Butyrolactone Production by Endophytic *Aspergillus terreus* from *Ocimum basilicum* L. and Screening of its Antimicrobial Potential

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***Abstract*-** Endophytic *Aspergillus terreus* was isolated from leaf of plant *Ocimum basilicum* L. Secondary metabolites were purified using chromatographic techniques HPLC, Preparative HPLC and mass was determined by LCMS. Through spectroscopic investigation and NMR, the structure was clarified. The chemical name of the synthesized compound was Butyrolactone ($C_{24}H_{24}O_8$) and the [M+H+] was observed as 441.15 that shows the molecular mass of the compound is 440.15amu. *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, and *Pseudomonas aeruginosa* were tested for butyrolactone's in vitro antibacterial activity, whereas *Candida albicans*, *Microsporum canis*, *Aspergillus flavus*, and *Alternaria solani* were tested for butyrolactone's antifungal activity. At a maximal concentration of 1000µg/ml of the sample, the chemical Butyrolactone exhibited moderate inhibition against *B. subtilis* (60 ± 3.12%). Antifungal activity showed Butyrolactone remained inactive against test fungi. However, weak activity was observed against *C. albicans* (17 ± 2.08%) and *A. flavus* (12 ± 1.18%) at 1000 µg/ml concentration of the compound. From the results it is concluded that plant possess *A. terreus* contains Butyrolactone that is rich source of secondary metabolites with interesting antibacterial potential.

***Index Terms*-** Antibacterial, Butyrolactone, *Aspergillus terreus*, *Ocimum basilicum*, secondary metabolites, antifungal

I. Introduction

Fungi are eukaryotic organisms that can be classified into different ecological groups that include parasitic fungi, endophytic fungi, soil fungi, fresh water fungi, and pathogenic fungi. They have the capability to produce biologically active secondary metabolites, some of them have noteworthy role in agricultural, human health issues with huge effects on social and commercial development (Gloer, 2007). Endophytes have been isolated from roots, herbs, trees, leaves, stem and marine algal thalli (*Fucus vesiculosus*, *Laminaria* sp., *Plumaria elegans*) sterilized the surface of parts of the plant by ethanol (70%) and sodium hypochlorite (Schulz et al., 1998). Endophytes have significant role in the production of biologically active secondary metabolites i.e., alkaloids, phenolic acids, saponins, tannins, terpenoids, quinones and steroids that exhibit different biological activities, antimicrobial, anti-insect and anticancer. The novel metabolites lead to the drug discovery against different diseases (Sushanto et al., 2016).

Compounds isolated from endophytic fungi have pharmacological importance and separate microbiological activities such as antibacterial, antidiebetic, anticancer and antifungal potential have been recounted (Lull et al. 2005). Natural products are also called as primary or secondary metabolites that have been produced by plants and microorganisms with molecular weight less than 3,000 daltons (Anulika et al. 2016). Primary metabolites are necessary for growth and reproduction of cell while secondary metabolites have no injurious effect on the producing organisms usually arbitrate antagonistic interactions competition and predation (Schafer and Wink, 2009). Fungi are the organisms that have the ability to produce natural products that have important value in agriculture, industries and in medicines (Calvo et al., 2002). Some of the natural products like mycotoxins are poisonous while antifungal compounds are advantageous (Demain and Fang, 2000).

The eminent class of antifungal compound that is structurally interrelated produced by numerous fungal species and fungal metabolites is strobilurins. The strobulins have the capability to inhibit plant pathogens of Ascomycetes, Basidiomycetes and Oomycetes. Fungi produce strobulins in all climate zones of world. Strobulins are fungicides used to control crop disease in the field (Anke, 1995 and Sauter et al. 1999).

The natural products are bases of novel compounds that are helpful in production of drug. As the secondary metabolites exist in the living system and the nature of drug-likeness and biological friendliness makes them as a good nominee for drug enlargement (Chin et al., 2006).

In the current study endophytic fungus was isolated from medicinal plant *Ocimum basilicum* L. and we concentrated on the purification and structural elucidation of compound produced by endophytic *Aspergillus terreus* L. and to explore its antibacterial and antifungal potential.

II. Material and Methods

General Experimental Procedure

The fungus *Aspergillus terreus* was isolated from a medicinal plant *Ocimum basilicum* L. and growth was optimized at 25-27 ◦C temperature. The growth of the fungus appeared at 27◦C. The extraction of secondary metabolites, full characterization and chemical structure of isolated compound were entirely done using different spectroscopic analysis, as before reported Nagia et al. (2012). The microbial activity (antibacterial and antifungal) of the isolated compound was also conducted.

Isolation and cultivation of fungus

The plant *Ocimum basilicum* L. was collected from Department of Botany, University of Peshawar, was cleaned with

tap water and any dust particle or diseased material was eliminated. The plant root, stem and leave were surface sterile with 75% ethanol for 1 minute and twice in disinfected purified water, then for 5 min in 0.1% mercuric chloride solution parts were dipped and washed thoroughly and carefully with sterile distilled water. After surface sterilization , plant part was cut into 0.5–1 cm fragments and inoculated on sabourad dextrose agar medium (four pieces each) for 4–10 days at 27 °C. Pure colonies were transferred onto new sabourad dextrose agar petriplates for further purification.

Extraction of secondary metabolites

Extraction of secondary metabolites from *Aspergillus terreus* L. was performed to collect secondary metabolites of fungus for carrying out biological assays such as antibacterial and antifungal bioassay. The mycelial culture was subjected to solvent extraction, after incubation period 200-250µl of 40% concentrated HCl was added to every flask. It was helpful to separate media components and improve their settlement. Mycelia were grinded by electrical blender and equal volume of ethyl acetate was added to each flask, mixed and shakes it for 30minutes. Filter the mycelia by cheese cloth and filter paper, organic layer was recovered in the beaker by using separating funnel. Brine solution (2M) was added to remove impurities and to dehydrate organic layer, sodium sulphate (Na_2SO_4) was added. The metabolites in crude form were transferred to rotary at 45°C for concentrating the metabolites. The extraction of metabolites is not an easy task to extract them in a single procedure due to chemical diversity of metabolites. Hence sample preparation is significant step to extract these metabolites, solvents such as ethyl acetate is used for extraction by Stadler et al. (2003). pH plays an important role for extraction, as 50% of the fungal products have an acidic property. Therefore extraction at low pH is required in most cases and can be accompanied with neutral extraction by Mansson et al. (2010) evaporator at 45°C for concentrating the metabolites. Then dried the crude extract by rinsing it with methanol.

Chromatographic purification

The fungal extract obtained after extraction using ethyl acetate followed by a series of chromatographic sanitizations containing silica gel column and thin-layer chromatography Dane et al. (2013).

Structural Characterization

The structure of isolated compound butyrolactone has been identified 1D (1H and 13C) and 2D (COSY, HMBC and HSQC) NMR techniques and MS spectral data and by comparison with the available literature. Chemical shifts in ppm were referenced to the internal tetra methyl cylane (TMS). Bruker 400 and 500 MHz NMR spectrometers equipped with DCH cyroprobesused for recording 1D and 2D NMR spectra.

Determination of Antibacterial activity

Antibacterial profiling of the pure compound was determined by using the agar disc diffusion method. Four sample concentrations 100, 250, 500 and 1000 g/ml were mixed in sterile DMSO (Dimethyl Sulphoxide) for antibacterial activity. The samples were impregnated (30) on pre-sterilized discs (5mm). Ciprofloxacin 25 disc (Cyrocin® - Highnoon Laboratories Ltd.) was used as positive control while sterile DMSO was used as negative control. The antibacterial profile of the crude extracts and pure compounds was assessed against four pathogenic bacterial species, i.e., *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi* and *Pseudomonas aeruginosa*, respectively.

$$Percentage growt\ in\ ibition = \frac{Zone of in\ ibition of sample}{Zone of in\ ibition of positive control} \times 100$$

Antifungal activity

Poisoned food method was employed for antifungal profiling of the pure compound. Primarily, this procedure is utilized to assess the antifungal potential of substance/chemicals against fungi. Four sample concentrations 100, 250, 500 and 1000 µg/ml were mixed in sterile DMSO (Dimethyl Sulphoxide) for antifungal activity. The samples were impregnated (30) on pre-sterilized discs (5mm). Miconazole 50 disc (Conaderm® - Harmann Pharmaceutical Laboratories (Pvt.) Ltd.) was used as positive control while DMSO was used as negative control. The antifungal profile of the pure compound was assessed against four pathogenic fungal species, i.e., *Candida albicans*, *Microsporum canis*, *Aspergillus flavus* and *Alternaria solani*, respectively. Growth inhibition was measured and percentage growth inhibition was calculated as follows:

$$inhibition = 100 - \frac{Percentage\ growth}{\frac{LinearGrowthinSampleContainingMedium}{LinearGrowthinControlMedium}} \times 100$$

III. RESULTS AND DISCUSSION

Fungal isolation and identification

Aspergillus terreus was identified on the basis of its golden/ brown growth on sabourad dextrose agar media. The pure culture of *A. terrus* shown in fig. 1. Observation of structure of fungal hyphae, colony pigmentation, spores shape and fruiting bodies are the main steps, examined by mycologists for identification of fungus (Kohlmeyer and Kohlmeyer, 1979).The isolated fungus was identified using slide culture method (Fig. 2). Microscopic study of *A. terrus* revealed its hyaline and septate hyphae with long and globose conidial heads. The conidia are small in size (2 µm), smooth and the production of aleurio conidia is the distinctive character (Lass and Cornelia, 2012).

Preliminary Antibacterial Testing

Based on preliminary antibacterial testing A. terreus was selected for isolation of biologically active compounds of natural products. Inhibition zone indicated the bioactivity of the isolated fungus. In the present study zone of inhibition was observed against *Escherichia coli* which showed the bioactivity of endophytic *Aspergillus terrus* L. (Goutam et al, 2016). Figure 3 showed the preliminary antibacterial activity against *Escherichia coli*. Zone of inhibition growth indicate the activity. Figure 4 shows microscopic image of *Aspergillus terreus.*

Liquid Chromatography Mass Spectrometry

LCMS analysis of the crude extract of *Aspergillus terrus* (shake) shows major peaks eluted at retention time 6.31, 8.5 and 8.72 min. The chromatogram of *A. terreus* shows all the peaks produce different metabolites (Fig. 5). It was observed from the LC chromatogram of *A. terrus* (Static) also produce metabolites. Result shows major peaks at 6.12, 7.15 and 7.6 min only. Different nutrients have change the properties so produce various bioactive secondary metabolites (Miao et al. 2006) thus frequently the nutrients are enhanced to produce particular compounds in the fungus grown at shaking position. Our results were confirmed through LCMS chromatogram that *A. terreus* (shake) produced distinct compounds. The compounds concentration varied with fungal growth at shake and static position. Diverse retention times and the masses of the peaks in LCMS analysis confirmed the difference.

Compound Butyrolactone

The structures of the compound are formed on the basis of spectroscopic estimates and data is compared with literature. The compound is eluted at retention time 6.31 shown in fig. 6. The result obtained from LCMS analysis shows the compound is Butyrolactone ($C_{24}H_{24}O_8$) and the [M+H+] was observed as 441.15 that shows the molecular mass of the compound is 440.15amu. The compound was searched in Dictionary of Natural Products for known compounds from *A. terreus* and was found similar to Butyrolactone. From the IR spectrum, a peak is obtained at 1722.6 cm-1 that shows the existence of carbonyl and two peaks at 1625 cm-1 and 1391cm-1 indicates the benzene ring. UVmax 274 and 320 nm represent the presence of benzene ring (Fig. 7).

Structural Characterization of Butyrolactone

The compound Butyrolactone obtained from *A. terreus* was carried out for structural characterization using 1D (1H and 13C) and 2D (COSY, HMBC and HSQC) NMR techniques. The structure of compound is shown in fig. 8.The compound is eluted at retention time 6.31. NMR spectra were employed to find out the molecular formula of the reported compound and the characterized compound is Butyrolactone.

The mass to charge (m/z) as determined is equals to 441.15 and the chemical formula is $C_{24}H_{24}O_8$. The compound displays 4 aromatic protons signals between 6.51 to 7.61 ppm. A doublet proton at 6.60 ppm with a coupling constant (J 8.9) shows signal for protons at 15 and 16 situation of aromatic ring, however a doublet proton at 6.80 ppm with a coupling constant (J 8.6) shows indication for protons at 3 and 5 position of aromatic ring. An incorporation of methoxy proton position 12 belongs to furan ring was obtained as a singlet at 3.81 ppm. Further there was one more singlet at 1.10 ppm that represents to methyl signals at 23 and 24 position. Carbon spectrum discovered total 22 signals. The numbers of aromatic carbon signals were8 between 115.59 to 138.11 ppm. The Methoxy carbon was recognized with indication at 53.10 ppm demonstrating the carbon at position 12. At position 23 and 24 Methyl carbons shows signals at 24.81 and 25.79 ppm. 1H-1H COSY presented a 3 bond coupling between H-2 to H-3 and H-5 to H-6. Similarly between H-15 to H-16 and H-20 to H-21.The HMBC correlation of the compound displayed coupling ofH-1 to C-3, H-6 to C-4, H-2 to C-4, H-3 to C-7, H-12 to C-11, H-15 to C-17 and C-19, H-16 to C-14, H-20 to C-18, H-24 to C-21 and H-23 to C-24. The result shows all the information of mass, 1H, 13C, COSY, HMBC and HSQC represented the isolated compound as Butyrolactone. Therefore, structural corroboration is worth to mention.

Compound was isolated from endophytic *A. terrus* as powder form yellow colour, eluted at retention time 6.31 with m/z 441.15 and the chemical formula is $C_{24}H_{24}O_8$. 1H NMR spectrum of compound eluted at retention time 6.31 min from *A. terrus* as shown in fig. 9. 1H-1H COSY (black lines bold) and HMBC correlations (black arrows) of compound eluted at 6.31 min shown in fig. 10. ^{13}C NMR spectrum of compound eluted at 6.31 min shown in fig. 11.

Antibacterial activity

The compound Butyrolactone revealed moderate inhibition against *B. subtilis* (60 ± 3.12%) and *S. aureus* (51 ± 1.01%) at maximum concentration of the sample (1000 µg/ml).Weak probation of growth was obtained towards *S. typhi* (11 ± 2.03%) while no activity was observed for *P. aeruginosa* by the compound. Antibacterial assay of the compounds of *A. terreus* are mentioned in table 1 and fig. 12. The compound butyrolactone showed moderate activity against *S. aureus* which is comparable to the results shown by Wu, Y. L. (2017) et al.

The compound Butyrolactone entirely inhibited the visible growth of the bacterial specie *B. subtilis* so it was considered as antibacterial. The current study showed that compound Butyrolactone has admirable antibacterial potential. This designates that increasing concentration of compound increases the rate of antibacterial potential.

Antifungal activity

Butyrolactone remained inactive against test fungi. However, weak activity was observed against *C. albicans* (17 ± 2.08%) and *A. flavus* (12 ± 1.18%) at 1000µg/ml sample of the compound. The antifungal assay of pure compound of *A. terreus* is shown table 2 and graphically mentioned in fig. 13. Butyrolactone presented antifungal effect against *Botrytis cinerea* in a concentration of 15–30 lg/ml and the minimum inhibitory concentration is 25. *Botrytis cinerea* grey mould of grapes is the leading rot causing pathogen (Zahavi et al. 2000).

Growth and chemical diversity of fungi is significantly pretentious by the nature of habitat due to adjustment of its particular environment that they have capability to produce diverse metabolites. In the current study endophytic fungi *A. terreus* was isolated from plant *O. basilicum* L. that was processed for isolation of secondary metabolites. Microscopic study of *A. terrus* L. revealed its hyaline and septate hyphae with long and globose conidial heads. The conidia are small in size (2 µm), smooth and the production of aleurio conidia is the distinctive character (Lass and Cornelia, 2012).

A number of biological, chemical and physiological interactions take between roots of plant, soil fauna and further microbial communities (Qu*et al*. 2020). Present research data suggests that fungi isolated from leaves of *O. basilicum* produces better metabolite production with antimicrobial properties. A study showed by Uzoh*et al*. 2018 reported that fungi isolated from plant rhizosphere delivers more chance to produce biologically active metabolites that possess antimicrobial potential between soil, roots and other communities of microbes.

The result obtained from LCMS analysis shows the compound is Butyrolactone ($C_{24}H_{24}O_8$) and the [M+H+] was

observed as 441.15 that shows the molecular mass of the compound is 440.15amu. The compound was searched in Dictionary of Natural Products for known compounds from *A. terrus* and was found similar to Butyrolactone. HPLC and LC-MS chromatogram reveals the variation of metabolites produced by a microorganism and is essential to detect and categorize the compounds to group of natural substances. Due to the elution of several peaks, retention time is not enough, UV-Vis spectra is also necessary to identify compounds or for their comparison to reference compounds (Fiedler, 1993). 2D NMR (1H-1H COSY) is commonly employed to deal with difficult NMR spectra as it is compulsory to assign all proton resonances and for the identification of protons that are coupled to each other. The COSY correlation conveys structural part, united to HMBC (1H-13C) that permit to consign all of the protons resonances in structural elucidation (Sato et al., 2012). The compound Butyrolactone revealed moderate inhibition against B. subtilis (60 ± 3.12%) and *S. aureus* (51 ± 1.01%) at 1000 µg/ml concentration. The compound butyrolactone showed moderate activity against *S. aureus* which is comparable to the results shown by Wu, Y. L. *et al.* 2017. Butyrolactone presented antifungal effect against *Botrytis cinerea* in a concentration of 15–30 lg/ml and the minimum inhibitory concentration is 25. *Botrytis cinerea*grey mould of grapes is the leading rot causing pathogen (Zahavi *et al*. 2000).

The purified compound might be considered as prospective antibacterial contestant to be used against the bacterial strains. Still, further studies are needed to confirm our findings and a number of selective toxicity assays desires to be perform before its solicitation as an antibacterial compound. The compound has the ability to inhibit the growth of bacterial strains by showing a prominent zone of inhibition.

CONCLUSION

The results revealed that Butyrolactone was isolated from *Aspergillus terreus* L. that have potential antibacterial activity. The data of the biological activity available here, earlier characterization of fungal metabolite, and forthcoming work in this path will display significant indication for probable drug development from endophytic fungal secondary metabolites in the treatment of various bacterial diseases.

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Table 1. Antibacterial profile revealed by Butyrolactone isolated from the ethyl acetate fraction of *A. terreus*

Bacterial spp.	Concentration ($\mu g/ml$)	% Growth inhibition by Butyrolactone ± SD
B. subtilis	100	7 ± 1.01
	250	13 ± 1.67
	500	20 ± 2.01
	1000	60 ± 3.12
S. aureus	100	2 ± 0.56
	250	11 ± 1.01
	500	17 ± 2.11
	1000	31 ± 1.01
S. typhi	100	0
	250	0
	500	3 ± 0.59
	1000	11 ± 2.03
P. aeruginosa	100	0
	250	0
	500	0
	1000	0

Table 2. Antifungal assay result of Butyrolactone from the ethyl acetate fraction of crude extract of *A. terreus*

Fungal spp.	**Concentration of sample ($\mu g/ml$)**	**% Growth inhibition by Butyrolactone ± SD**
C. albicans	100	3 ± 0.98
	250	5 ± 1.03
	500	11 ± 1.48
	1000	17 ± 2.08
M. canis	100	0
	250	0
	500	0
	1000	0
A. flavus	100	0
	250	0
	500	4 ± 0.88
	1000	12 ± 1.18
A. solani	100	0
	250	0
	500	0
	1000	0

Figure 1. Pure Culture of *A. terreus*

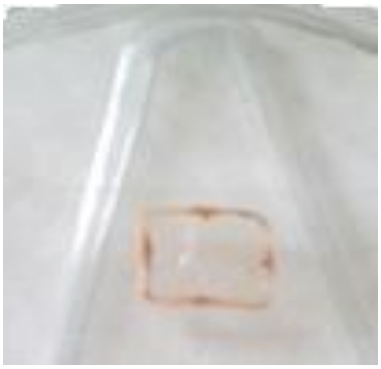


Figure 2. Slide culture method

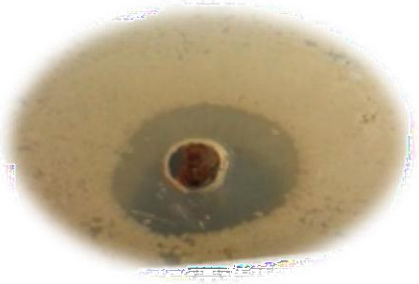


Figure 3. Zone of inhibition by *A.terreus*against *E.coli*

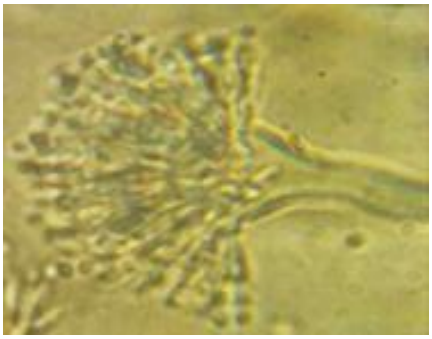


Figure 4. Microscopic image *A. terrus*

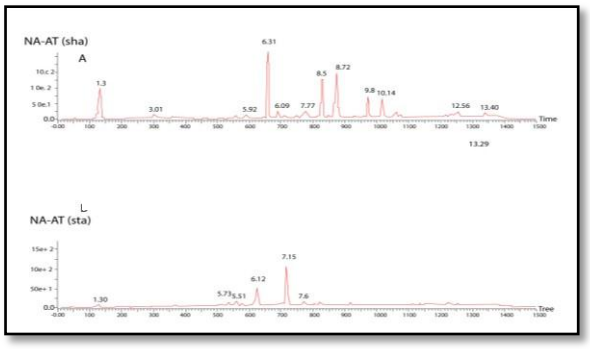


Figure 5. LC Chromatogram of *Aspergillus terrus* shake (A) and static (B)

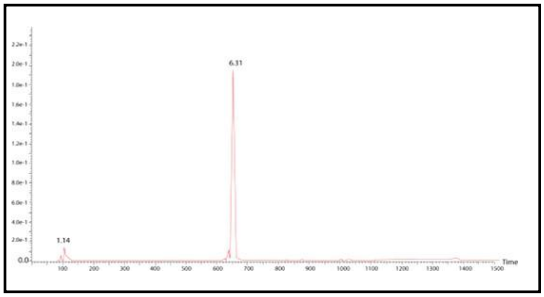
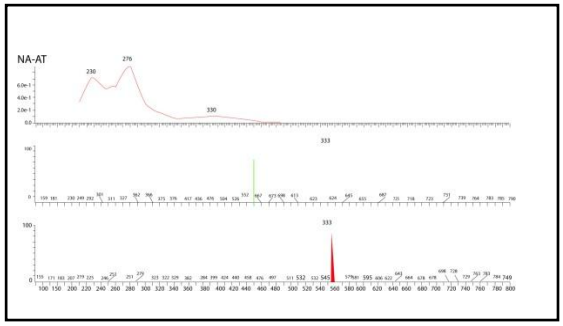


Figure 6. Purified compound eluted at 6.31 min



7. UVmax ES (+) and ES (-) of compound Eluted at 6.31min

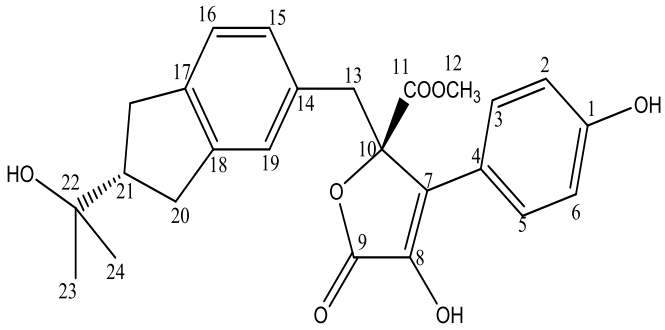


Figure 8. Structure of Butyrolactone

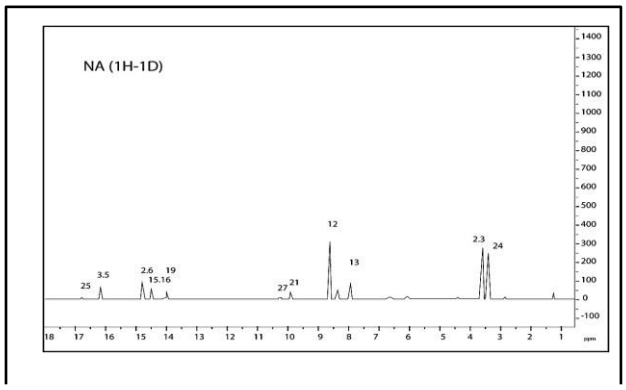


Figure 9. [1]H NMR spectrum of compound eluted at retention time 6.31 min from *A. terrus* (Numbers shows the position of protons in structure)

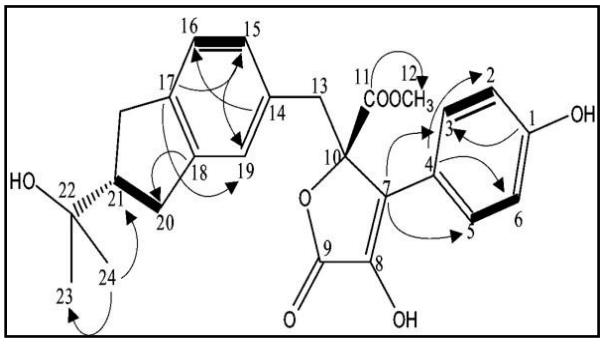


Figure 10. 1H-1H COSY (black lines bold) and HMBC correlations (black arrows) of compound eluted at 6.31 min

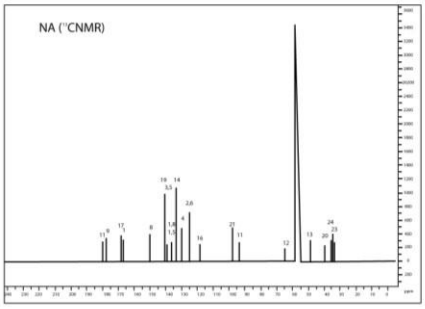


Figure 11. [13]C NMR spectrum of compound eluted at 6.31 min(Numbers represent the position of carbons in structure

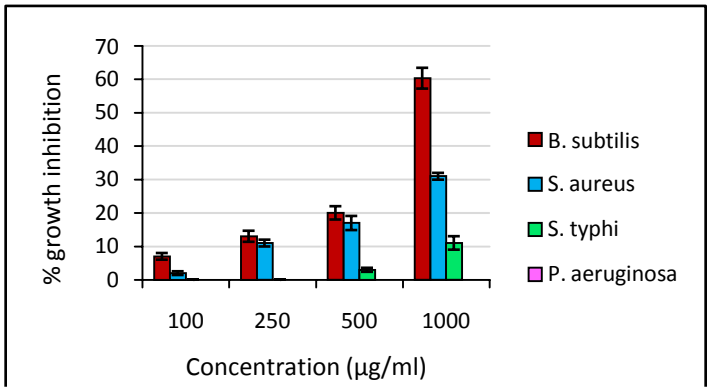


Figure 12**.** Antibacterial profile of Butyrolactone

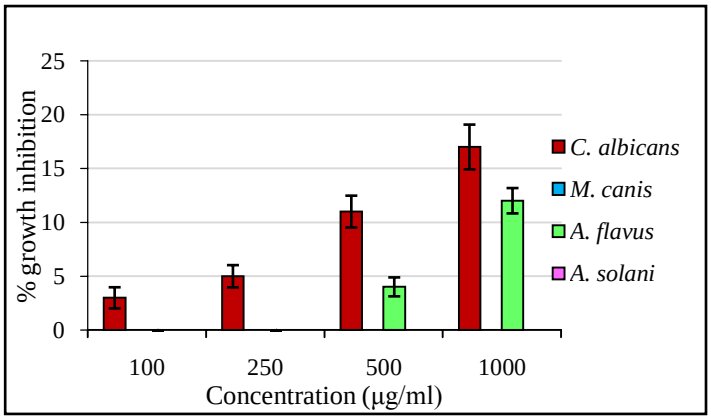


Figure 13.Antifungal profile of Butyrolactone

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