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Chemical investigation and biological activity of phytoconstituents from methanol extract of *Abutilon indicum* leaves

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ABSTRACT

Eudesmic acid, ferulic acid and caffeic acid have been isolated from the methanol extract of leaves of the plant Abutilon indicum. The concerted use of IR, ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy and chemical methods allowed the identification of these compounds. The antibacterial and antifungal activity has also been studied for these isolated compounds.

Keywords: Malvaceae, Abutilon indicum, methanol extract , Eudesmic acid, ferulic acid, caffeic acid.

INTRODUCTION

Abutilon indicum (Linn) belonging to family Malvaceae and commonly known as "Country Mallow" (English), "Kanghi (Hindi) and "Atibala" (Sanskrit) is distributed throughout the hotter parts of India. From ancient times, this plant has been used as ayurvedic medicine with greater benefits[1] (Table 1). In the literature, *Abutilon indicum* is ascribed to have wide range of medicinal applications. The aqueous extract of the plant was tested for hepatoprotective activity against carbon tetrachloride and paracetamol induced hepatotoxicities in rats[2]. The alcohol and water extracts of leaves of *Abutilon indicum* showed significant hypoglycemic effects in normal rats[3]. Seven flavanoids are isolated from flowers of *A.* indicum by Matalawska et al.[4]. Eugenol (4-allyl-2-methoxyphenol) isolated from *A. indicum* has a significant analgesic activity[5]. U-galactose and D-mannose is isolated from gum obtained from seeds by Singh and coworkers [6]. Sharma[7] isolated two sesquiterpene lactones from roots of *A indicum*. Muthu [8] et.al has reported the use of paste of whole plant which is applied topically to treat cuts and wounds. Jain [9] et.al reported the use of seed extract and stem powder in birth control and sexual diseases. Antimicrobial activity in seeds has been reported by Srinivasan [10] et.al. The methanolic extract of A. indicum exhibited some estrogenic potential of antifertility substances [11]. Gossypetin – 8 & 7- glycosides and cynidin 3- rutinoside are also isolated by Sebestian[12].The seeds are reported as laxative[13]. Pushpagadan[14] has described the ethno-medicobotanical investigations in Kerala for use of leaves of *A.indicum* in malarial fever, cough, cold, chest pain. Gallic acid shows analgesic activity in animal models [15]. From roots non drying oil consisting of fatty acids like linoleic, oleic, palmitic, stearic, lauric, myristic, caprylic and unusual fatty Acids having C-17 carbon skeleton are yielded. This oil showed significant analgesic activity[16]. Caryophyllene and its oxide, cineole, pincene, geraniol, geranyl acetate, endesmol, farnesol and borneol are identified in oil [17].

Literature on traditional medicinal plants is very meagre. In India plenty of plants are being used as drugs due to their medicinal properties. The plant kingdom still holds many species which contains substance of medicinal values, yet to be discovered [18]. Twenty medicinal plants used extensively by the tribals of Satpuda region in Maharashtra state have been screened qualitatively[19] to find out the active principles. Similar work by our coworkers based on phytochemical screening and antimicrobial studies of *Butea monosperma* has been reported on the indigenous plants found in this area [20],[21],[22] .This fact prompted us to undertake phytochemical investigation of the *Abutilon indicum* for the research. After thorough investigation and literature search it was

observed that less work has been done on this plant especially on its leaves. In our earlier reports[23] we have reported the preliminary phytochemical screening and antimicrobial studies of *Abutilon indicum* extracts using different solvents. The need of the hour is also to screen a number of medicinal plants for promising biological activity. Here we are reporting the isolation and identification of the phytoconstituents present in the methanol extract of *Abutilon indicum* leaves along with their biological activities.

Table 1: Medicinal Uses of Abutilon indicum

Part of the plant	Uses
Fruit	In Piles, gonorrhea and cough treatment. Fruit decoction mixed with ammonium chloride is given orally to treat hemorrhagic septicemia.
Leaves	Decoction of the leaves is used in toothache and tender gums. Internally for inflammation of bladder and in treatment of ulcer.
Bark	As Febrifuge, anthelmentic, alexerteric, astringent and diuretic.
Seed	As laxative, expectorant, in treatment chronic cystitis, gleets, gonorrhea and piles.
Roots	As Demulcent, diuretic, in chest infection and urethritis

EXPERIMENTAL SECTION

Plant materials

The leaves of *Abutilon indicum* were collected from the local areas of Shahada Tahsil in Nandurbar District (M.S.) and authenticated by Dr. S. K. Tayade, Taxanomist, Dept. of Botany, P.S.G.V.P.M's ASC College, Shahada, Dist: Nandurbar (M.S.). A voucher specimen has been deposited in the author's laboratory.

Extract Preparation

Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in air tight bottles. Dried powder of the leaves were extracted successively in soxhlet apparatus using petroleum ether, chloroform and methanol. The extracts were made to powder by using rotary evaporator under reduced pressure. Leaves of *Abutilon indicum* yielded 0.62%, 0.42% and 4.5% w/w powder extract with petroleum ether, chloroform and methanol respectively.

The methanol soluble fraction was subjected to CC on silica gel column using n-hexane with a gradient of Chloroform upto 100 % followed by MeOH which afforded various fractions. The fraction A was subjected to CC on silica gel column using gradient elution with chloroform and methanol (9.2 : 0.8) to get 3 major spots. The sub fraction A_1 was subjected to preparative TLC using Chloroform : Ethyl acetate : Methanol (7:5: 2: 0.5) to afford compound C**1** (55 mg).The fraction B when subjected to preparative TLC showed two spots. The major spot on recovery by PTLC yielded compound C**2** (46 mg).The fraction C was subjected to preparative TLC using Chloroform : Methanol (6 : 4) yielded compound C**3** (36 mg).

Analysis and Measurements

Spectral Analysis

Solvents for extraction and chromatography were distilled and dried by standard methods. Thin layer chromatography was carried out on plates coated with silica gel with gypsum binder and fluorescent indicator viewed under ultraviolet lamp (254 and 366 nm). Silica gel pre-coated plates (Merck, Germany) were also used. Column chromatography was performed using silica gel (60 - 120 mesh). Infra-red (IR) spectrum was recorded on Schimadzu IR. The GC/MS spectra were recorded on GC/MS ATURIN 2000R mass spectrometer (Varian), interphased with Varian Model 3800 GC/MS. Conditions: capillary column: DB5-MS, 30 m length, 0.25 mm ID, 0.5 µm film thickness, injector 250°, temp. programme: 60 - 300°C, 15° min-1; split ratio: 1:20; carrier gas He, mass spec.: 6 – 30 min, mass range: 55 - 450 m/z. The m/z parameters of the spectra were matched on the already existing computer library coupled to the analyzer. The ^{1}H-NMR and ^{13}C-NMR spectra were taken on 300 MHz and 75 MHz respectively on Mercury plus 300 MHz Make Varian USA model at Univerity of Pune. Elemental analysis were done on Flash EA 1112 series (Make-Thermo Finnigan, Italy at SAIF,IIT Indore.

Test Organisms

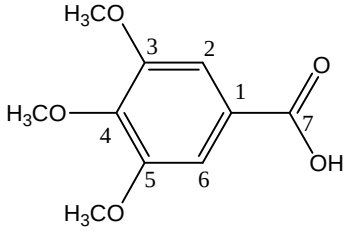
Preparation of extracts: The various fractions isolated from methanol extracts were dried in vaccum dessicator and were stored at 4^0C in a labeled sterile screw capped bottles. Microorganisms were obtained from National Chemical Laboratory, Pune, India.Microorganisms were maintained at 4^0C on nutrient agar slants. Among four microorganisms investigated, two Gram +ve bacteria were *Staphylococcus aureus* and *Bacillus subtilis*, while two Gram -ve bacteria were *Escherichia coli* and *Pseudomonas aeruginosa*.

The antimicrobial assay was performed by Agar diffusion method. The disc was saturated with 100 µl of test compound, allowed to dry and was introduced on upper layer of seeded agar plate. The plates were incubated overnight at 37^0C.Microbial growth was determined measuring the diameter of zone of inhibition. The results were compared with standard antimicrobics viz. Chloramphenicol (10 µg/disk) and Amphotericin –B

Microbiological media for bacteria used: Nutrient agar (Hi- media)
Composition (gL^{-1}) : Sodium chloride, 5.0; Beef extract 10.0; Peptone 10.0 (pH 7.2)
Inocullum size : 1x 10^8 bacteria per ml.
Concentration of compound : 0.1 mg (Prepared in DMF)
Method used : Kirby-Bauer Agar diffusion assay [25] (Well method, size 6 mm)
Dilution of the drug : Stock prepared in DMF (0.1 mg per ml)

RESULTS AND DISCUSSION

COMPOUND C1: The elemental analysis of compound C**1** corresponded to molecular formula $C_{10}H_{12}O_5$ .The IR spectrum displayed characteristic absorption bands at 3200-2860 cm^{-1} for hydroxyl group and 1680 cm^{-1} for carbonyl carbon, indicating presence of a carboxylic group in the molecule. The absorption band near 1585, 1504, 1465 cm^{-1} is for C=C stretching vibration and the band at 1322 cm^{-1} is for O-H bending vibration of aromatic compound. The band at 856 cm^{-1} indicates aromatic substitution. The ^{1}H-NMR spectrum of compound **1** exhibited one singlet at δ 7.4 in the aromatic region for H-2 and H-6. It showed a broad singlet for one proton at a downfield value of δ 12.2 indicating presence of OH group at C-7. It also displayed a sharp singlet of 9 H for three OCH_3 groups at δ 3.9. On the basis of these spectral data compound C**1** was characterized as Eudesmic acid.

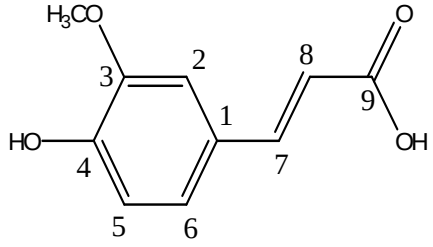


C1 (Eudesmic acid)

COMPOUND C2: The elemental analysis of compound C**2** corresponded to molecular formula $C_{10}H_{10}O_4$.The IR spectrum exhibited sharp absorption band at 3437 $cm^{-1}$for OH group of carboxylic acid, an absorption band at 1689 $cm^{-1}$for –C=O of carboxylic acid and an absorption band near 1620 $cm^{-1}$for -C=C- stretching. The band at 1176-1323 cm^{-1} showed presence of methoxy group (–C-O stretching frequency).

The ^{1}H-NMR spectrum displayed two ortho-coupled doublets (J = 8.0 Hz) each for 1H, at δ 6.3 (H-8) and δ 6.95 (H-5) and a broad singlet for 1 H at δ 7.26 (H-2) in the aromatic region indicating the presence of a trisubstituted aromatic ring in the molecule. The ^{1}H-NMR spectrum also displayed two doublets (J = 15.0 Hz), each for 1H, at δ 7.7(H-7) and 6.3(H-8). The large value of coupling constant indicated the presence of *trans*- disubstituted ethylene moiety in the molecule. The ^{1}H and ^{13}C chemical shifts of olefinic protons and carbons [δ 126.68 (C-7) and 123.6 (C-8)] were similar to those of *trans*-cinnamic acid [24].The downfield signal for three hydrogens at δ 3.9 indicates that methyl group is attached to electron withdrawing oxygen atom of OCH_3 group. The ^{13}C-NMR spectrum of **2** exhibited presence of nine carbon atoms in the molecule. The ^{13}C chemical shifts of a carbon at δ 193.59 indicated the presence of carboxylic functional group in the molecule. The upfield chemical shifts of one of the ethylenic carbon (C-8) and proton (H-1) indicated that the carboxylic group is located at C-8 position. The ^{13}C-chemical shifts of carbon atoms at δ 147.07 (C-3), 148.39 (C-4), indicated that the hydroxyl group are attached at C-3 and C-4 positions. The position of ethylenic function was determined by chemical shift of C-1 carbon at δ 126.68 and the downfield chemical shifts of C-7 carbon and H-7 proton of ethylene moiety.

On the basis of these spectral data compound C**2** was characterized as Ferulic acid.



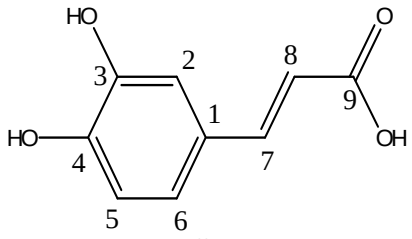
C**2 (Ferulic acid)**

COMPOUND C3: The elemental analysis of compound C**3** corresponded to molecular formula $C_9H_8O_4$ .The IR spectrum exhibited sharp absorption band at 3433 $cm^{-1}$for OH group of carboxylic acid, an absorption band at 1643 $cm^{-1}$for –C=O of carboxylic acid and an absorption band near 1620 cm^{-1} for -C=C- stretching frequency. The band at 1276 cm^{-1}showed presence of methoxy group (–C–O stretching frequency).

The ^{1}H-NMR spectrum of **3** displayed two ortho-coupled doublets (J = 8.0 Hz) each for 1H, at δ 6.85 and δ 6.92 and broad singlet at δ 7.08 for H-2 in the aromatic region. This indicated the presence of a trisubstituted aromatic ring in the molecule. The ^{1}H-NMR spectrum also displayed two doublets (J = 15.0 Hz), each for 1H, at δ 7.54 (H-7) and δ 6.24 (H-8). The large value of coupling constant indicated the presence of *trans*- disubstituted ethylene moiety in the molecule.

The ^{1}H and ^{13}C chemical shifts of olefinic protons and carbons δ 144.09 (C-7) and δ 126.73 (C-8) were similar to those of *trans*-cinnamic acid. The ^{13}C-NMR spectrum of **3** exhibited presence nine carbon atoms in the molecule. The ^{13}C chemical shifts of a carbon at δ 169.12 indicated the presence of carboxylic functional group in the molecule. The upfield chemical shifts of one of the ethylenic carbon (C-8) and proton (H-1) indicated that the carboxylic group is located at C-8 position. The ^{13}C-chemical shifts of carbon atoms at δ 145 (C-3), 147.3 (C-4), indicated that the hydroxyl groups are attached at C-3 and C-4 positions. The position of ethylenic function was determined by chemical shift of C-1 carbon at δ 121.49 and the downfield chemical shifts of δ 144.09 for C-7 carbon and H-7 proton of ethylene moiety.

On the basis of these spectral data, compound C3 was characterized as caffeic acid.



C3 (Caffeic acid)

Compound Characterisation:

Compound C1 : Amorphous solid, m.p. 236-238^{0}C (MeOH); Elemental Analysis: C=56.9723%, H=5.5749%, (calc. $C_{10}H_{12}O_5$); Molecular weight 212.19.IR (υ max ^{KBr}) :cm^{-1} 3200-2860 (OH), 2839, 1680 (-C=O), 1585, 1504, 1465 (C=C stret. vib.), 1322 (O-H bend. vibr.), 856 etc. ^{1}H-NMR (300 MHz, DMSO) : δ 12.2 (1H,bs,OH), 7.4 (2H,*s*,H-2,6), 3.9 (9H,*s*, OCH_3-3,4,5); ^{13}CNMR (75 MHz, DMSO): δ 124 (C-1), 107.2 (C-2,6), 152.7 (C-3,5), 142.6 (C-4), 171.78 (C-7), 56 (C of OCH_3).

Compound C2 : Amorphous solid; m.p.168-172^{0}C (MeOH) ; Elemental Analysis: C=61.83%, H=5.15%, (calc. $C_{10}H_{10}O_4$); Molecular weight 194.18; El-MS: m/z 180 [M]+, 163,135, 109, 92, 81, 75, 65, 45. IR (υ max ^{KBr}): cm^{-1} 3437 (OH), 1689(-C=O), 1620(C=C), 1516 (-C-O stret.), 1323 (-C-O stret.), 1176 (OH), 852,802 etc. ^{1}H-NMR: (300 MHz, $CDCl_3$): δ 7.7 (1H, *d*, *J* = 15.6 Hz, H-7), 7.26 (1H, *s*, H-2), 7.1 (1H, *m*, H-6), 6.95 (1H, *d*, *J* = 8 Hz ,H-5), 6.3 (1H, *d*, *J* = 8 Hz, *J* = 15 Hz, H-8), 5.9 (1H , bs , 4-OH), 3.9 (3H , s , 3- OCH_3).^{13}C-NMR (75 MHz, $CDCl_3$): δ 114.7 (C-1), 86.73 (C-2), 147.07(C-3), 148.3 (C-4), 109.47 (C-5), 114.37 (C-6), 126.68 (C-7), 123.6 (C-8), 193.5 (C-9), 55.98 (3-OCH_3).

Compound C3: Amorphous solid, m.p. 194-198^{0}C (MeOH); Elemental Analysis: C=59.98%, H=4.44%, (calc. $C_9H_8O_4$) ; Molecular weight 180.15. EI+- MS: m/z 300 $[M+1]^+$, 283, 163, 128, 121, 110, 93, 65, 43,etc. IR (υ max ^{KBr}): cm^{-1} 3433 (OH), 1643(-C=O), 1620 (C=C), 1523(-C-O stret.), 1276, 1118 (OH), 817 etc. ^{1}H-NMR: (300 MHz, $CDCl_3$): δ 8.33 (4-OH), 8.12 (3-OH), 7.08 (1H, *d*, H-2), 6.85 (1H,*d*, *J* = 8 Hz, H-5), 6.92 (1H, *d*, *J* = 8 Hz, H-6), 6.24 (1H, *d*, *J* = 15 Hz, H-8), 7.54 (1H, *d*, *J* = 15 Hz, H-7). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 121.49 (C-1), 114.25 (C-2), 145 (C-3), 147.3 (C-4) , 115.33 (C-5), 115.45 (C-6), 144.09 (C-7), 126.73 (C-8), 169.12 (-C=O).

Biological activity :

The compound C1, C2 and C3 were evaluated for antibacterial activity against medicinally important bacteria viz. *Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa* and for antifungal activity against *Candida albicans* and *Aspergillus niger* .The in vitro antibacterial activity was performed by agar diffusion assay (Disc diffusion method). All compounds showed some degree of antibacterial activity against the tested bacterial strains. The results of antibacterial and antifungal activity of compounds are shown in Table 2,while the photographs of the plates are also shown in Fig 1. and Fig.2

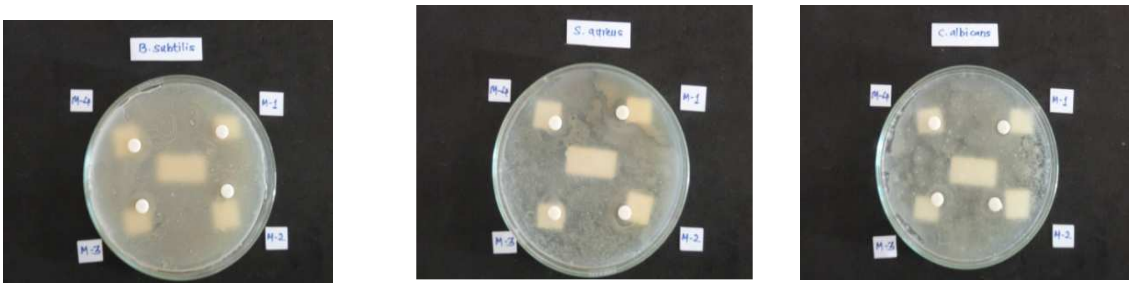
Table 2: Antibacterial and antifungal studies of compounds C1, C2 and C3 from methanol extracts of *Abutilon indicum*

Com No.	*Sample code*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococus aureus*	*Bacillus subtilis*	*Candida albicans*	*Aspergillus niger*
C1	M6	15.6	9.56	9.29	-	-	-
C2	M1	12.5	16.7	12.01	-	7.70	13.45
C3	M5	11.34	9.45	9.59	-	7.30	12.11
Chloramphenicol		28.67	24.44	29.63	26.30	NA	NA
Amphotericin-B		NA	NA	NA	NA	14.23	15.34

' - ' means no Zone of inhibition. Diameter in mm calculated by Vernier Caliper.

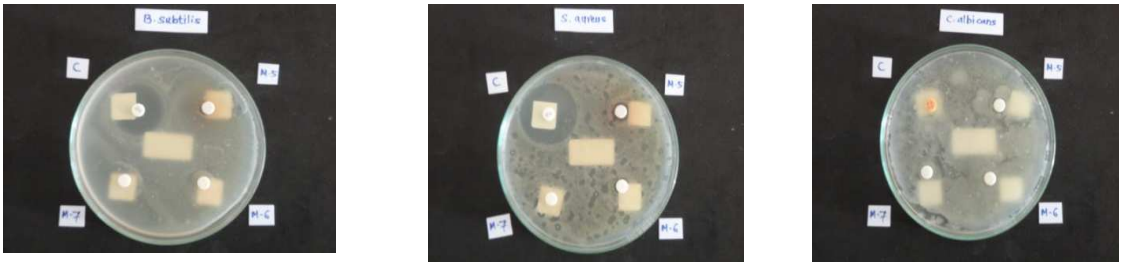
The compound **C1** showed maximum inhibition zone for *E.coli* whereas the compound C**2** showed maximum inhibition zone for *P.aeruginosa*. None of the three compounds of methanol extracts could inhibit *Bacillus subtilis*. But *Pseudomonas aeruginosa* was the most succeptible bacteria, while *Bacillus subtilis* was the most resistant bacteria which was comparable to that of standard Chloramphenicol (Chart 1).

Compound **C2** and **C3** showed good antifungal activity against *Asperigillus niger* and *Candida albicans* which was comparable to that of standard Amphotericin -B. (Chart 2)



A. Bacillus subtilis **B- Staphylococcus aureus** **C- Candida albicans**

Fig. 1- Zone of inhibition shown by compound C2 (MKP-M1) from methanol extracts of *Abutilon indicum* on *B. subtilis* , *S. aureus, and C. albicans.*



D-Bacillus subtilis **E- Staphylococcus aureus** **F- Candida albicans**

Fig 2:- Zone of inhibition shown by C1 (MKP-M6) and C3 (MKP-M5) from methanol extracts of *Abutilon indicum* on *B. subtilis, S. aureus, and C. albicans.*

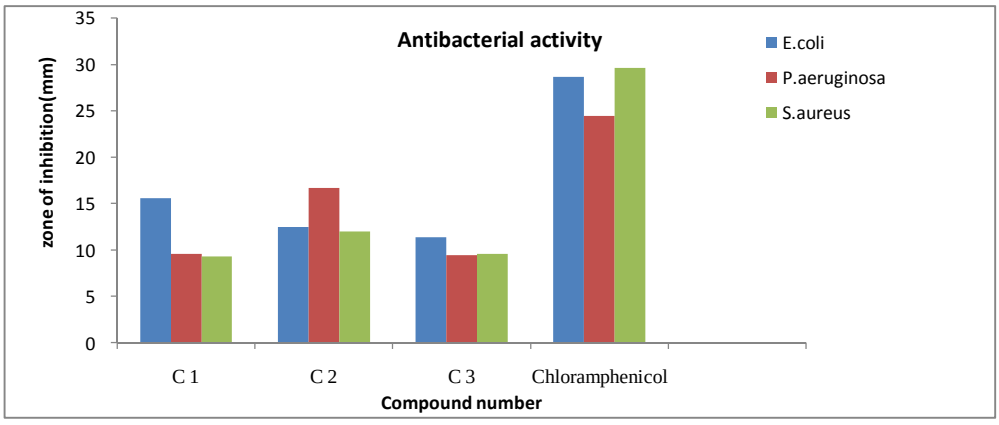


Chart 1: Graph showing the antibacterial activity of isolated compounds (potent) in comparison with the standard at 10 microgram per disk.

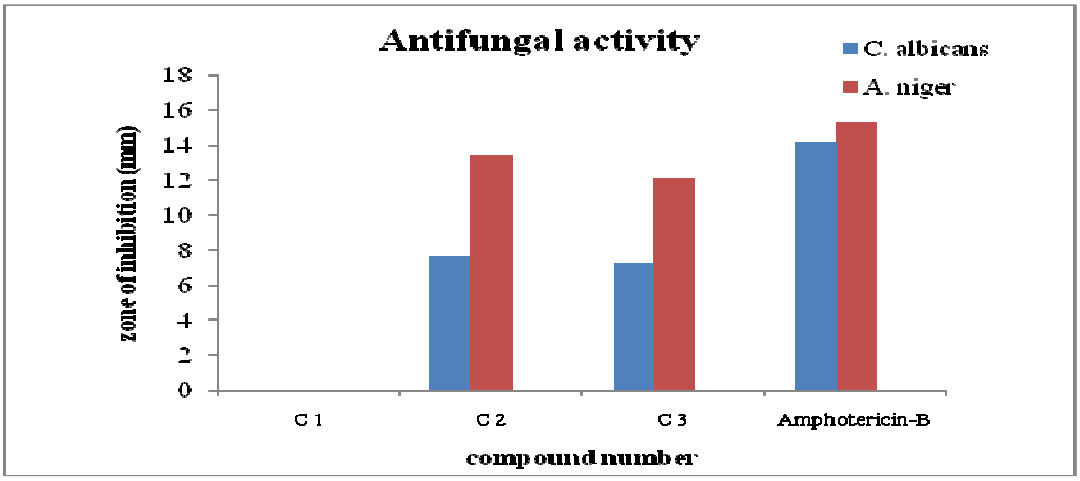


Chart 2: Graph showing the antifungal activity of isolated compounds (potent) in comparison with the standard at 100 microgram per disk.

The presence of antibacterial substances in higher plants is well established [26].Plants have provided a source of inspiration for novel drug compounds as plant derived medicines have made significant contribution towards human health. Successive isolation of botanical compounds from plant material is largely dependent on type of solvent used for extraction .Our results showed that Gram +ve bacteria *Escherichia coli* was most succeptible bacteria followed by *Pseudomonas aeruginosa*. Various workers have already shown that Gram +ve bacteria are more succeptible towards plant extracts as compared to Gram –ve bacteria [27],[28] . This difference may be attributed to the fact that cell wall in Gram +ve bacteria is of single layer where as Gram –ve cell wall is multilayered structure [29].The investigated plants did not show strong antibacterial activity; however, negative results do not mean absence of bioactive constituents nor is that the plant is inactive . Active compounds may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed [30]. Lack of activity can thus only be proven by using large doses [31].

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