RESEARCH ARTICLE

Determination and Isolation of Valuable Bioactive Compound (lupeol) from *Portulacaria afra* Jacq.

Ali H. H. AL-temimi*, Enas J. Kadhum

Department of Pharmacognosy and Medicinal Plant, College of Pharmacy, University of Baghdad, Baghdad, Iraq

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ABSTRACT

Portulacaria afra is a small succulent tree, previously belonging to the Portulacaceae family, but with further studies, the plant transferred to the Didieracea family. *P. afra* was used as an ornamental, vegetable, and ethnomedicinal plant. Uses of the plant by rural South Africans to treat chronic skin conditions and rashes, alleviate exhaustion, and aid in treating TB and diarrhea have been documented in folklore. According to pharmaceutical research, plant extracts offer a wide range of remedial outcomes, such as antidiabetic, antifungal, antibacterial, anticancer, antioxidant, and anti-inflammatory. The study aims to determine some bioactive constituents responsible for pharmacological activities and traditional usefulness. Thin-layer chromatography (TLC) is used for detecting lupeol by specific reagents; a p-anisaldehyde sulfuric acid reagent and 10% methanolic sulfuric acid. And high-performance liquid chromatography was used to detect pentacyclic triterpenoids (lupeol) in the n-hexane. The lupeol was isolated by preparative layer chromatography (PLC). Testing the efficacy of the separation method, the isolated compounds have been identified and characterized by different chromatographic and chemical analyses (TLC, ATR-FTIR, LC-CMS-APCI$^+$, and ^{1}H-NMR).

Keywords: ^{1}H-NMR, ATR-FTIR, LC-CMS-APCI$^+$, Lupeol, *Portulacaria afra*, PLC.

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INTRODUCTION

The value of medicinal plants belongs to the bioactive secondary metabolites which exert pharmaceutical response upon administration as Alkaloids, flavonoids, terpenoids, glycosides, and tannins. Each has antibacterial, antiparasitic, antidiabetic, antioxidant, anti-inflammatory, and anticholinesterase properties.[1] Before being moved to the Didieracea family due to morphological and anatomical research, RNA sequences, and geographic distribution, *Portulacaria afra* was formerly classified as part of the Portulacaceae family. Madagascar, East Africa, and southern Africa are all areas where the plant thrives.[2] The most common name of the plant is spekboom, elephant food, and pork bush, while in Iraq called dumooa Altefel. The plant has reddish stems and small oval green leaves with rare blooming tiny pink flowers, reproduced by cutting rather than seeds.[3] Spekboom is edible and is added to a salad as a vegetable in South Africa because of its high water content and sour tart taste.[4] *P. afra* has many uses in folk medicine in South Africa; sores, chronic rash, eczema, increased milk in breastfeeding women, fever, heartburn, quenching thirst in dehydrated people, hypertension, rheumatism, and diuretics.[5] Moreover, *P. afra* has many phytoconstituents that impact many pharmacological properties as antibacterial, antioxidant, antifungal, anti-inflammatory, and anticancer.[6] The biological activities belong to the secondary metabolites in the spekboom, fatty acids, vitamins, minerals, alkaloids, phenolics, glycosides, and flavonoids.[7] Besides synthesizing the water-soluble nitrogen-containing pigments, betalains include red-violet betacyanins and yellow betaxanthins. The mentioned pigments are found exclusively in the caryophyllales order of plants betalain pigment that does many activities. As antioxidant, antilpidimic.[8] Bate smith describes the phenolic content in the plant as quercetin, cyanidin, kaempferol, sinapic acid, and ferulic acid.[9] Steroids approved in Caryophyllales by Patterson and Shihua, the type detected is Δ 5 sterols.[12] In this study, the use of PLC for the isolation of lupeol, isolated compound has many valuable pharmaceuticals uses as an anticancer and antioxidant.

METHODS

Plant Material

The complete plant of the Didieracea family species *P. afra Jacq*. The plant does collect from a Baghdad farm in the April of 2021. Dr. Sukaena Abass of the University of Baghdad's

*Author for Correspondence: alihasan_husain@yahoo.com

Department of Biology in the College of Sciences confirmed the plant's identity. The plant was adequately cleaned, dried in the shade, then reduced to a fine powder in a mechanical grinder for extraction.

Extraction and Fractionation

A total of 100 gm of the powdered plant is extracted with 750 mL of 85% aqueous ethanol in the soxhlet apparatus for 24 hours. The crude extract is dissolved in distilled water and fractionated with solvents of varying polarities. Starting with (n-hexane, chloroform, ethyl acetate, and n-butanol) in a separatory funnel to extract the active ingredients. Over 3 days, the crude extract was mixed with the solvents in three separate volumes. Then, the three volumes of each solvent were combined. The anhydrous sodium sulfate was used to dry the extracts, filtered, and evaporated using a rotary evaporator, except the n-butanol extract was evaporated directly.[13]

Identification and Screening of Different Bioactive Constituents by Thin-Layer Chromatography (TLC)

The n-hexane fraction of *P. afra* whole plant was examined by TLC to detect the components present in the plant. The stationary phase of (TLC) consists of Readymade silica gel plates GF254 nm (20 x 20 cm) of 0.25 mm thickness (MACHEREY-NAGEL) were used as the adsorbing stationary phase. As a first step, the TLC plates are activated at 110°C for 30 minutes before being used to establish the sample's baseline and front line. The secondary metabolites were identified using TLC with various solvent systems. In 100 mL of the mobile phase was prepared in a jar with a dimension of (20 x 9 x 20 cm). The jar closed with a glass lid and lined with filter paper from inside, allowed to saturate with the solvent system for 1-hour. The mobile phases used were S4 Toluene: chloroform: ethyl acetate (5: 4: 1)[14] and S6 Hexane: dichloromethane (5: 5)[15] with some modification. The chemical reagents used to detect lupeol are anisaldehyde-sulfuric acid reagent (AS) and 10% methanolic H_2SO_4.

Qualitative Analysis of the Compounds by High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) does use to examine the components identified by TLC qualitatively. Throughout this process, the retention times of the sample were investigated and compared to those of the standard. One gram of the n-hexane fraction is dissolved in 5 mL of methanol. Approzimately, 1-mL of the standards is dissolved in 2 mL of high-purity methanol. The HPLC examination was carried out with an SYKAM model 2010. The column was C18-ODS (25 cm in length, 4.6 mm in diameter, and a particle size of 5 m). Autosampler (model number S 5200), fraction collector (model number FOXY R1), and ultraviolet detector (model number UV-vis 10A-SPD spectrophotometer). The conditions used for the identifications of lupeol are Isocratic elution.[11] Acetonitrile: methanol (90:10), the temperature: 38°C, UV detection at 210 nm, and the flow rate is 1.5 mL/min, and the injection volume is 2 mL.

Isolation of Lupeol with Preparative Layer Chromatography

The S4 (Toluene: chloroform: ethyl acetate) (5: 4: 1) mixture was the most effective solvent system for isolation. The stationary phase is a readymade preparative TLC GF254 (MERCK). Spot the sample on the plate's baseline at a distance of cm^{-1}, then position the standard in the plate's corner. When detecting lupeol, a destructive reagent is used. Hence the TLC plate must be cut vertically to the baseline from the side where the standard compound is placed. The TLC plate cut must be done with clearance around the sample area. The plate is placed in jar, and mobile phase is run until the plate reaches a length of 18 cm, removing the TLC plate from the jar and allowing it to dry. After spraying the reagent on the cut plate and placing it in an oven preheated to 110°C for five minutes, a visual comparison may be made between the generated sample and the reference location on the cut plate. Next, the cut plate was placed next to the original TLC plate to match the band, and a steel spatula was used to scrape the surface. The powdered silica was collected in a conical flask, and then the chloroform was used to extract the lupeol from the silica.

Identifications of the Isolated Compounds by Chemical and Chromatographic Analysis

The separated chemicals are examined by Attenuated transition total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR) type (BRUKER\GERMANY), liquid chromatography-compact mass spectrometry by atmospheric pressure chemical ionization in positive mode (LC-CMS-APCI+) single multifunctional quadrupole mass spectrometer type (EXPRESSIONs\ADVION). The mobile phase was constituted of methanol (A) and water containing 0.1% formic acid (B) at a (90:10) ratio running in an isocratic flow of 1.0 mL/min,[16] and Proton nuclear magnetic resonance (^{1}H-NMR) type (BRUKER\GERMANY), Using a Euro-vector EA 3000A NMR spectrometer (400MHz). The compounds dissolved in DMSO Dimethylsulfoxide and run on the NMR device.

RESULT AND DISCUSSION

Plant Material

The drying process must be conducted in a carefully monitored environment to prevent any unwelcome chemical reactions. Therefore, dry it rapidly without excessive heat, ideally in a well-circulating breeze. Therefore, with adequate ventilation, *P. afra* whole plant drying should be accomplished in the shade.

Extraction and Fractionation

The soxhlet apparatus is utilized for the hot extraction method of *P. afra*. (100 gm) amount of ground dry plant material is packed in a thimble for extraction with aqueous (85% ethanol). The extract is evaporated in the rotary evaporator type (DLAB\USA) at 40°C. the yield is (17 gm) brown sticky crude extract. The result is dissolved in 350 mL distilled water (D.W) and shaken with a stirrer until complete solubilization of the quote. The water extract is fractionated with an equal volume of n-hexane. Lastly, the yield for the n-hexane is (1.9 gm).

TLC for n-hexane Fraction

The mobile phases used for pentacyclic triterpenoid (lupeol) are (S4) and (S6). The compound was identified and isolated from the n-hexane fraction of the whole plant *P. afra* cultivated in Iraq. After spraying the plates with 10% methanolic H_2SO_4 and heating them in an oven for 5 minutes at 110°C. Figure 1 shows the TLC chromatogram of the pentacyclic triterpenoid lupeol matching the standard. And the retardation factor values (R_f) for both mobile phases were (0.75) and (0.3) for S4 and S6, respectively.

Qualitative Analysis of n-hexane Fraction by High-Performance Liquid Chromatography (HPLC)

The HPLC chromatogram of the n-hexane fraction shows the presence of the pentacyclic triterpenoids lupeol as the compound detection in the TLC analysis proved by two mobile phases. The HPLC chromatogram of the lupeol standard and the n-hexane fraction are present in Figure 2 shows the retention time (R_t) values of the n-hexane fraction and the lupeol standard 30.10 and 30.11 minutes, respectively.

Isolation of lupeol by preparative layer chromatography

From the results of the chemical analysis, the TLC chromatogram with two mobile phases, and the HPLC chromatogram of the n-hexane fraction with lupeol standard, the isolation of lupeol can be done by preparative layer chromatography with the best mobile phase S4 = (Toluene: chloroform: ethyl acetate) (5: 4: 1). The mobile phase allowed to reach 18 cm from the plate. However, because lupeol cannot be seen on TLC plates, a destructive reagent must be used to detect the compound (lupeol). Therefore, a glass cutter slices the preparative layer chromatography plate (0.5 mm thickness) on one side. Then the cut glass plate is derivatized with 10% H_2SO_4 and heated in an oven at 110°C to avoid the chemical from being damaged during the detection procedure. Then, The visible band on the derivatized cut glass plate was compared to the band on the remaining sliced plate to establish a match and the scratch of the invisible band containing the lupeol. The R_f value of the band is (0.75). The band was selected by pencil and scrapped with a spatula. The extraction from scrapped silica was done by using chloroform. The plates are shown in Figure 3.

Identification of the Isolated Compounds with TLC

Two mobile phases were used to compare the lupeol standard to the isolated sample. With an R_f value of (0.55) for S4 and (0.3) for S6. The chromatograms are shown in Figure 4**.**

Identification by Attenuated Total Reflection-Fourier Transforms Infrared Spectrometry (ATR-FTIR) for the Determination of the Lupeol Separated by Preparative Layer Chromatography

Each compound's unique ATR-FTIR vibrations and bands are shown in Figure 5; the FTIR provides some insight into the functional groups in the compound's structure. The wavelengths employed range from 4000 to 400 cm^{-1}, and the instrument utilized is the TENSOR 27 model from (BRUKER, Germany), which comes equipped with fully integrated OPUS software. For lupeol, the comparison between the isolated and the standard compound gives the more characteristic peaks, for the isolated compound the stretching vibrations of the (OH-) group at carbon number three 3537.82 cm^{-1}, the stretching vibration of protons linked to the olefin group for (C_{20}-C_{29}) at 3068.24 cm^{-1}, the two vibrations of the proton attached to the alkane group at 2925.52, 2858.15, the band 1589.17 cm^{-1} the characteristic vibration of the alkene group, bending vibrations of the methylene groups at 1458.71 cm^{-1}, besides bending vibrations of the methyl group at 1376.19 cm^{-1}, and the (C-O)

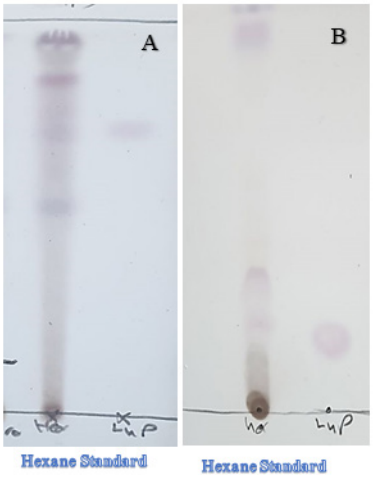

Figure 1: TLC chromatogram of the lupeol standard versus the n-hexane fraction. The mobile phases are: (A): S4 and the (B): S6.

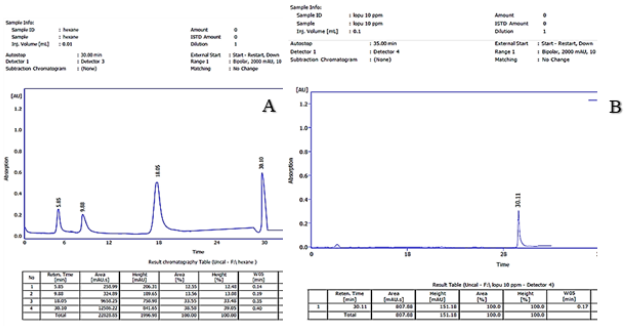

Figure 2: HPLC chromatograms A: n-hexane fraction of the whole plant of *P. afra* cultivated in Iraq B: lupeol standard. By using acetonitrile: methanol (90: 10) as the mobile phase.

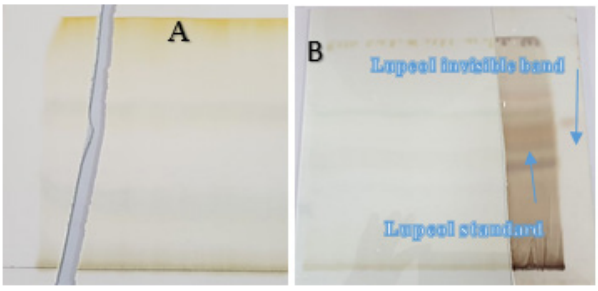

Figure 3: Preparative layer chromatography of the n-hexane fraction. The mobile phase used is S4, A: the plate cut before derivatization, B: the cut plate after derivatization with 10% methanolic H_2SO_4.

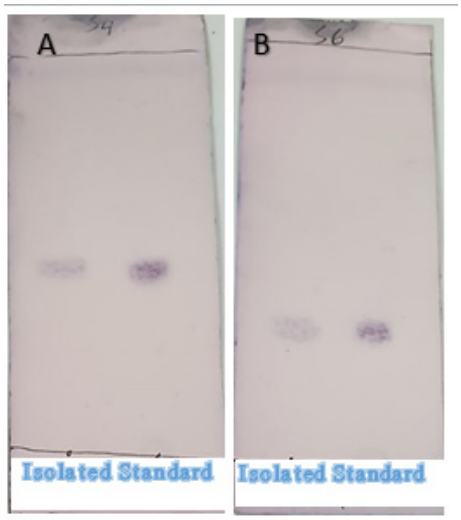

Figure 4: TLC chromatogram of the isolated (ISO) lupeol and the lupeol standard (ST). The two mobile phases used are, A: S4 and B: S6.

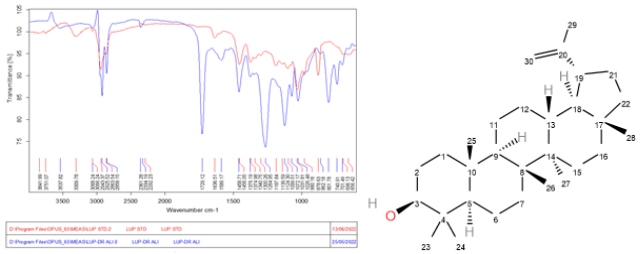

Figure 5: ATR-FTIR spectra for the isolated lupeol in (blue) and lupeol standard in (red).

bond stretching vibrations at 1026.48 cm^{-1}. The same value appears with the standard lupeol and is approximately the same as the literature.[19]

Liquid Chromatography-Compact Mass Spectrometry of the Isolated Sample

The isolated and the standard lupeol were further analyzed by liquid chromatography- compact mass spectrometry by atmospheric pressure chemical ionization LC-CMS-APCI$^+$ in positive mode. This technique will give a fingerprint for the Identification of lupeol. The lupeol standard at retention time (0.51 min) the fragmentation pattern gives the pseudo molecular ion m\z = 411 $[M+H-CH_3]^+$ by losing one vicinal hydrogen to the carbocation to form the base peak at m\z= 410 $[M+H-CH_3-H]^+$. Previous studies and the NIST library indicate the presence of molecular ions at m\z= 411.[(10)] All of the steroids and lupeol (except lupeol has many methyl groups that fragment more than other groups), when evaluated, generated mainly $[M–H_2]^-$, $[M–CH_4]^-$, $[M–H_2–CH_4]^-$, and $[M–H_2–H_2O]^-$ fragment ions in ESI-MS/MS investigations, with the losses of H_2 and CH_4 being the most abundant. There was also a later release of a second CH_4 group from the steroid if more than one CH_3 group was present on the steroid.[18] The pseudo molecular ion peak at m\z = 339 is determined by the cleavage pattern of the isolated material at retention time

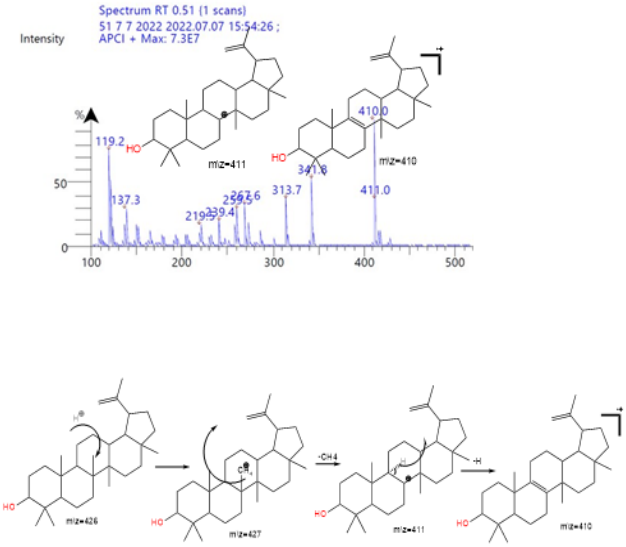

Figure 6: LC-CMS-APCI$^+$ chart and the proposed fragmentation pattern for lupeol standard.

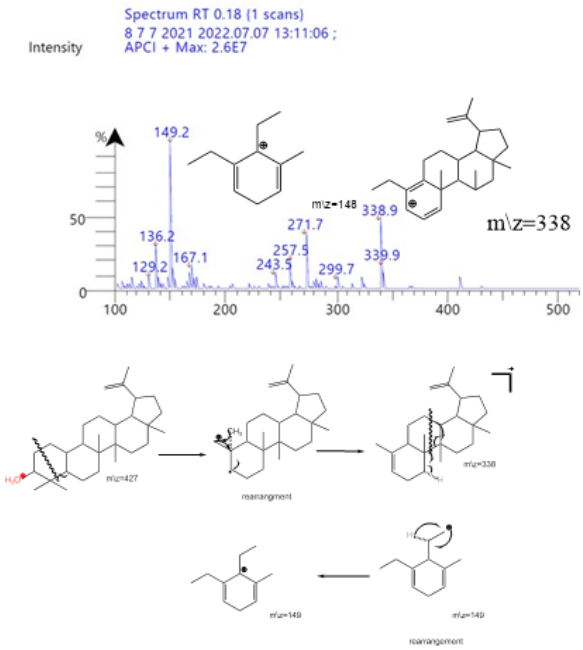

Figure 7: The proposed fragmentation pattern and LC-CMS-APCI+ for the isolated lupeol.

(0.18 min). The formulas $[M+H-C_5H_9-H_2O-H]^+$ at m\z = 339 and $[M+H-C_5H_9-H2O-2H]^+$ at m\z = 338 suggest the loss of water at C_3 and the rupture of the ring A at C_1-C_2 and C_4-C_5 will be the initial steps in the fragmentation process. Further fragmentation of the molecular ion m\z = 338 at the C_{12}-C_{13} bond and C_8-C_{14} of ring C yields the base peak of m\z = 149 as illustrated in Figures 6 and 7.

^{1}H-NMR for the Isolated Compound by the PLC

The compound isolated by the PLC method was subjected to the proton nuclear magnetic resonance for further Identification. The spectrum shows seven methyl groups with three protons

Table 1: H^1 NMR spectra of the isolated compound from the PLC compared to the lupeol from the literature (at 400 MHz, DMSO, δ in ppm, and j in Hz)

Carbon atom	*δ value in ppm*	*Reference* [22]
1	H_a= 0.9 (s), H_b=1.77 (o)	0.9, 1.65 (o)
2	H_a =1.53 (s), H_b= 1.64 (o)	1.52, 1.67 (o)
3	H= 3.33 (dd, J = 3.5, 1.8 Hz)	3.2 (dd)
5	H=0.66 (s)	0.67 (o)
6	H= 1.35 (o)	1.37, 1.52 (o)
7	1.44	1.39 (o)
9	1.28 (s)	1.25 (o)
11	1.23	1.2 (o), 1.4 (o)
12	1.04 (o)	1.06, 1.62 (o)
13	1.64 (o)	1.66 (o)
15	1.04 (o)	1.05, 1.6 (o)
16	1.34 (s)	1.35, 1.45 (o)
18	1.35 (o)	1.36, 1.37 (o)
19	2.3 (d, j = 11.8 Hz)	2.4 (m), 1.45 (o)
21	1.28	1.3 (o), 1.91 (m)
22	1.15 (s)	1.18, 1.37 (o)
23	0.9 (s)	0.9 (s)
24	0.76 (s)	0.76 (s)
25	0.83 (s)	0.83 (s)
26	1.04 (o)	1.03 (s)
27	0.92 (s)	0.94 (s)
28	0.86 (s)	0.79 (s)
29	H_a = 4.68 (d, J = 56.0 Hz,) H_b = 4.54	H_a = 4.69 (d) H_b = 4.57
30	1.77 (o)	1.67 (o)

(s = singlet, d =doublet, dd = doublet doublet, m =multiplet, o =overlap)

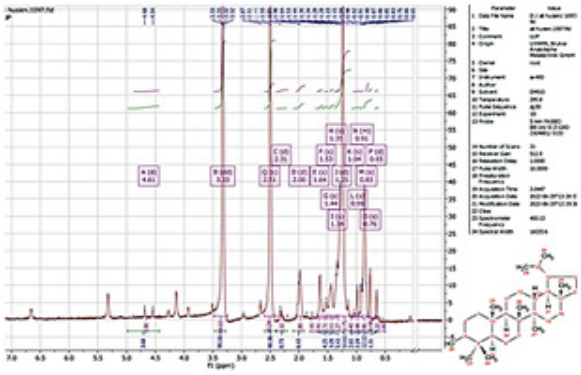

Figure 8: Chart of ^{1}H-NMR of the lupeol isolated by preparative layer chromatography

at δ (0.76, 0.83, 0.86, 0.90, 0.92, 1.04, and 1.77 ppm). Each one was integrated with three protons. And a doublet peak appears at δ 2.3 ppm, a characteristic of beta-oriented H-19. Olefin proton $H29_a$ and $H29_b$ give characterized doublet peaks at δ 4.68 and δ 4,54 ppm. The alfa 3-proton gives special doublet peaks at δ 3.33 ppm; all the mentioned values of the peaks have an agreement with the structure of lupeol compounds.[17] Figure 8 and Table 1 show the spectra of the H^1 NMR for the isolated compound by the PLC.

CONCLUSION

The study indicates the ability to extract valuable bioactive compounds from *P. afra*. The isolation can be done with high-performance liquid chromatography and preparative layer chromatography. Furthermore, the isolated bioactive compounds are separated from the spekboom for the first time, and the characterization gives acceptable results with the corresponding standard. The traditional use in hypertension, rheumatism, and a chronic rash of *P. afra* may result from the many secondary metabolites found in the plant, such as the isolated compound lupeol.

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