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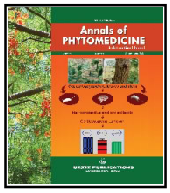

Original Article : Open Access

Bioassay guided fractionation, isolation and synthesis of potent antimicrobial agents from *Alpinia galanga* (L.) Willd.

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Antimicrobial activity

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Abstract

The air-dried and powdered methanol solvent extraction of the rhizomes of *Alpinia galanga* (L.) Willd. is subjected to bioassay guided fractionation and isolation yielded two compounds, namely; p-coumaryl diacetate (1) and 1'-S-1'-acetoxychavicol acetate (2). The isolated known compounds have been identified based on the physical, spectral data of IR, 1H, 13C NMR and mass spectroscopy and comparison with an authentic sample. Finally, the isolated p-coumaryl diacetate (1) and 1'-S-1'-acetoxychavicol acetate (2) were confirmed by synthesis. The crude methanol extract, identified known compounds 1 and 2 were tested for antimicrobial property against *Malassezia furfur*, which were exhibited with MIC values 1000, 62.5 and 7.81 µg/ml, respectively.

1. Introduction

Common dandruff is measured by excessive formation of scales of white greyish color, depositing on the scalp area and is common problem for most of the population ethnicity and both genders. The nature and origin of dandruff has been consistently controversial (Shemer *et al*., 2000). *Pityrodporium ovale* is strongly suspected to play important role in the manifestation of the seborrheic dermatitis (De Angelis *et al*., 2005: Ro *et al*., 2005). Clinically, white greyish flakes of scalp skin are very often visible on the hair and shoulders. The remaining indication of dandruff is itching with scalp soreness. Commonly three major etiological factors are causing for dandruff like Malassezia fungi, sebaceous secretions and individual sensitivity (De Angelis *et al*., 2005: Ro *et al*., 2005). The genus Malassezia is mainly belongs to Basiodiomycetous yeast and is classified in the Malasseziales (Gueho *et al*., 1996 : Chang *et al*., 1998). These are generally identified in the places of rich source in sebaceous glands mainly these are lipid dependent. The *Malassezia furfur* is one of the important microorganisms of dandruff and it is a member of normal fungal flora on the human skin scalp, because it may be isolated from the scalp (Kurtzma *et al*., 1998). For growth of *M. furfur* is mainly requires essential lipids and proteins, responsible for dermal inflammation and tissue damage (Boekhout *et al*., 2003). As fungal resistance to synthetic antibiotics is in rise (Mathew *et al*., 2017), an alternative in the way of herbal medicine in controlling the same is the need of the hour. The current study was an attempt to pick out the potential antimicrobial components from *A. galanga* is an alternative strategy. Earlier reports on this plant showed various biological properties, such as antiviral (Mehrotra *et al*., 2020), antibacterial, antidiabetic, antiprotozoal, antioxidant, antifungal, immunomodulatory, DNA binding studies (Rekha *et al*., 2019) antiplatelet and hypolipidemic (De-pooter *et al*., 1985; Kiuchi *et al*., 2002). Our main interest on the isolation of biologically active molecules from medicinal plants for antimicrobial agents and we have undertaken the air-dried rhizomes of *A.galanga* and synthesis of isolated known compounds for its antimicrobial action against *M. furfur*.

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2. Materials and Methods

2.1 General experimental procedures

IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. NMR spectra were recorded on a Bruker 300 NMR spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) with chemical shifts given in ppm (Ä). Silica gel (Merck, 100-200 mesh and 230-400 mesh) was used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). TLC was performed using Merck pre-coated silica gel F_{254} plates. Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v).

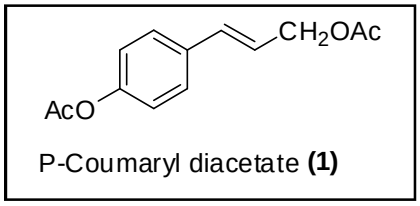
2.2 Plant materials

The air-dried rhizomes of *Alpinia galanga* (L.) Willd. (Family: Zingiberaceae) (1.0 kg) were purchased from market in Hyderabad, Telangana, India in December 2019. A voucher specimen (AG-001) of this plant was deposited in the Department of Chemistry, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

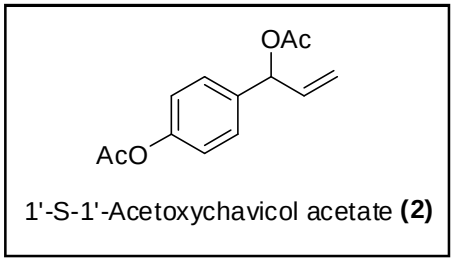
Figure 1: ***A. galanga*** **rhizomes.**

2.3 Extraction and isolation

The air-dried and powdered rhizomes of *A. galanga* (0.8 kg) was extracted with methanol (3 × 3.0 liter) by using Soxhlet apparatus. The filtrate of the solvent was concentrated vacuum distillation under reduced pressure by rotary evaporator at 35°C to yield 40 g. It is represented as methanol crude extract. The crude methanol extract was submitted antimicrobial activity and it showed significant antimicrobial activity against *M. furfur* - MTCC 1374 with MIC 1000 µg/ml. Portion of the methanol extract (25 g) was dissolved in minimum amount of methanol (MeOH) and adsorbed over silica gel (60 g, 100-200 mesh, Merck). 250 g of silica gel (100-200 mesh) was taken in silica gel column and on the top of column crude material was loaded. Initially, column was eluted with direct step gradient mixtures as eluents like hexane: chloroform (1:1, 1:2, 1:3 and 1:4), chloroform and finally with the mixtures of $CHCl_3$: EtOAc (9:1) collected ten fractions. Based on the TLC pattern, similar fractions are combined it and made into three fractions; Fr.1 (4.52 g), Fr. 2 (10.85 g), and Fr.3 (2.14 g). The separated three fractions (1-3) were submitted for antimicrobial activity against *M. furfur*. Fractions 1 and 2 were showed more potent against antimicrobial activity against *M. furfur* with the MIC 250 µg/ml. Fraction 1 (3.0 g) was further purified by using silica gel column chromatography eluted with hexane/ ethyl acetate (8:2) to give p-coumaryl diacetate (1, 1.2 g). In the TLC analysis, Fraction 2 was showing one major compound and some minor pigments appeared. This was further purified by small silica gel column chromatography using the direct solvent mixture like hexane: chloroform (7:3) yielded a compound 2 as colorless oil (3.5 g). The isolated pure compounds were characterized by various spectral data and it was exactly matched with reported literature of p-coumaryl diacetate (1) and 1'S-1'-acetoxychavicol acetate (2) (Noro *et al.*, 1988). Finally, isolated and known compounds 1 and 2 were confirmed by synthesis. Compounds 1 and 2 have been submitted for antimicrobial activity against *M. furfur* exhibited with the MIC values 62.5 and 7.81 µg/ml.



P-Coumaryl diacetate **(1)**



1'-S-1'-Acetoxychavicol acetate **(2)**

Structures of compounds 1 and 2

2.4 p-coumaryl diacetate (1)

Compound 1 is a pale yellow oil, calculated for $C_{13}H_{14}O_4$, 234.2397; IR (CCl_4) ν max, cm^{-1}: 1759, 1651, 1238.^{1}H NMR (CDCl3, 300 MHz) δ 7.31 (2H, d, J = 8.2 Hz), 7.01 (2H, d, J = 8.2 Hz), 6.58 (1H, d, J = 16.5 Hz), 6.19 (1H, dt, J = 16.5, 6.1 Hz), 4.71 (2H, dd, J = 6.1, 1.3 Hz), 2.31 (3H, s), 2.12 (3H, s); ^{13}C NMR (CDCl3, 75 MHz) δ 170.4, 169.2, 150.8, 134.3, 132.8, 127.7, 122.7, 120.7, 64.7, 21.3, 21.0. NMR data were identical to the literature values (Noro et al., 1988 : Khalijah et al., 2010).

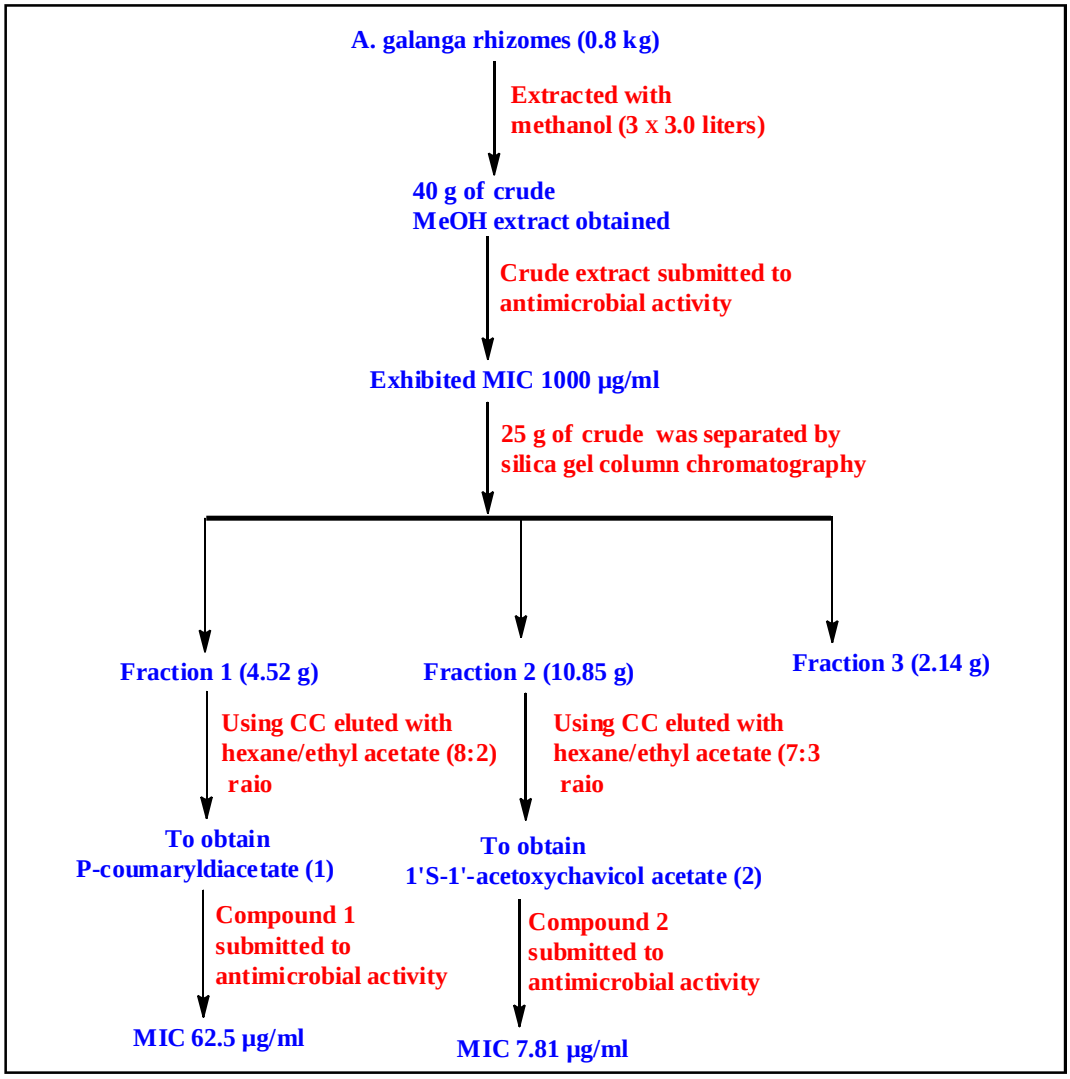
2.5 1'S-1'-acetoxychavicol acetate (2)

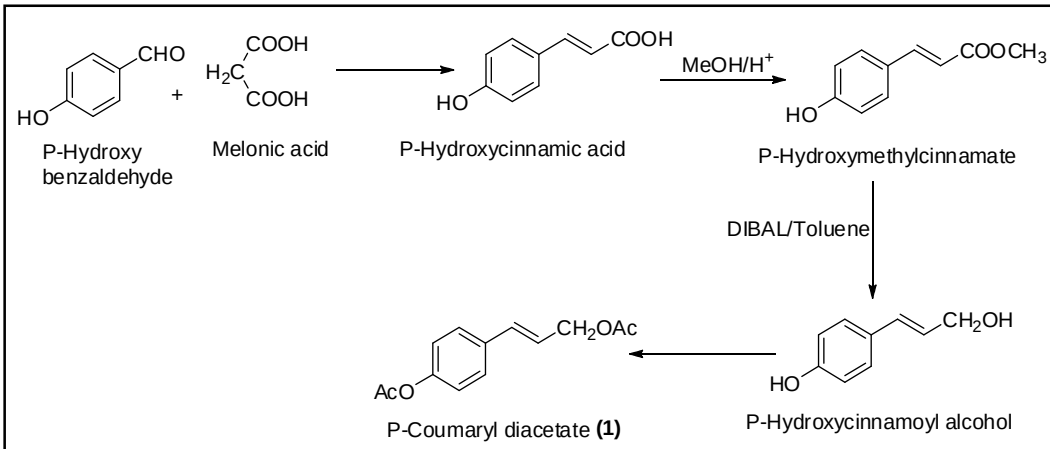
Compound 2 is a colorless oil, calculated for $C_{13}H_{14}O_4$, 234.2479; Found 234, 192, 150, 149, 132, 77, 42. IR (CCl_4) ν max, cm^{-1}: 1761, 1645, 1234.^{1} H (CD_3OD, 300 MHz): δ 7.35 (2H, d, J = 8.5 Hz), 7.06 (2H, d, J = 8.5 Hz), 6.25 (1H, d, J = 6.1 Hz), 6.01 (1H, ddd, J = 16.2, 10.4, 6.1 Hz), 5.27 (1H, dd, J = 16.2, 1.3 Hz), 5.21 (1H, d, J = 10.4, 1.3 Hz), 2.25 (3H, s), 2.07 (3H, s); ^{13}C (CD_3OD, 75 MHz): δ 170.1, 169.4, 150.2, 136.4, 135.2, 126.9, 122.5, 114.9, 75.2, 19.2, 19.3.

2.6 Synthesis of p-coumaryl diacetate (1)

Take p-hydroxybenzaldehyde (61 gm, 0.5 moles) and malonic acid (104 gm, 1 mole) in pyridine solvent (75 ml) at room temperature and added 1.6 ml of piperidine as catalyst. The total solution was refluxed at 105-110^{0}C for 6 h in oil bath. After usual work up to obtain crude solid as P-hydroxycinnamic acid in 58 g, 71 % yield. In the second step, take P-hydroxycinnamic acid (16.4 g, 0.1 moles) in MeOH (250 ml) and added concentrated sulfuric acid (2.0 ml) and reflux at 90-100^{0}C for 4-5 h. After completion of reaction, the solution was cooled down to room temperature and poured in to crushed ice in a beaker to obtain white precipitate. The white precipitate was filtered and washed with cold water thoroughly to obtain P-hydroxymethylcinnamate as color less solid 15.42 g, 87 % yield. Taken P-hydroxymethylcinnamate (1.78 g, 0.01 mole), in freshly distilled toluene (50 ml) in 100 ml RB flask, cooled in ice cold water bath (-5 to 0^{0}C) and added 40 ml of 1.0 M DIBAL (0.04 moles) in toluene solution by syringe over the period of 10 min). The reaction mixture was continued for another one hour and maintained the same temperature. The reaction mixture was quenched with ethanol (5 ml) and the solvent was partially removed in *vacuo* at 40^{0}C and about 50 ml of water was added and the aq. layer was extracted with ethyl acetate (thrice). The combined extract was dried over $MgSO_4$ and concentrated under vacuum to obtain as buff colored P-hydroxycinnamoyl alcohol (1.28 g, 85%). The obtained P-hydroxycinnamyl alcohol (85 g, 0.566 moles) and Ac_2O (231 ml, 4 eq) was taken in 1 lit. Round bottom flask and added 50 ml pyridine at room temp. The reaction mixture was refluxed for 4 h and then cooled to room temp. The reaction mixture was added to crush ice water in a 2 lit.conical flask to obtain white waxy type solid. This

was further filtered and the resulting solid was dried in *vacuo* to obtain crude and purified by vacuum liquid chromatography (VLC) using hexane: EtOAc (8:2) as solvent system to obtain *p*-coumaryl diacetate (1) in 118 g, 89% yield).

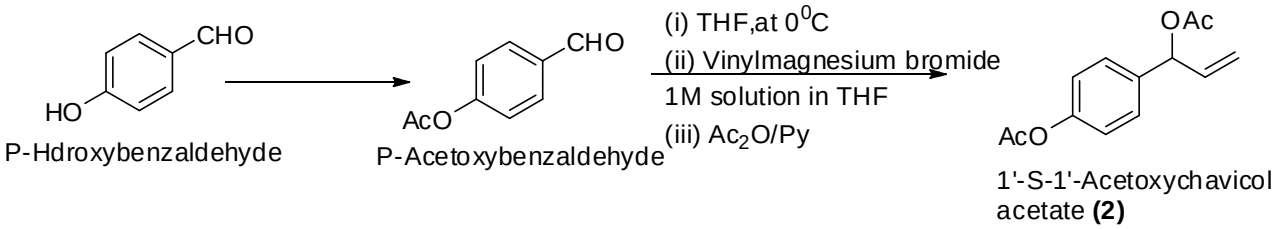




Scheme-1: Sythesis of P-Coumaryl diacetate **(1)**

2.7 Synthesis of 1'S-1'-acetoxychavicol acetate (2)

The target compound P-coumaryl diacetate was completed in four steps. In the 1st step, P-hydroxycinnamic acid was prepared from P-hydroxybenzaldehyde and malonic acid. Second step, the P-hydroxycinnamic acid was converted to its methyl ester by MeOH and H_2SO_4 and the resulting P-hydroxy methylcinnamate was reduced with DIBAL to obtain P-coumaryl alcohol. This was further acetylation with Ac_2O to obtain P-coumaryl diacetate. Take 188 g of p-hydroxybenzaldehyde in round bottom (RB) flask, added 306 g of acetic anhydride, and 300 ml of pyridine. The reaction mixture was refluxed up to 100-105 °C for 5 h. Reaction mixture progress was observed by TLC (thin layer chromatography) for every one hour. After completion of the reaction, this was cool down to room temperature and poured in to ice cold water. The mixture was extracted with ethyl acetate to get P-acetoxybenzaldehyde as colorless oil, 228 g, yield is 90%. Taken P-acetoxybenzaldehyde (4.0 g, 0.024 mol) in 250 ml of two necked RB flask and add 20 ml of THF. Then, the flaks was cooled to below 0°C by using ice-salt mixture, then added drop wise of vinyl magnesium bromide 1 M solution in THF (30 ml, 0.03 mol) and stirred at same temp for 15 min. Temperature has been raised slowly to room temperature, and the reaction mixture was stirred for another 5 h. Reaction progress was monitored by TLC and quenched with 0.5 M HCl and extracted with ethyl acetate (EtOAc). The ethyl acetate extract was concentrated under reduced pressure and acetylated with acetic anhydride to get colorless liquid compound (2), 1'-acetoxy chavicol acetate and this was exactly matched with isolated compound by TLC.



Scheme-1: Sythesis of 1'-S-1'-Acetoxychavicol acetate **(2)**

3. Discussion

The antimicrobial assay (antidandruff) was conducted based on the reported method which was done previously (Rao *et al*., 2012). The cell culture of *M. furfur* MTCC-1374 was collected from Gene Bank, Institute of Microbial Technology, Sector 39-A, chandigarh, India. Bacterial and yeast cell culture was nourished by Tryptone soya agar (TSA) and sabouraud dextrose agar (SDA), respectively. Stock solution preparation: The antimicrobial stock solution was prepared concentrations at least 1,000 mg /ml or ten times the higher concentration whichever is greater. A sample weighing 15 mg was dissolved in 500 ml of dimethyl sulfoxide (DMSO) and it was further diffused in 1 ml of propylene glycol. The total solution was makeup to 15 ml with tryptone soy broth and vortex for about a minute. The control was used as without any test sample. Preparation of bacterial inoculum: Three to five well isolated colonies of the same morphological type of the fresh stock cell culture was picked on solid agar and transferred into a tube consist of 5 ml of tryptone soy broth (TSB). The broth culture was incubated at 37°C on overnight. The turbidity of the grown stock culture was monitored by spectrophotometer at 600 nm wavelength. Approximately, the culture suspension was adjusted to get with the cell concentration of about 108 cfu/ml. Yeast cultures: Fresh grown stock culture was on solid agar, at least three to five well-isolated colonies of the same morphological type were picked and transferred into a tube containing 5 ml of sabouraud dextrose broth (SDB). The broth culture was incubated at 32°C overnight. The turbidity of the grown culture was measured by a spectrophotometer at 600 nm. Approximately, the culture suspension was adjusted to get with the cell concentration of 106 cfu/ml.

3.1 Macro tube dilution method

The sample stock solution of 500 ml was serially diluted in tubes containing 500 microliters of TSB until concentration of 0.488 mg/ml was reached. 500 microliter of cell suspension was transferred into aseptically test tube and incubated at 37°C for 24 h. The sterile tube containing TSB without sample was inoculated with 500 microliters of cell suspension and used as a positive control. Similarly, a sterile TSB tube containing a sample without cell suspension was used as sample control. After 24 h of incubation, an aliquot was taken from each test tube on tryptone soy agar plates and incubated at 37°C for 48 h to validate the growth. The MIC (minimum inhibitory concentration) was calculated from the lower concentration of the sample that completely inhibits the growth of tested organisms. A similar assay was conducted for yeast with SDB and incubated at 32°C for 72 h.

4. Conclusion

Bioassay guided fractionation and isolation of A. galanga obtained two known compounds identified as, p-coumaryl diacetate (1) and 1'-S-1'-acetoxychavicol acetate (2). The known compounds 1 and 2 were exhibited potent antimicrobial activity against M. furfur with MIC 62.5 and 7.81 µg/ml. This is the first time reported antidandruff activity from this plant. The isolated compounds 1and 2 were confirmed by synthetic methods. Compounds 1 and 2 are having two acetyl groups, trans C=C double bond in 1 and exocyclic double bond in 2. In compound 1 one of the acetyl groups was attached to methylene carbon and in 2 the acetyl group was attached methine carbon atom. Based on the above information, compound 2 is more potent than 1 due to double bond nature and acetyl group attached carbon play a crucial role antimicrobial activity against *M. furfur.*

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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