Research Journal of Pharmacognosy (RJP) 11(2), 2024: 19–25
Received: 17 Oct 2023
Final revision: 24 Dec 2023
Accepted: 27 Jan 2024
Published online: 31 Jan 2024
DOI: 10.22127/RJP.2024.420686.2249

Original article

Evaluation of Wound Healing and Cytotoxic Activities of Anacardic Acid (13:0) Isolated from *Pistacia vera* Hull Extract

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Abstract

Background and objectives: *Pistacia vera* fruit is a popular nut belonging to Anacardiaceae family. Traditionally, the hulls have been used as herbal remedies for treatment of oral and skin wounds, peptic ulcers and hemorrhoids. **Methods:** In this study, anacardic acid (13:0) was elucidated by EI-MS, FTIR, 1D-NMR and 2D-NMR data analysis from active fraction. Cytotoxic activity was assessed against normal NIH/3T3 cells, and several cancerous human cells, including human breast cancer (MCF-7), hepatocarcinoma (HepG-2) and gastric cancer (MKN-45) using MTT assay. The wound healing activity of this compound was evaluated using in vitro scratch-wound healing assay on NIH/3T3 cells. **Results:** Anacardic acid (13:0) was toxic at the concentrations tested against all cell lines (6.25-100 µg/mL). The selectivity index showed no selective cytotoxicity (SI< 2); however, anacardic acid (13:0) revealed significant wound healing effects through the migration of NIH/3T3 cells at the concentrations of 1.25-5 µg/mL. **Conclusion:** These results suggested that anacardic acid (13:0) from *P. vera* hull has cytotoxic activity on human cancer cell lines and can also be useful as a bioactive molecule in wounds treatment. However, more in vitro and in vivo studies need to be done to confirm the efficacy and cytotoxicity of anacardic acid (13:0).

Keywords: anacardic acid; cytotoxicity; *Pistacia vera*; wound

Citation: Sarkhail P, Hashemi Gh, Souri E. Evaluation of wound healing and cytotoxic activities of anacardic acid (13:0) isolated from *Pistacia vera* hull extract. Res J Pharmacogn. 2024; 11(2): 19–25.

Introduction

Pistacia vera L. hull, one of the largest by-products in pistachio industry is rich of nutrients and medicinal agents and possesses multiple therapeutic properties. Different parts of pistachios have been traditionally used as antibacterial, antiviral, anti-inflammatory and antitumor agents [1-5]. For example, in Iranian traditional medicine, the hull and gum mastic (oleoresin) of *P. atlantica* and *P. vera* are used for periodontal ailments and toothache treatment, oral and skin wounds, stomachache, peptic ulcers and haemorrhoids [6,7]. The gum mastic from *P. lentiscus* has been used for its antitumor action and antimicrobial effect in many traditional medicines [8], and it has been already vindicated that the gum has anti-*Heliobacter pylori* activity and anticancer effects against prostate and colorectal cancer [9].

Pharmacological investigations have confirmed that pistachio hull has several potential health benefits such as wound healing, antimicrobial, antioxidant, and anti-angiogenic activities [6,10].

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A few reported studies described the beneficial effects of pistachio hulls in different cancer cell lines such as SKMEL-3, HepG-2, MCF-7, and colon cancer cell lines [11-14]. Pistachio hull is known as a good source of fatty acids, alkylphenolic acids, triterpenoids, resins, polyphenolics, flavonoids, essential oil and bitter substances and some of these compounds were found to play an important role in different beneficial biological activities [15-18].

A previous study has suggested that the non-polar extract of *P. vera* hull is rich of bioactive compounds with wound healing activity, and 3-epimasticadienolic acid was found to reduce the scratch area through fibroblast stimulation and modulation of inflammatory response [19]. Based on prior research findings, this study aimed to identify the major compound from *P. vera* hull $CHCl_3$ extract that may be responsible for the wound healing activity. In addition, due to numerous reports regarding the anticancer effects of pistachios hull, the cytotoxic effects has also been evaluated.

Material and Methods

Ethical considerations

This study was approved by the ethics committee of Tehran University of Medical Sciences (TUMS) and received ethical Code number: IR.TUMS.TIPS.REC.1400.012.

General experimental procedures

The UV spectra were recorded at the wavelength of 200–400 nm using a Shimadzu UV-160A spectrophotometer (Japan). FT-IR spectrum was performed on Nicolet Magna 550-IR (USA) in the range of 4000–400 cm^{-1}. EI-MS spectrum was recorded on a mass spectrometer (Agilent Technologies, USA) with electron ionization at 70 eV and quadruple analyzer. All the NMR spectra were recorded on a Bruker Avance 400 MHz (Germany) using tetramethylsilane (TMS) as the internal standard. A BioTek Synergy HT Microplate Reader (Germany) was used to measure absorption in MTT assay.

Chemicals

Silica gel 60 G (70–230 mesh) was used as the stationary phase in column chromatography and pre-coated silica gel plates (Kieselgel 60 F-254, 0.5 mm, Merck, Germany) were used for preparative thin layer chromatography (PTLC). Thin layer chromatography (TLC) was performed on silica gel F-254 (Merck, Germany) pre-coated aluminum sheets. All other solvents and chemicals used in experiments were obtained from Merck (Germany). The reagents used for the cytotoxicity activity were of high purity and were purchased from Sigma-Aldrich (Germany). Cisplatin was provided from Mylan, USA as a 10 mg/mL solution. The cell lines were obtained from the cell bank of Iranian Biological Resource Center (IBRC), Iran.

Plant material

Pistacia vera L. fruits (Anacardiaceae) were purchased from the local market in Tehran, Iran and validated by the corresponding author. A voucher specimen (No. 1391-3) was deposited at Pharmaceutical Sciences Research Center (PSRC) laboratory, Tehran University of Medical Sciences.

Extraction and isolation

The separated pink-white hulls were dried at vacuum (50 °C). Twenty grams of crispy hulls were crushed with an electric blender and extracted with MeOH (80%). The dried extract (3.72 g) was successively partitioned to the $CHCl_3$, EtOAc and n-BuOH fractions. Five fractionations from $CHCl_3$ extract were achieved by chromatographic techniques as described by Sarkhail et al. [19]. Subsequently, PTLC technique was carried out to separate three sub-fractions (Fr4.I, Fr4.II and Fr4.III). After monitoring the subfractions by TLC with mobile phase (hexane: ethyl acetate 3.5:1.5), a major compound was detected in subfraction Fr4.II, which was visualized under UV light (254 and 366 nm). This compound was separated and purified from Fr4.II with PTLC and was identified by EI-MS, FTIR, 1D-NMR and 2D-NMR data analysis spectrometry. Before cytotoxic assay, final purification was carried out by crystallization in MeOH to yield pure compound (5 mg).

Cytotoxicity assay

Cell growth inhibitory assay was performed by MTT method [20] on four cell lines, including MCF-7, HepG-2, MKN-45, and NIH/3T3. In brief, the cell lines were cultured in DMEM, including 10% FBS and 1% antibiotic solution (100 U/mL penicillin and 100 µg/mL streptomycin). Then they were incubated at 37 °C in 5% CO_2 and humidity of 80%. After 24 h, the

culture medium was removed and replaced with fresh medium. The cells were then treated in triplicate with various concentrations (6.25 -100 µg/mL) of active compound and cisplatin as the positive control [21] for 48 h. After washing medium with phosphate buffer, 50 µL of MTT (0.5 µg/mL) was added and the cells were incubated for 3 hours at 37 °C. The formed purple formazan crystals were dissolved in DMSO for 30 min and the absorption was read using microplate reader at 570 nm. The background absorbance at 690 nm was subtracted from the absorbance at 570 nm. Data was represented as percent of control. The 50% inhibition concentration (IC_{50} value) was determined from dose-response curves.

In vitro scratch-wound healing assay

The ability of migration of NIH/3T3 cells into the wounded area was considered using scratch assay method [22]. Different concentrations of active compound (1.25, 2.5 and 5 µg/mL) and allantoin (at 50 µg/mL) as the positive control were used [23]. After 48 hours, the cells were fixed with 4% paraformaldehyde for 15 min and stained by DAPI for 1 min. Finally, the images were taken by a fluorescence microscope (Olympus BX51, Japan) and the changes in wound area were analyzed using "image J" software.

Statistical analysis

The results were expressed as the means of experiments in triplicate ± SEM. The group means were compared using the ANOVA test followed by Tukey post hoc test for multiple comparisons and $p<0.05$ was considered significant.

Results and Discussion

Fractionation and purification of the $CHCl_3$ extract of *Pistacia vera* (Anacardiaceae) hull by silica gel column chromatography and plate chromatography resulted in the isolation of a white amorphous powder. Its molecular formula, $C_{20}H_{32}O_3$, was identified by EI-MS m/z 320 $[M]^+$ corresponding to its ion molecular mass. The main fragment ions were observed at m/z: 302 $[M^+ - H_2O]^+$, 273, 175,160, 152 $[M^+ - C_{12}H_{25}]$, 147, 134, 105, 77, 43 (supplementary Figure S1). The UV spectrum of this compound showed absorption maxima at 322, 248, 216 and 232 nm and IR (cm^{-1}): 3200-3500 (O-H stretching vibration), 3104 and 3056 (aromatic ring), 2924 and 2854 (aliphatic C−H stretching vibration), 1647 (carboxyl C=O stretching band), 1608 (aromatic C=C stretching bands), 1450 (aliphatic C−H bending vibration), 1384, 1303, 1246, 1211, 819, and 711, indicating the presence of acidic, hydroxyl and aromatic ring. The spectral data of ^{1}H-NMR and ^{13}C-NMR are shown in supplementary Table S1. The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum displayed three aromatic protons at δ_H 7.35 (1H, t, J = 7.8 Hz, H-4), 6.86 (1H, d, J = 8.3 Hz, H-5) and 6.77 (1H, d, J = 7.6 Hz, H-3) typical of a 1, 2, 6-trisubstituted benzene ring. The presence of a methylene group bonded to aromatic ring was recognized by a triple downfield signal at δ_H 2.96 (2H, t, J = 10 Hz, H-1'(Ar-CH_2)). A multiple signal at δ_H 1.58 was attributed the methylene group attached to H-1'and the broad bond at δ_H 1.25-1.35 (protons signals overlapped), characterizing a chain CH_2 moiety corresponding to 20 protons. In addition, a triplet up field peak at δ_H 0.88 (3H, t, J = 7.5 Hz, H-13') was attributed to three hydrogens of a terminal methyl group present in this compound (supplementary Figure S2).

The ^{13}C-NMR spectrum in conjunction with the DEPT spectrum revealed 20 carbon resonance (supplementary Figures S3 and S4), including one carboxylic acid carbonyl group at δ_C 175.79, three aromatic methines (δ_C 115.89, 135.45, 122.78), three quaternary aromatics (δ_C 110.38, 147.82, 163.67), 13 methylene (CH_2, δ_C 22.72-36.52) and one methyl group at δ_C 14.15. The HSQC and ^{1}H-^{1}H COSY spectral correlations (presented in supplementary Figures S5 and S6) of this compound confirmed the resonances due to coupling of two aromatic protons that formed by coupling of one proton to both an ortho and a meta proton [δ_H 6.86 (d, J = 8.3 Hz, H-5) / δ_C 115.89), δ_H 6.77 (d, J = 7.6 Hz, H-3) / δ_C 122.78] and coupling of one proton to both ortho protons [δ_H 7.35 (t, J = 7.8 Hz, H-4) / δ_C 135.45]. In the HMBC spectrum, ^{1}H ^{13}C correlations of this compound finalized the structure (supplementary Figure S7), showing 3J correlations from the triplet proton δ_H 7.35 to the ^{13}C signals at δ_C 163.67 (C-2), 147.82 (C-6); of the signal at δ_H 6.86 with the ^{13}C signals at δ_C 110.38 (C-1), 122.78 (C-5), and 163.67 (C-2) and proton signal δ_H 6.77 with δ_C 110.38 (C-1), 115.89 (C-5). The methylene position at δ_H 2.96 was also confirmed with HMBC correlation of the signal with the ^{13}C signals at δ_C 31.93 (C-2'), 147.82 (C-6), 122.78 (C-5), and 110.38 (C-1). No range couplings

were observed in the HMBC spectrum to the δ_H 5.35 and 2.00 signals. The signals at δ_H 5.35 and at δ_C 129 are related to impurity peaks. Based on the above evidence and previous reported data [24,25] the structure of this compound was established as 2-hydroxy-6-tridecyl-benzoic acid (Figure 1), which commonly known as anacardic acid (13:0) or ginkgoneolic acid.

Anacardic acids (AAs) are known as natural salicylic acid derivatives that occur in several plants such as pistachio shells, nuts of ginkgo cashew nuts and nutmeg. Previous studies have described that AAs possesses high antioxidant activity. In addition, they showed antibacterial, larvicidal, antitumor, and inhibitory activity of several enzymes, including tyrosinase, xanthine oxidase, histone acetyltransferase (HAT), lipoxygenase, and cyclooxygenase (COX) [26-30]. However, to date, investigation of the biological activities of AA (13:0) is very limited [25].

In the present study, the cytotoxic activity of AA (13:0) and cisplatin (positive control) at a concentration range of 6.25-100 µg/mL against cancerous cell lines, and non-cancerous fibroblast cell line were determined by MTT assay. This compound showed an IC_{50} values of 18.90 µg/mL (towards MCF-7), 26.10 µg/mL (against HepG-2), to 17.73 µg/mL (towards MKN-45). It also displayed cytotoxic activity against NIH/3T3 normal cells with an IC_{50} value of 18.69 µg/mL (supplementary Figure S8). However, in comparison with cisplatin (IC_{50} < 6 µg/mL), the compound showed a lower cytotoxic effect against cancerous cell lines. On the other hand, the main objective of chemotherapy is to use compound with greater selectivity index (SI) value, which reflects the minimal cytotoxicity of a compound against normal cells and maximal cytotoxicity toward cancer cells. SI values lower than 2 suggest general toxicity of the compound [31]. The SI values in supplementary Table 2 indicate that AA (13:0) displays low cytotoxic selectivity and cannot be used as a (herbal) drug at these concentrations range. A previous research has revealed that the cytotoxic effect of AAs, rich of monomers C15:1, C17:1, and C13:0, against MCF-7 cells (the IC_{50} value of 23.8 µg/mL), which was almost similar to our results. The researchers also revealed the anti-proliferative activity against CNE-2Z, MD-MB-231, and H1975 cells with IC_{50} values ranging from 14.9 to 23.1 µg/mL [32]. The AAs from *P. lentiscus* fruit oil could induce apoptosis in normal NHDF cells (at concentrations above 50 µg/mL). According to the previous studies, the AAs C15:1, C13:0, and C17:1 have been identified as hazardous agents and showed significant toxic and side effects which lead to hepatotoxicity and skin irritation [33-35]. Previous reports have explained that the antitumor effects of AAs can be due to numerous mechanisms, including angiogenesis inhibition, anti-migratory, activation of apoptosis-inducing factor, inhibition of estrogen receptor α DNA binding, and intrinsic pathway executioner such as cytochrome c, nuclear factor-κB (NF-κB) inhibition [34-38].

According to the results, the wound healing activity of AA (13:0) was determined through scratching methods on NIH/3T3 cells at the concentrations ≤ 5 µg/mL that showed survival rates above 100%. Interestingly, all doses significantly (p<0.001) reduced wound area (31.92, 20.06 and 29.12% of the control) and this reduction was comparable with that of the positive control (allantoin showed down to 33.67% of control), however, this effect was not in a concentration dependent manner at the concentrations of 1.25, 2.5 and 5 µg/mL (Figure 2).

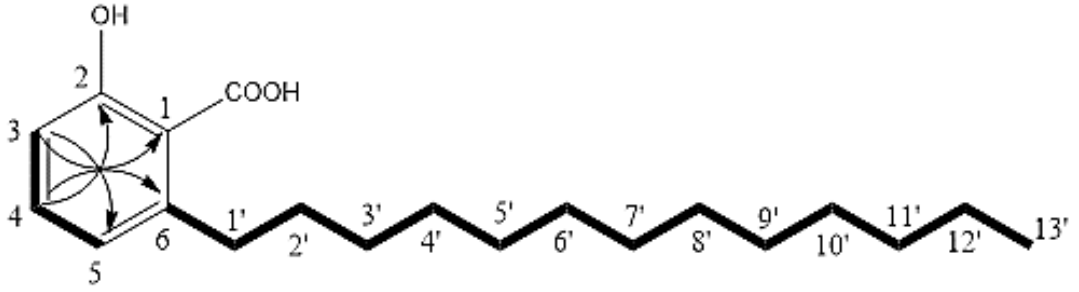

Figure 1. The key COSY (▬) and HMBC (→) correlations of anacardic acid

As a result, anacardic acid (13:0) was found to be one of the compounds responsible for the wound healing effect of $CHCl_3$ fraction from *P. vera* hull. In a previous study, the compound 3-epimasticadienolic acid from $CHCl_3$ fraction showed significant wound reduction activity at the concentrations higher than 20 µg/mL [19], while AA (13:0) can significantly reduce scratch wound area at the concentrations between 1.25 and 5 µg/mL. This result showed that the cell migration in the normal fibroblast cells increased at low concentrations (≤ 5 µg/mL) of anacardic acid (13:0), unlike its anti-migration effect on cancerous cells [32,38].

Conclusion

The findings of this study showed that anacardic acid (13:0) extracted from the $CHCl_3$ extract of *P. vera* hull has cytotoxic activity against three human cancer cell lines. In addition, AA (13:0) showed wound healing activity in vitro normal fibroblast cells for the first time. Thus, AA (13:0) can be useful as a bioactive molecule in wounds treatment after validation of the efficacy and cytotoxicity by in vitro and in vivo studies.

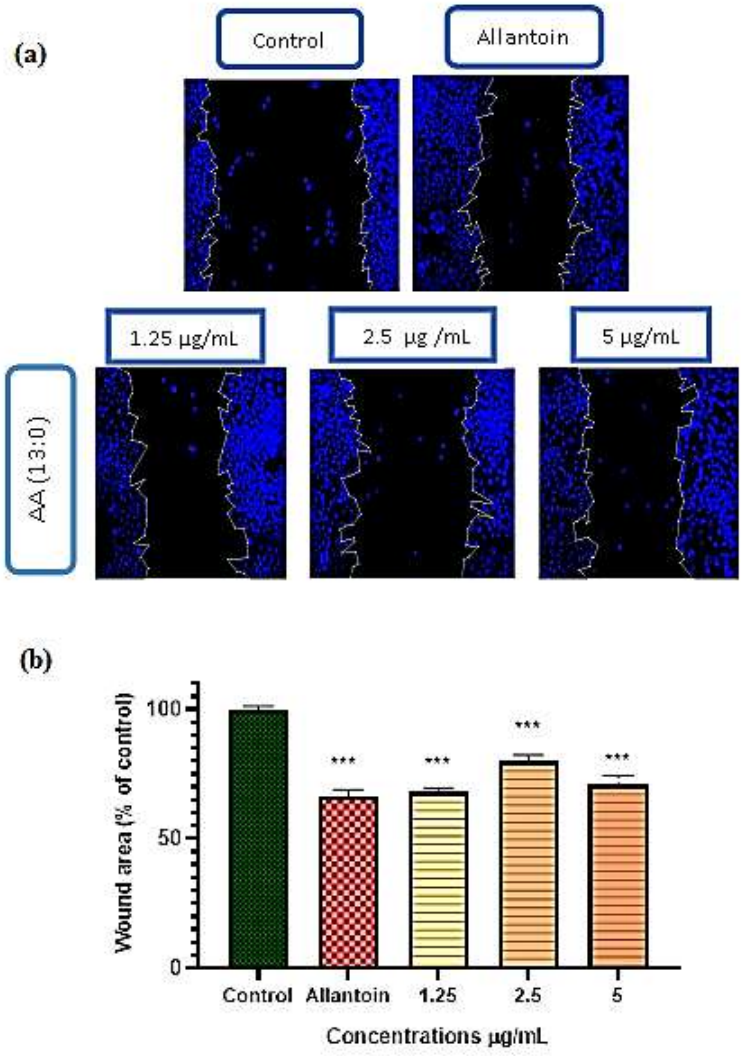

Figure 2. Qualitative (a) and quantitative (b) scratch-wound healing assay results after 48 h on NIH/3T3 fibroblast cells exposing to three different concentrations of anacardic acid (13:0) (1.25, 2.5 and 5 µg/mL) and allantoin (50 µg/mL); Images of the fields were collected by fluorescence microscopy (magnification ×20) and analyzed with ImageJ software. *** Significant differences compared to control at $p<0.001$; bars represent mean ± SEM, (n=3)

Acknowledgements
This research was funded by Medicinal Plants Research Center, Tehran University of Medical Sciences (TUMS) under grant number 99-1-120-46613. We thank the Stem Cell Technology Research Center (Bone Yakhteh) for technical support in the cytotoxic assays.

Author contributions
Parisa Sarkhail designed and supervised the study, and was also involved in phytochemical experiments and data analysis; Ghazal Hashemi accomplished the experiments and collected the data; Effat Souri provided professional advice and contributed to the analysis of data. All authors contributed to the writing, revising and editing of the manuscript.

Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

DMEM; Dulbecco's modified Eagle's medium; DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride; EC_{50}: half maximal effective concentration; FBS: fetal bovine serum; HepG-2: human liver cancer cell line; MCF-7: human breast cancer cell line; MKN-45: human gastric cancer cell line; MTT: 3-4,5- dimethylthiazol-2,5 biphenyl tetrazolium bromide; NIH/3T3: murine normal fibroblast cell line; CNE-2Z: human nasopharyngeal carcinoma cell line; MD-MB-231: breast cancer cell lines; H1975: lung cancer cell line; NHDF: normal human dermal fibroblasts