Medicinal & Aromatic Plants
ISSN: 2167-0412

Karthikeyan et al., Med Aromat Plants 2016, 5:3
DOI: 10.4172/2167-0412.1000239

Research Article Open Access

Isolation, Characterisation and Antifungal Activity of β-Lapachone from *Tecomaria capensis (Thunb.)* Spach Leaves

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Abstract

β-Lapachone (3, 4-dihydro-2,2-dimethyl-2H-naphthol [1,2-b] pyran-5,6-dione), was originally isolated from the lapacho tree (*Tabebuia avellanedae*) of South America. It is a lipophilic ortho-naphthoquinone. β-Lapachone has antimicrobial, antifungal, antiviral antitumor and anti-trypanosomal activities. In a number of tumors (e.g., breast, colon, lung, and pancreatic cancers) with high NAD (P) H expression levels of: quinone oxidoreductase (NQO1), β-lapachone activates a novel apoptotic response. β-Lapachoneis a major constituent of the species *Tecomaria capensis* (Thunb.) Spach. On silica gel GF_{254} TLC plates and the chromate plate analytical thin layer chromatography was accomplished. It was developed with Toluene: Ethyl acetate (7:3 v/v) as mobile phase. In this study isolation of β-Lapachone was achieved by preparative TLC. The compound thus isolated was characterised by Mass, H^1 NMR and FT-IR spectral analysis. Mass spectral data shows molecular ion peak of m/z=242. Antifungal activity was measured against *Candida albicans* and *Aspergillus nigrus* fromethyl acetate extracts of selected species.

Keywords: *Tecomaria capensis* (Thunb.) Spach leaves; β-Lapachone; FT-IR; H^1NMR; MASS; Antifungal activity

Introduction

Naphthoquinones display very substantial pharmacological properties; like antipyretic, antibacterial, antifungal, insecticidal, anti-inflammatory and antiviral properties. Cardiovascular and reproductive system related pharmacological effects have been demonstrated too [1]. β-Lapachone (3,4-dihydro-2,2-dimethyl-2H- naphthol[1,2-b] pyran-5,6-dione), a lipophilic ortho-naphthoquinone, was originally isolated from the lapacho tree (*Tabebuia avellanedae*) of South America [2-5]. Beta-lapachone can be easily synthesized by chemical transformation of lapachol, also extracted from the bark of plants of the Bignoniaceae family or from lomatiol, isolated from seeds of lomatia [6]. it is reported to present a significant anti-neoplastic activity against human cancer cell lines originating from leukemia [7], prostate [8], malignant glioma [9], hepatoma [10], colon [11,12], breast [13], ovarian [14], pancreatic [15,16] tumors, at concentrations in the range of 1-10 μM (IC50). Other studies have shown that in combination with taxol, beta-lapachone is an effective agent against human ovarian and prostate xenografts in mice. This naphthoquinone was reported to have, anti-Trypanosoma properties [17-19]. Previous studies showing antifungal activity of lapachol and beta-lapachone against C. *neoformans* are to some extent contradictory because of the different experimental protocols used [20]. Nevertheless, these data indicate that new studies are necessary before proposing antifungal tests on humans. This study was aimed to isolate, characterize and evaluation of antifungal activity of β-lapachone against *Candida albicans* and *Aspergillus nigrus* from ethyl acetate extracts of the species. Development of suitable extraction and isolation procedure which aids to get the purified material. Further the study also deals with characterisation of β-lapachone in the sample extract by Mass and H^1 NMR and FT-IR spectral analysis for structure identification.

Materials and Methods

Instruments

Double beam UV-Visible Spectrophotometer (Systronics model 2203) with matched cuvettes was used in this study. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), and an ultrasonic bath sonicator (spectra lab, model UCB 40).

Chemicals and reagents

Methanol, Ethanol and Sulphuric acid was purchased from Merck Pvt. Ltd. Mumbai, India. And chloroform was procured from Sd-fine Pvt. Ltd. Mumbai, India.

Collection and identification of plant materials

The leaves of *Tecomaria capensis* (Thunb.) Spach. were collected from Guntur district, AP and it was authenticated by professor Dr. S. M. Khasim, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur. The specimen (No: ANU/00129/2009/AP) was deposited in the Department of Botany and microbiology for future reference.

Determination of various physicochemical constants

The determination of several physico chemical constants were done as per the protocol given in the practical pharmacognosy Mr. S. B. Gokhale, Dr. C. K. Kokate Nirali Prakashan, 07-Aug-2008 [12].

Preparation of extracts

The techniques commonly used in the field of photochemistry were extraction, isolation and structural elucidation of natural products, as well as chromatographic techniques. The solvent extraction of any botanical materials may yield very less quantity of volatile oils and a large yield of non-volatile components like resins, pigments, waxes and fatty acids.

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Received January 27, 2016; **Accepted** February 27, 2016; **Published** February 29, 2016

Citation: Karthikeyan R, Sai Koushik O, Kumar PV (2016) Isolation, Characterisation and Antifungal Activity of β-Lapachone from *Tecomaria capensis (Thunb.)* Spach Leaves Med Aromat Plants 5: 239. doi:10.4172/2167-0412.1000239

Successive solvent extraction

This extract was prepared by using soxhlet apparatus. About 150 gm of dried flower powder was taken in a muslin cloth bag. The purified ethanol was passed through the tube where the powder bag was kept. The solvent was passed through siphon tube so that it reaches the round bottom flask in which porcelain chips were provided. The vapours containing the constituents pass through the condenser and reach the tube containing powder bag and the process was repeated. This was continued for 24 hours. Then the round bottom flask containing extract was transferred to a beaker and was allowed to evaporate in a water bath.

Preliminary phytochemical identification

The following chemical tests were performed to identify the phytochemical constituents present in pet.ether, n.hexane, chloroform, ethyl acetate, ethanol and aqueous extracts of *Tecomaria capensis*.

Isolation and purification of active compounds

Analytical TLC: Analytical TLC was carried out on preparative TLC plates (5 × 5 cm with 0.2 mm thickness, silica gel GF_{254}, Merck, Darmstadt, Germany) cut from the commercially available sheets. An aliquot of a sample solution of crude extract was spotted onto the silica gel plate and allowed to dry for a few minutes. Afterwards, the chromatoplate was developed with toluene and ethyl acetate in ratio 7:3, fractions were isolated and similar fraction were clubbed as mobile phase in a previously saturated glass chamber with eluting solvents for some time at room temperature. The developed plate was dried under normal air and the spots were visualised by spraying with a solution of 0.5%w/v ferric chloride and dried under oven. The (retention factor) values of isolated compounds and standard were calculated and compared.

Preparative TLC for purification: A streak of crude extract was applied manually on a preparative TLC glass plate (20 cm × 20 cm; 1500 µm thickness) with inorganic fluorescent indicator binder (Analtech, Sigma-Aldrich, Steinheim, Germany). After air drying, the plate was developed, using the same mobile phase as used in the analytical TLC, in a pre-saturated glass chamber. In each experiment, two plates were used in parallel. One of the plates from each set of experiment was sprayed with as described above, and the bands were scraped off carefully from the plate. The scratched sample was dissolved in HPLC grade methanol and centrifuged at 12000 rpm for 15 min in order to remove silica. The supernatant was collected, filtered from 0.22 µm filter, and dried under reduced pressure. Further, all the dried samples were passed under nitrogen gas for 5 min and then dissolved in methanol for further characterization and quantitative HPLC analysis. The entire purification process was carried out under dark or dim light conditions.

Column chromatography: 10 grams of extract was defatted using n-hexane for 6 hours for the removal of fatty materials then the material was dried and dissolved in ethyl-acetate (100 ml) about 500 grams of silica G* was activated and column chromatography was runned from the mixture of toluene and ethyl acetate in ratio (7:3 v/v), fractions were isolated and similar fraction were clubbed.

Characterization of purified compound: It is very helpful record which would give information about functional group present in the organic compounds. Mechanism of bond stretching and bending is happened when electromagnetic radiation ranging from 500 cm^{-1} to 4000 cm^{-1} passed through sample. Instrument used was ABD BOWMEN Spectrometer. ESI mass spectra was acquired from isolated compound and characterized by using JOEL Gcmate Mass spectrometer. Proton nuclear magnetic resonance spectra were acquired using 400 MH_Z NMR spectrometer employing TMS as an internal standard and deuterated methanol was used as solvent.

Antifungal activity

The Antifungal activity of the isolated β-lapachone from ethyl acetate extract was tested against *Aspergillus niger* and *Candida albicans*. The antifungal sensitivity pattern for the isolates was studied by the disc diffusion method. Potato dextrose agar media was made and the plates were swabbed with the cultures of respective fungi grown in nutrient broth. Sterile discs of 6 mm diameter were impregnated with 25 µg/ml, 50 µg/ml, 100 µg/ml each separately. Blank disc impregnated with DMSO was used as negative control and discs of *Fluconazole* (30 µg/ml) as positive control. The plates were then incubated at 37°C for 24 hrs. The antifungal activity was assessed by measuring the zone of inhibition. The relative antifungal activity of the extract was calculated by comparing its zone of inhibition with the standard drugs. Inhibition was recorded by measuring the diameter of inhibition zone at the end of 24 h [13].

Results and Discussion

Complete extraction of β-Lapachone was achieved by successive solvent extraction with ethyl acetate. Preliminary phytochemical study reveals that the ethyl acetate extract contain alkaloids, flavonoids, cardiac glycosides, coumarin glycosides, proteins, tannins, steroids, inulin, terpenoids, volatile oil and mucilage showed in Tables 1 and 2. Several mobile phase combinations were tried and mixture of toluene and ethyl acetate in ratio 7:3 was found optimum for isolation of β-Lapachone. The R_f values of sample compound was found as 0.3625 cm. TLC profile of compound was represented in Figures 1 and 2. Isolation of β-Lapachone from the extract was achieved by preparative thin layer chromatography. Characterization of isolated compound was done by studying FT-IR V_{max} (KBr) cm^{-1}: 1730, 1655, 1375, 1222.5; Its FT- IR values showed the following functional groups # 1730(C=O), 1655(C=C), 1375(CH_3), 1222.5(C-O). H^1NMR^{1}H NMR (δ ppm): 7.3280-7.8891(4H, m), 2.5070(2H, s), 1.9955(2H, s), 1.399(6H, s)Its ^{1}H NMR displayed a signal of 4H multiplet 7.3280-7.8891 referred to aromatic ring, 2H singlet 2.5070 and 1.9955 referred to two methylene group, 3H singlet 1.399 referred for two methyl groups. ^{13}C NMR from the ^{13}C NMR showed 15 carbons, 137.92, 137.40, 136.71, 136.04, 133.89, 127.49 referred to benzene ring, 164.33, 112.21 referred to ethylene group, 181.11, 177.20 referred to carbonyl group, 78.80 referred to fully substituted C, 35.14, 15.94 referred to CH_2, 27.74, 27.74 referred to CH_3. Mass spectral data shows molecular ion peak m/z: 242.09. H^1 NMR and ^{13}C NMR spectra show different kinds of protons in the compound which was seen in the spectra and its assignment resembles the complete structure of β-lapachone shown in Figure 2. Zone of

S No	Physico-chemical constant	Values
1	Moisture Content (Loss on Drying)	1%
2	Foreign Matter	2%
3	Alcohol Soluble Extraction	0.25 gm
4	Water Soluble Extraction	0.36 gm
5	Ether Soluble Extraction	0.04 gm
6	Total Ash Value	0.93
7	Acid Insoluble Ash Value	0.01
8	Sulphated Ash Value	0.06
9	Water Soluble Ash Value	0.05

Table 1: Determination of Various Physico Chemical Constant.

Phyto constituents	Pet. Ether	n-hexane	Chloroform	Ethylacetate	Ethanol	Water
Alkaloids	--	--	--	--	++	--
Flavinoids	--	--	++	++	++	++
Cardiac Glycosides	++	++	++	++	++	--
Saponin Glycosides	++	++	++	++	--	--
Coumarin Glycosides	--	--	--	++	++	--
Tannins	--	--	--	--	++	--
Steroids and Terpinoids	--	++	++	++	++	--
Carbohydrates	--	--	--	++	--	--
Protein	--	--	--	++	++	--
Inulin	--	--	--	--	++	++
Volatile oil	++	++	++	++	++	--
Waxes	--	--	--	--	--	--
Mucilage	--	--	--	--	++	++

Table 2: Results of Preliminary Phytochemical Identification.

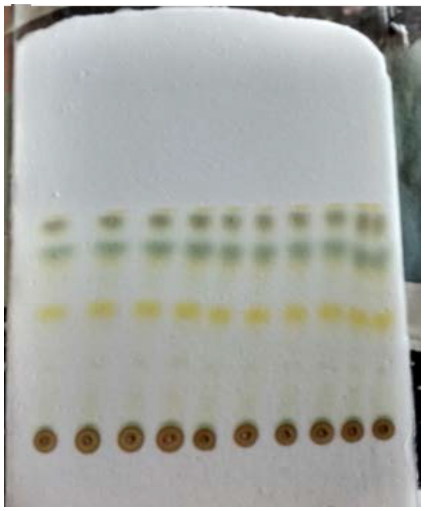
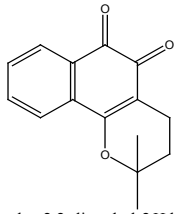


Figure 1: Analytical TLC of isolation of β-lapachone.



3,4-dihydro-2,2-dimethyl-2*H*-benzochromene-5,6-dione

Figure 2: Chemical structure of β -Lapachone structure.

inhibition of β-lapachone against the selected fungi are seen in Table 3. The antifungal activities of the different extracts of the isolated compound β-lapachone at different concentrations were determined. The two different concentrations (25, 50 and 100 µg/ml and in DMSO) exhibited antifungal activity against *Candida albicans* and *Aspergillus niger*. The solvent doesn't interfere in Anti-fungal activity. The positive control Flucanzole showed significant activity against both the fungi *Candidaalbicans* was 19 ± 0.816 mm and for *Aspergillus niger* it showed inhibition zone of 18 ± 0.481 mm. At 25 µg/ml β-lapachone showed zone of inhibition against *Candida albicans* was 15 ± 0.821 mm, 16 ± 0.471 mm (50 µg/ml) and 100 µg/ml with the zone of 18 ± 0.512 mm respectively. For *Aspergillus niger* it showed zone of inhibition of 16 ± 0.471 mm and 100 µg/ml with the zone of 18 ± 0.512 mm. The isolated

S No	Sample Treatment (µg/ml)	Name of the fungi	
		Candida albicans	***Aspergillus niger***
1	β-lapachone 25 50 100	 15 ± 0.821 mm *16 ± 0.471 mm *18 ± 0.512 mm	 14 ± 0.821 mm *16 ± 0.491 mm *16 ± 0.372 mm
2	Flucanzole (positive control) (30)	*19 ± 0.816 mm	*18 ± 0.481 mm
3	DMSO (negative control) (25 µl)	NZI	NZI

SD ± mean; *P Value>0.001 is considered as significant; NZI – No zone of inhibition

Table 3: Zone of inhibition of β –Lapachone against the different fungi.

β-lapachone showed dose–dependent and significant activity against all two fungi. The antifungal results are shown in Table 3.

Conclusion

Isolation, characterization of β –Lapachone was achieved successfully which will be helpful for the standardization of active constituent in herbal formulations. The Isolated β –Lapachone has significant anti-fungal action and showed comparable action with the standard Flucanzole. β –Lapachone showed significant activity against *Aspergillus niger* and *Candida albicans* so it can be used as narrow spectrum of anti-fungal agent.

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgements

The authors would like to thank to Dr. L. Rathaiah, Honorable Chairman, Vignan group of institutions, Vadlamudi, Guntur for providing the necessary facilities to carry out this research work.

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