra were recorded using a JEOL JNM-ECZ 600R spectrometer. Normal-phase silica gel column chromatography was performed using Wakogel® 60N (FUJIFILM Wako Pure Chemical, Osaka, 63–212 μm). Thin-layer chromatography (TLC) was performed using TLC plates precoated with $60F_{254}$ silica gel (Merck, Darmstadt, Germany; 0.25 mm, ordinary phase). High-performance liquid chromatography (HPLC) was performed using an SPD-10A*vp*

UV–vis detector (Shimadzu, Kyoto, Japan), an LC-10AD*vp* pump (Shimadzu), an SCL-10A*vp* system controller (Shimadzu), and LabSolutions LC software (version 1.25, Shimadzu) with a COSMOSIL 5C_{18}-AR-II column (Nacalai Tesque, 250 × 4.6 mm i.d. and 250 × 10 mm i.d.) for analytical and preparative purposes, respectively. The solvent ratios were calculated based on the volume. FT-IR, UV–Vis, and HR-ESI-MS spectra were recorded using spectroscopic-grade methanol (Wako Pure Chemical, Japan). Bacto Agar and Nutrient Broth were obtained from Becton Dickinson Microbiology Systems (San Jose, CA, USA). Sodium ammonium hydrogen phosphate tetrahydrate was purchased from Merck (Darmstadt, Germany). MNU was prepared as previously described [15]. Genistein was purchased from TCI (Tokyo, Japan).

2.2. Plant material

Plumbago auriculata was purchased from TAKII & Co. Ltd. (Kyoto Prefecture, Japan) (voucher specimen number: SOCU-2025-2). Voucher specimens were deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Sanyo-Onoda City University, Yamaguchi, Japan. The plant material was examined and identified by Prof. Hiroyuki Tanaka (PhD), Department of Pharmacognosy and Kampo, Faculty of Pharmaceutical Sciences, Sanyo-Onoda City University. The deposited voucher specimens, including roots and leaves, were shade-dried and stored.

2.3. Extraction and bioassay-guided fractionation

Dried roots (236.0 g) of *P. auriculata* were extracted three times with acetone for 3 h using a modified procedure based on a previously reported method [16]. Solvent evaporation yielded an acetone extract (PAA, 5.9 g, 2.50%). Additionally, a chloroform-soluble fraction (PAC, 1.2 g, 0.51%) and a residual fraction (PAAR, 4.7 g, 1.99%) were obtained from the acetone extract. The PAC fraction was subjected to normal-phase silica-gel column chromatography (100.0 g, *n*-hexane: chloroform [1:0 → 1:1 → 0:1]), yielding five fractions (PAC1–5). Compound **1** (500.0 mg, 0.21%) was obtained as an orange, amorphous solid from PAC3. Compound **2** (25.5 mg, 0.01%) was obtained as an orange amorphous solid after purification of PAC4 (1.0 g) using HPLC [H_2O:MeCN:AcOH (60:40:0.3)].

2.4. Plumbagin (1)

Orange amorphous solid; ^{1}H NMR (chloroform-*d*, 600 MHz) δ_H 6.80 (1H, m, H-2), 2.17 (1H, d, *J* = 1.8 Hz, Me-3), 7.25 (1H, d, *J* = 1.8, 7.8 Hz, H-5), 7.61 (1H, d, *J* = 7.8 Hz, H-6), 7.63 (1H, dd, *J* = 1.8, 7.8 Hz, H-7), 11.97 (1H, s, OH-8) and ^{13}C NMR (chloroform-*d*, 150 MHz) δ_C 190.2 (C-1), 135.4 (C-2), 149.6 (C-3), 16.5 (Me-3), 184.8 (C-4), 132.0 (C-4a), 119.3 (C-5), 136.1 (C-6), 124.1 (C-7), 161.1 (C-8), 115.1 (C-8a); ESI-MS; *m*/*z*: 189 $[M + H]^+$.

2.5. cis-Isoshinanolone (2)

Orange amorphous solid; $[a]_D^{25}$ +29.5 (*c* 0.2, MeOH); ^{1}H NMR (chloroform-*d*, 600 MHz) δ_H 2.56 (1H, dd, *J* = 4.2, 18.0 Hz, H-2), 2.87 (1H, dd, *J* = 10.8, 18.0 Hz, H-2), 2.43 (1H, m, H-3), 1.17 (1H, d, *J* = 7.2 Hz, Me-3), 4.74 (1H, d, *J* = 2.4 Hz, H-4), 6.92 (1H, br s, H-5), 7.48 (1H, t-like, *J* = 8.4 Hz, H-6), 6.94 (1H, br d, *J* = 1.8 Hz,

H-7), 12.42 (1H, s, OH-8) and ^{13}C NMR (chloroform-*d*, 150 MHz) δ_C 204.8 (C-1), 40.7 (C-2), 34.4 (C-3), 16.2 (Me-3), 71.1 (C-4), 145.0 (C-4a), 118.6 (C-5), 136.9 (C-6), 118.1 (C-7), 162.6 (C-8), 115.09 (C-8a); ESI-MS; *m/z*: 193 $[M + H]^+$.

2.6. Bacterial mutation assay

The bacterial mutation assay was performed as described by Maron et al. and Brusick et al. [17, 18]. Dr. T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) kindly provided *S. typhimurium* TA1535. A solution of MNU (1.5 μmol/50 μL of DMSO) was added to a test tube and supplemented with 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL), a solution (50 μL) with various concentrations of each fraction, and a culture of *S. typhimurium* TA1535 (0.1 mL), and the mixture was thoroughly mixed. Next, top agar (2 mL) was added, and the mixture was poured onto a minimal glucose agar plate. Revertant colonies were counted after incubation at 37°C for 44 h. The experiments were performed in triplicate and repeated three times with similar results. The results are expressed as mean ± standard error (SE). Plates without MNU or plant extracts were used as negative controls. MNU (1.5 μmol/50 μL) yielded 1316 ± 72 colonies. All tested plates were microscopically examined for thinning, absence of background lawn, and/or presence of microcolonies, which are considered indicators of toxicity induced by the test material. Neither MNU nor the plant extracts exhibited toxicity toward *S. typhimurium* TA1535 in the antimutagenicity test. Mutagenic activity in the presence of the extracts was expressed as the percentage of mutagenicity (% = Rs/R × 100), where Rs is the number of His + revertants/plate for plates exposed to MNU and plant extracts, and R is the number of His + revertants/plate of MNU. The number of spontaneous revertants was subtracted to obtain Rs and R. Thus, the mutagenicity of MNU in the absence of plant extracts was defined as 100%.

2.7. Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) or GraphPad Prism 8.43 software (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Bioassay-guided isolation of antimutagenic naphthoquinones from P. auriculata

The acetone extract of *P. auriculata* roots was partitioned with chloroform to obtain PAC and PAAR. PAC was subjected to normal-phase silica gel column chromatography, followed by repeated HPLC to obtain two naphthoquinone derivatives, plumbagin (**1**) [19] and *cis*-isoshinanolone (**2**) [20] (Fig. 1 for compound structures). Antimutagenic activity was evaluated *in vitro* using the Ames assay with *S. typhimurium* TA1535. The individual activities of PAA, its fractions (PAC and PAAR), and isolated compounds (**1** and **2**) are summarized in Table 1. *Plumbago* species and other members of the Plumbaginaceae family are rich sources of bioactive compounds, including naphthoquinones, flavonoids, and sterols [11]. The primary objective of this study was to isolate and characterize naphthoquinone derivatives from *P.*

auriculata roots and to evaluate their antimutagenic potency. Our results confirmed that *P. auriculata* contains naphthoquinones, such as plumbagin, which is consistent with previous reports [21]. Furthermore, PAA exhibited antimutagenic activity against MNU in the Ames test using *S. typhimurium* TA1535 (IC_{50}: 44.7 ± 9.89 μg/plate), comparable to that previously reported for licorice extracts [6]. Among the fractions, PAC exhibited stronger antimutagenic activity than PAAR. Compound **1** exhibited greater potency (IC_{50}: 12.9 ± 0.86 nmol/plate) than compound **2** (IC_{50}: >2.0 μmol/plate), suggesting that the distinct stereochemistry of their quinone ring structures may influence their target interactions [2]. As a positive control, the flavonoid genistein was employed, and its antimutagenic activity against MNU showed an IC_{50} value of 1.29 ± 0.02 μmol/plate. No toxicity was observed at any of the concentrations tested. However, several limitations of this study should be acknowledged. First, the antimutagenic activity was evaluated only by the Ames test, which, although widely accepted, does not fully reflect the complexity of mammalian cellular responses to mutagens. Second, the mechanistic basis underlying the observed activity of plumbagin (**1**) and *cis*-isoshinanolone (**2**) was not investigated in detail, therefore, the precise molecular targets and pathways remain to be clarified. These limitations suggest that further studies using mammalian cell-based systems, DNA damage assays, and broader metabolite profiling will be necessary to confirm the protective effects and to elucidate the mechanisms involved. These findings provide the first evidence that *P. auriculata* is a source of nanomolar-active antimutagenic naphthoquinones, supporting its potential as a chemopreventive agent.

Table 1
Antimutagenic activity of the *Plumbago auriculata* extract, fractions, and compounds against *N*-methyl-*N*-nitrosourea (MNU)

Dose (µg/plate)	**Revertants/plate (Mutagenicity %)**					
	0	**15.625**	**31.25**	**62.5**	**125**	**250**
PAA	1570 ± 147 (100.0%)	1213 ± 141 (77.1%)	862 ± 52 (54.6%)	706 ± 53 (44.6%)	525 ± 50 (33.0%)	223 ± 88 (12.6%)
Dose (µg/plate)	0	50	100			
PAC	1505 ± 29 (100.0%)	455 ± 48 (29.5%)	105 ± 9 (6.0%)			
PAAR	1505 ± 29 (100.0%)	1447 ± 46 (96.1%)	1260 ± 34 (83.5%)			
Dose (µg/plate)	0	25	50			
PAC 1	1570 ± 16 (100.0%)	1579 ± 44 (100.6%)	1329 ± 72 (84.5%)			
PAC 2	1570 ± 16 (100.0%)	1353 ± 67 (86.0%)	1371 ± 54 (87.2%)			
PAC 3	1570 ± 16 (100.0%)	30 ± 5 (0.7%)	5 ± 2 (0.0%)			
PAC 4	1570 ± 16 (100.0%)	1518 ± 53 (96.6%)	1289 ± 66 (81.9%)			
PAC 5	1570 ± 16 (100.0%)	1465 ± 54 (93.2%)	1398 ± 46 (88.9%)			
Dose (nmol/plate)	0	6.25	12.5	25	50	100
1	1515 ± 56 (100.0%)	997 ± 124 (65.4%)	736 ± 16 (47.9%)	546 ± 26 (35.2%)	276 ± 67 (17.1%)	57 ± 13 (2.5%)
Dose (µmol/plate)	0	0.125	0.25	0.5	1.0	2.0
2	1330 ± 87	1356 ± 136	1240 ± 123	1242 ± 140	1062 ± 70	835 ± 120

Dose (µg/plate)	Revertants/plate (Mutagenicity %)					
	0	**15.625**	**31.25**	**62.5**	**125**	**250**
	(100.0%)	(102.0%)	(93.2%)	(93.3%)	(79.7%)	(62.4%)
Dose (µmol/plate)	0	0.25	0.5	1.0	1.5	2.0
Genistein	1270 ± 96 (100.0%)	1190 ± 103 (93.7%)	1112 ± 38 (87.6%)	816 ± 58 (64.3%)	520 ± 37 (41.0%)	338 ± 72 (26.6%)

4. Conclusion

This study provides the first evidence that *P. auriculata* roots contain antimutagenic naphthoquinones, plumbagin (**1**) and *cis*-isoshinanolone (**2**), isolated through bioassay-guided fractionation. Purified plumbagin (**1**) potently inhibited mutagen-induced revertant formation at nanomolar concentrations, a level of activity rarely reported for natural compounds. PAC exhibited stronger antimutagenic activity than PAAR, and compound **1** (plumbagin) was more active than compound **2**, suggesting that stereochemical differences in the quinone ring influence their biological interactions. No toxicity was observed at any of the concentrations tested. These findings highlight *P. auriculata* as a previously underrecognized source of highly active antimutagenic constituents and underscore the chemopreventive potential of naphthoquinones beyond their classical cytotoxic properties.

Declarations

Ethical Approval

Not applicable.

Statement of Informed Consent

No human participants were included in this study; therefore, informed consent was not required.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. All experimental data supporting the findings, including raw values from Ames tests and compound characterization (HPLC profiles, NMR spectra), are archived at Department of

Pharmacognosy, Faculty of Pharmaceutical Sciences, Sanyo-Onoda City University, Yamaguchi, Japan and can be provided upon request for verification and replication purposes.

Declaration of conflicting interest

The authors declare no potential conflicts of interest regarding the research, authorship, or publication of this study.

Funding statements

Not applicable.

Authors contribution

Takuya Muraoka: Writing–original draft, project administration, conceptualization. Daisuke Imahori: Data curation. Hiroyuki Tanaka: Data curation. Keiko Inami: Writing–review and editing, Supervision, Conceptualization.

Acknowledgements

We would like to thank Editage (www.editage.com) for the English language editing.

References

1. Wu Z, Xia F, Lin R. Global burden of cancer and associated risk factors in 204 countries and territories, 1980–2021: a systematic analysis for the GBD 2021. J Hematol Oncol. 2024;17(1):119. 10.1186/s13045-024-01640-8.
2. Kumar S, Gautam S, Sharma A. Antimutagenic and antioxidant properties of plumbagin and other naphthoquinones. Mutat Res. 2013;755(1):30–41. 10.1016/j.mrgentox.2013.05.007.
3. Seong H, Izutsu R, Osaki M, Okada F. Cancer prevention: past challenges and future directions. Genes Environ. 2025;47(1):4. 10.1186/s41021-025-00326-y.
4. Greim H, Albertini JR. Cellular response to the genotoxic insult: the question of threshold for genotoxic carcinogens. Toxicol Res. 2015;4(1):36–45. 10.1186/s13045-024-01640-8.
5. Goyal K, Goel H, Baranwal P, Dixit A, Khan F, Jha NK, Kesari KK, Pandey P, Pandey A, Benjamin M, Maurya A, Yadav V, Sinh RS, Tanwar P, Upadhyay TK, Mittan S. Unravelling the molecular mechanism of mutagenic factors impacting human health. Environ Sci Pollut Res Int. 2022;29(41):61993–2013. 10.1007/s11356-021-15442-9.
6. Tatsuzaki J, Jinwei Y, Kojo Y, Mine Y, Ishikawa S, Mochizuki M, Inami K. Antimutagenicity screening of extracts from medicinal and edible plants against *N*-methyl-*N*-nitrosourea by the Ames assay. Nat Prod Res. 2014;36(2):39–46.
7. Zhang LH, Jenssen D. Site specificity of *N*-methyl-*N*-nitrosourea-induced transition mutations in the hprt gene. Carcinogenesis. 1991;12(10):1903–9. 10.1093/carcin/12.10.1903.

8. Akram M, Riaz M, Wadood AWC, Hazrat A, Mukhtiar M, Zakki SA, Daniyal M, Shariati MA, Khan FS, Zainab R. Medicinal plants with antimutagenic potential. Biotechnol Biotechnol Equip. 2020;34(1):309–18. 10.1080/13102818.2020.1749527.
9. Edenharder R, Worf-Wandelburg A, Decker M, Platt KL. Antimutagenic effects and possible mechanisms of action of vitamins and related compounds against genotoxic heterocyclic amines from cooked food. Mutat Res. 1999;444(1):235–48. 10.1016/s1383-5718(99)00098-4.
10. Malik S, Kaur K, Prasad S, Jha NK, Kumar V. A perspective review on medicinal plant resources for their antimutagenic potentials. Environ Sci Pollut Res Int. 2022;29(41):62014–29. 10.1007/s11356-021-16057-w.
11. Abdu A, Prakash A, Kondal R, Sharma S, Bhagat M, Pal R, Singh H, Singh B, Kaur S. Comprehensive review on Plumbago indica: Traditional, pharmacological insights and conservation strategies. J Appl Pharm Sci. 2024;14(11):1–16. 10.7324/JAPS.2024.200049.
12. Yin Z, Zhang J, Chen L, Guo Q, Yang B, Zhang W, Kang W. Anticancer Effects and Mechanisms of Action of Plumbagin: Review of Research Advances. Biomed Res Int. 2020;2020:6940953. 10.1155/2020/6940953.
13. Shukla B, Saxena S, Usmani S, Kushwaha P. Phytochemistry and pharmacological studies of *Plumbago zeylanica* L.: a medicinal plant review. Clin Phytosci. 2021;7:34. 10.1186/s40816-021-00271-7.
14. Saji A, Antony VT. *Plumbago auriculata* Lam. Int J Pharm Sci Rev Res. 2015;33(2):281–4.
15. Inami K, Asada Y, Harada T, Okayama Y, Usui N, Mochizuki M. Antimutagenic components in *Spatholobus suberectus* Dunn against *N*-methyl-*N*-nitrosourea. Genes Environ. 2019;41:22. 10.1186/s41021-019-0137-4.
16. Kapadia NS, Isarani SA, Shah MB. A simple method for isolation of plumbagin from roots of *Plumbago rosea*. Pharm Biol. 2005;43(6):551–3. 10.1080/13880200500220888.
17. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res. 1983;113(3–4):173–215. 10.1016/0165-1161(83)90010-9.
18. Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. Mutat Res. 2000;455(1–2):29–60. 10.1016/S0027-5107(00)00064-6.
19. Kaewbumrung S, Panichayupakaranant P. Isolation of three antibacterial naphthoquinones from *Plumbago indica* roots and development of a validated quantitative HPLC analytical method. Nat Prod Res. 2012;26(21):2020–3. 10.1080/14786419.2011.652927.
20. Bringmann G, Günther C, Ochse M, Schupp O, Tasler S. Synthesis of enantiopure naphthoquinones by oxidative coupling: a biomimetic approach. Phytochemistry. 1999;51(5):693–9. 10.1016/S0031-9422(99)00053-0.
21. Deshpande J, Labade D, Shankar K, Kata N, Chaudhari M, Wani M, Khetmalas M. In vitro callus induction and estimation of plumbagin content from Plumbago auriculata Lam. Indian J Exp Biol. 2014;52:1122–7.

Figures

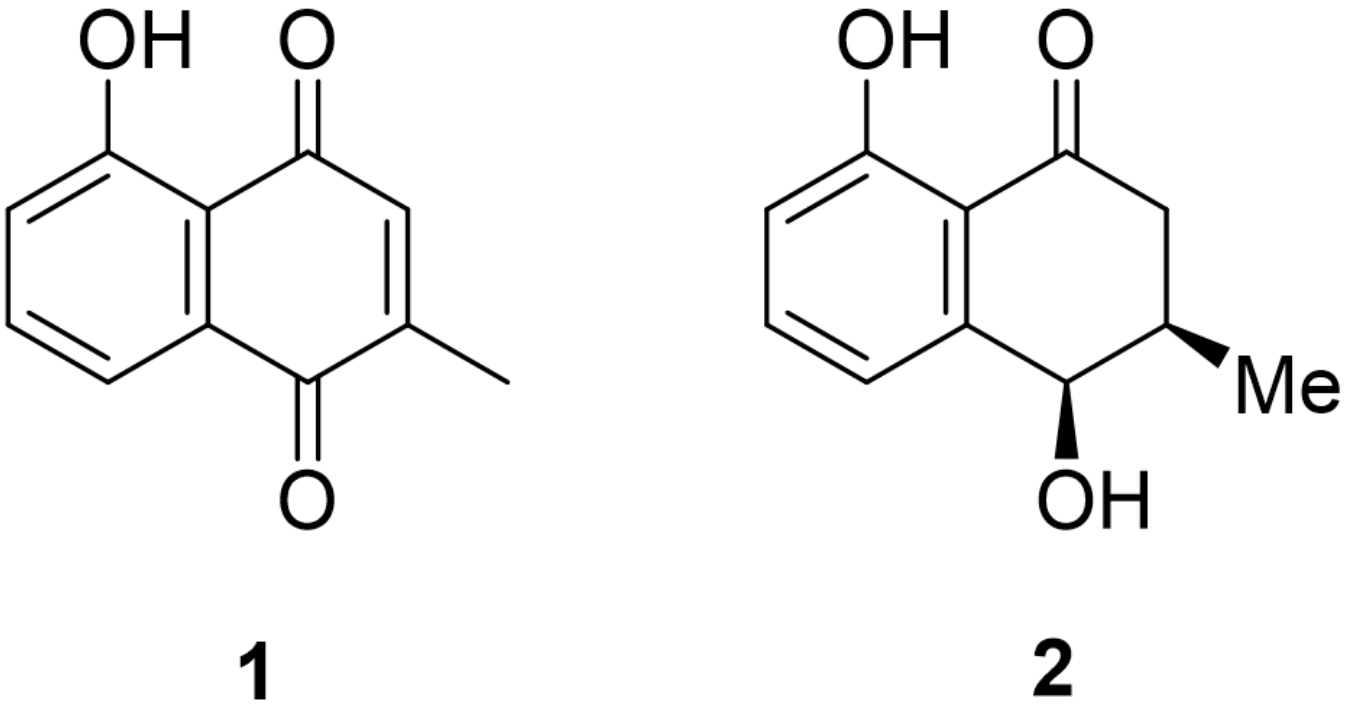

Figure 1

Chemical structures of compounds isolated from *Plumbago auriculata* roots