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Full Length Research Paper

Antifungal activity of crude extracts of some medicinal plants against *Fusarium* sp., the pathogen of dirty panicle disease in rice

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Antifungal activity of the ethanolic crude extracts of twenty-four medicinal plants namely, *Boesenbergia pandurata*, *Curcuma longa*, *Zingiber officinale*, *Alpinia galanga*, *Zingiber cassumunar*, *Amonum xanthioides*, *Kaempferia galanga*, *Curcuma aromatica*, *Curcuma xanthorrhiza*, *Kaempferia parviflora*, *Amonum krervanh*, *Syzygium aromaticum*, *Allium sativum*, *Allium ascolonicum*, *Cymbopogon citratus*, *Cymbopogon nardus*, *Leptochloa chinensis*, *Eupatorium odoratum*, *Piper betle*, *Synedrella nodiflora*, *Cassia siamea*, *Sorghum bicolor*, *Rosmarinus officinalis* and *Origanum vulgare* were tested against *Fusarium* sp. (the pathogen of dirty panicle disease in rice) by poisoned food technique at 0, 1,000, 2,500, 5,000, 7,500 and 10,000 ppm. The inhibition of mycelial growth was evaluated. All the used twenty-four crude extracts showed significant antifungal activity against *Fusarium* sp. The result showed that the *S. aromaticum* and *O. vulgare* crude extracts showed 100% inhibition of mycelial growth at all concentrations, whereas, *S. nodiflora* and *S. bicolor* crude extracts at 10,000 ppm gave the lowest inhibition of 42 and 32%, respectively.

Key words: Antifungal activity, dirty panicle disease, *Fusarium* sp., medicinal crude extract, rice.

INTRODUCTION

Fusarium species are important fungal pathogens causing seed borne diseases of many crops worldwide. The dirty panicle disease is a major disease problem caused by *Fusarium* sp. around the locations of rice production in Thailand and the tropical location in the world (Ou, 1985). Abdelmonem (2000) reported this disease from different location caused by several pathogens: *Fusarium* sp., *Alternaria* sp., *Cercospora* sp.

and *Curvularia* sp. The panicle dirty disease control in rice production had five methods namely mechanical, cultural, biological, chemical and integrate method. The chemical control is the best method of control for dirty panicle disease, whereas this method is harmful to environmental condition, product residues and human health.

Although, with the application of several fungicides,

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disease can be controlled but the toxicity effects on products in human health and environmental issues are studies. Nowadays, the farmers use the biological control for dirty panicle control in rice. Natural plant products have the potential as safe alternatives for chemical fungicides in rice disease managements. The medicinal herb crude extracts for the seed borne pathogen control have attracted wide interest. In general, several researches have been conducted on medicinal herb crude extracts and essential oils to control plant disease (Nwachukwu and Umechuruba, 2001; Kritzinger et al., 2002; Palhano et al., 2004; Velluti et al., 2004).

Many experiments reported on some Zingiberaceae species for the antimicrobial activity, Saleem et al. (2011) studied the antimicrobial activity of essential oil of two plants (*Curcuma longa* and *Curcuma aromatica*) against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and found that the essential oil of two plants can completely inhibit the growth of three pathogens. Husein et al. (2009) working on antimicrobial activity of crude extracts from *Curcuma xanthorrhiza* against three pathogen namely *E. coli*, *S. aureus* and *Bacillus cereus*, found that the ethanol extract inhibited *S. aureus* and *B. cereus* and ethyl acetate extract inhibited *E. coli*. Kummee et al. (2008) found that the ethanol extract of *Kaempferia parviflora* exhibited strong antifungal activity against three pathogens, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum gypseum* with MIC values of 62.5, 125 and 250 µg/ml, respectively. Johnny et al. (2011) tested rhizome extracts of *Alpinia galanga* extracted by methanol, chloroform and acetone against *Collectotrichum capsici* at 0.01 0.10 1.00 and 10.0 µg/ml concentrations and found that methanol, chloroform and acetone extracts showed the highest of inhibition of radial growth at 10.0 µg/ml concentrations. Sawatdikarn (2011) studied the antifungal activity of crude extracts of six Zingiberaceae species namely *Boesenbergia pandurata*, *Zingiber officinale*, *Zingiber cassumunar*, *Amonum xanthioides*, *Kaempferia galanga* and *Amonum krervanh* against *Curvularia* sp. (the pathogen of dirty panicle disease in rice), selected crude extracts of *B. pandurata* at 1,000 ppm showed the highest of mycelial growth inhibition of 57.8% and the crude extracts of *A. Krervanh* at 1,000 ppm showed the lowest of mycelial growth inhibition of 43.7%.

Many experiments showed that the lemongrass (*Cymbopogon citratus*) essential oil inhibited the mycelial growth of some pathogens: *Collectotrichum gloeosporioides* (Palhano et al., 2004), *Fusarium verticillioides*, *Fusarium proliferatum* and *Fusarium graminearum* (Velluti et al., 2004) and *Fusarium moniliforme* (Nwachukwu and Umechuruba, 2001). Istianto and Emilda (2011) found that the essential oil of citronella grass (*Cymbopogon nardas*) can completely inhibit the mycelial growth of *F. oxysporum*.

Kritzinger et al. (2002) tested crude extracts of clove (*Syzygium aromaticum*) for antifungal activity against two plant pathogens (*F. oxysporum* and *F. equiseti*), the clove crude extract at 1,000 ppm showed 100% inhibition on mycelial growth of *F. oxysporum* and *F. equiseti*. Moghtader et al. (2011) from Iran reported that essential oil of rosemary (*Rosmarinus officinalis*) aerial parts at 1½ oil dilution inhibited the growth of *Aspergillus flavus*.

Many experiments reported some plants crude extracts and essential oil have antimicrobial activity. Lee et al. (2001) tested essential oils of *Origanum vulgare* for their antimicrobial activities against four plant pathogens (*Botrytis cinerea*, *Collectotrichum gloeosporioides*, *Pythium altimum* and *Rhizoctonia solani*), selected essential oils of *Origanum vulgare* showed the inhibition of mycelial growth for 65 68 78 and 92% of *B. cinerea*, *R. solani*, *C. gloeosporioides* and *P. altimum*, respectively. Bansod and Rai (2008) tested plant extracts (*Allium sativum*) for their efficacy against *Aspergillus fumigatus* and found extract of *A. sativum* at 100 µg/ml to completely inhibit the mycelial growth of *A. fumigatus*. Owalabi et al. (2010) reported that the pure extract of *Eupatorium odoratum* can completely inhibit the mycelial growth of *Bacillus cereus* and *Aspergillus niger*. Ali et al. (2010) noted the inhibitory effect of extract of *Piper betle* on the mycelial growth of four pathogens namely *Aspergillus flavus*, *A. niger*, *A. fumigatus* and *A. paraciticus* and found that the extract showed maximum inhibition of the mycelial growth of the pathogens. Ogbekor and Adekunle (2005) tested 21 plants extracts on the mycelial growth against *Corynespora cassiicola* (the pathogen of leaf spot of para rubber) under laboratory condition and found extract of *Synedrella nodiflora* showed the inhibition of mycelial growth for 34.8%. Nanasombat and Teckchuen (2009) screened 20 species for their antimicrobial activity and found that the methanolic extracts of *Cassia siamea* exhibited strong antibacterial activities namely *B. cereus*, *Listeria monocytogenes* and *S. aureus*. Soetan et al. (2006) reported on the seed extract of *S. bicolor* on the mycelial growth of *S. aureus* under laboratory condition and found that 25 mg/ml seed extracts of *S. bicolor* showed the highest of inhibition on the mycelial growth of the pathogen.

No information was found on the inhibition of mycelial growth of *Fusarium* sp. (the pathogen of dirty panicle disease of rice). The objective of this research was to evaluate twenty-four medicinal herb crude extracts on the mycelial growth of *Fusarium* sp. in central area, Thailand.

MATERIALS AND METHODS

This work was conducted at Department of Applied Science, Faculty of Science and Technology, Phranakhon Si Ayutthaya Rajabhat University, Phranakhon Si Ayutthaya during 2010-2011 to determine the antifungal activity of 1) *Boesenbergia pandurata* 2)

Curcuma longa 3) *Zingiber officinale* 4) *Alpinia galanga* 5) *Zingiber cassumunar* 6) *Amonum xanthioides* 7) *Kaempferia galanga* 8) *Curcuma aromatica* 9) *Curcuma xanthorrhiza* 10) *Kaempferia parviflora* 11) *Amonum krevanh* 12) *Syzygium aromaticum*, 13) *Allium sativum* 14) *Allium ascolonicum* 15) *Cymbopogon citratus* 16) *Cymbopogon nardus* 17) *Leptochloa chinensis* 18) *Eupatorium odoratum* 19) *Piper betle* 20) *Synedrella nodiflora* 21) *Cassia siamea* 22) *Sorghum bicolor* 23) *Rosmarinus officinalis* and 24) *Origanum vulgare* against *Fusarium* sp. (the pathogen of dirty panicle disease in rice) in sterile distilled water and ethanol by using food poisoning technique (Prasad et al., 2010).

The plant's name is the name of the different families (8 family), the plant name in number 1-11 is the member of Zingiberaceae, number 12 is the member of Myrtaceae, number 13-14 are the members of Amaryllidaceae, number 15-17 and 22 are the members of Poaceae, number 18 and 20 are the members of Asteraceae, number 19 is the member of Piperaceae, number 21 is the member of Fabaceae and number 23 and 24 are the members of Lamiaceae.

Preparation of rice seeds and isolation of pathogen

Rice seeds were obtained from three location in Central areas : Phranakhon Si Ayutthaya, Aungthong and Prathumthani Province. *Fusarium* sp. from the rice seeds were isolated and maintained on Petri dishes containing potato dextrose agar (PDA) and incubated at 25°C for 3 days before the tests.

Collection and preparation of plants samples

Twenty-four medicinal herb crude extracts namely : *B. pandurata*, *C. longa*, *Z. officinale*, *A. galanga*, *Z. cassumunar*, *A. xanthioides*, *K. galanga*, *C. aromatica*, *C. xanthorrhiza*, *K. parviflora*, *A. krevanh*, *S. aromaticum*, *A. sativum*, *A. ascolonicum*, *C. citratus*, *C. nardus*, *L. chinensis*, *E. odoratum*, *P. betle*, *S. nodiflora*, *C. siamea*, *S. bicolor*, *R. officinalis* and *O. vulgare* was extracted by 90% ethanol and tested for antifungal activity on *Fusarium* sp.

Twenty-four medicinal crude extracts used in this study was obtained from three locations in Phranakhon Si Ayutthaya province, Bangban, Wangnoi and Bangpa-in, where medicinal herb is produced and exported. Fresh rhizomes of 11 species namely *B. pandurata*, *C. longa*, *Z. officinale*, *A. galanga*, *Z. cassumunar*, *A. xanthioides*, *K. galanga*, *C. aromatica*, *C. xanthorrhiza*, *K. parviflora* and *A. krevanh* were collected from Bangban, fresh stems of *C. citratus*, *C. nardus* and *L. chinensis* were collected from Wangnoi, fresh leaves of *E. odoratum*, *P. betle*, *S. nodiflora*, *C. siamea*, *S. bicolor*, *R. officinalis* and *O. vulgare* were collected from Bangpa-in, fresh bulbs of *A. sativum* and *A. ascolonicum* were collected from Wangnoi and fresh bud parts of *S. aromaticum* were collected from Bangpa-in. They were washed with tap water and air dried for three days to eliminate surface moisture. Then each part of the medicinal plants were packed to envelop and kept in oven at 80°C until dried. Each dried parts were grinded separately in an electric grinder to obtain powder which was then kept in plastic bags before the tests.

Preparation of crude extracts

One hundred grams of the dried powdered plant were soaked in 1,000 ml of 90% ethanol. These mixtures were refluxed followed by agitation at 200 rpm for 1 h. The ethanolic extracts were squeezed and filtered by muslin cloth. The crude extracts were placed in a wide tray to evaporate ethanol and water plant extracts were added (Prasad et al., 2010).

Mycelial growth test

Food poisoning technique

Diffusates were added in PDA and poured into Petri dishes. To the PDA medium was added only with ethanol and water served as the control treatment. Each Petri dishes was inoculated with 5 mm plug of pure isolate taken from margins of actively growing culture of pathogen. All Petri dishes were incubated at 25°C.

The screening of crude extracts for antifungal activity was conducted using the agar dilution method. Different crude extracts were tested using food poisoning technique. Each tested crude extracts was used at different concentrations: 0 (control treatment), 1,000, 2,500, 5,000, 7,500 and 10,000 ppm. The Petri dishes were incubated at room temperature for 7 days. The efficacy of treatment was assessed from all the four plates by measuring fungal colony development (cm). The mycelial growth inhibition (M) with respect to the control treatment was calculated from the formula (Sheng-Yang et al., 2005)

$$M = [(A-B) / A] \times 100$$

Where A is the colony diameter of the control treatment and B is the colony diameter of the treated crude extracts.

Statistical analysis

For statistical analyses, Duncan multiple range test was used to compare the average.

RESULTS AND DISCUSSION

The twenty-four medicinal plant crude extracts showed inhibition on mycelial growth of *Fusarium* sp. at different concentrations (Table 1). The crude extracts of *S. aromaticum* (Figure 1) and *O. vulgare* (Figure 2) showed 100% inhibition on mycelial growth at all concentrations whereas, the *S. nodiflora* (Figure 3) and *S. bicolor* (Figure 4) crude extracts at 10,000 ppm gave the lowest inhibition of 42 and 32%, respectively.

The clove (*S. aromaticum*) and origano (*O. vulgare*) crude extracts showed 100% inhibition on mycelial growth at all concentrations (Figure 1) and the crude extracts of these species can be used for *Fusarium* sp. control (the pathogen of dirty panicle disease in rice) at all concentration (1,000-10,000 ppm). These results are in agreement with that the researches of Kritzinger et al. (2002), Lee et al. (2001) and Prasad et al. (2010). The crude extracts of *S. nodiflora* (Figure 3) and *S. bicolor* (Figure 4) showed inhibition of mycelial growth of *Fusarium* sp. Which is similar to the researches of Ogebor and Adekunle (2005) and Soetan et al. (2006).

The *Z. officinale* and *P. betle* had 100% of inhibition on mycelial growth of *Fusarium* sp. at 2,500-10,000 ppm (Table 1). These results have been confirmed by some researches, for examples Sawatdikarn (2011) noted that *Z. officinale* crude extract at 1,000-10,000 ppm inhibited mycelial growth of *Curvularia* sp. (the pathogen of dirty

Table 1. Efficacy of different concentration of some medicinal plants crude extracts on mycelial growth inhibition of *Fusarium* sp.

Medicinal herb crude extracts	Mycelial growth inhibition (%)				
	1,000 ppm	2,500 ppm	5,000 ppm	7,500 ppm	10,000 ppm
<i>Boesenbergia pandurata</i>	53 ^d	64 ^d	73 ^c	100 ^a	100 ^a
<i>Curcuma longa</i>	71 ^b	76 ^c	82 ^b	100 ^a	100 ^a
<i>Zingiber officinale</i>	62 ^c	100 ^a	100 ^a	100 ^a	100 ^a
<i>Alpinia galangal</i>	21 ^f	28 ^g	43 ^g	64 ^d	76 ^c
<i>Zingiber cassumunar</i>	51 ^c	82 ^b	100 ^a	100 ^a	100 ^a
<i>Amomum xanthioides</i>	8 ^g	20 ^g	29 ^h	100 ^a	100 ^a
<i>Kaempferia galanga</i>	71 ^b	83 ^b	86 ^b	100 ^a	100 ^a
<i>Curcuma aromatica</i>	83 ^b	88 ^b	100 ^a	100 ^a	100 ^a
<i>Curcuma xanthorrhiza</i>	76 ^b	78 ^c	79 ^c	80 ^b	100 ^a
<i>Kaempferia parviflora</i>	46 ^e	48 ^e	50 ^e	51 ^e	54 ^e
<i>Amonum krevanh</i>	53 ^d	69 ^c	80 ^c	84 ^b	87 ^b
<i>Syzygium aromaticum</i>	100 ^a	100 ^a	100 ^a	100 ^a	100 ^a
<i>Allium sativum</i>	26 ^f	39 ^f	52 ^f	57 ^e	61 ^d
<i>Allium ascolonicum</i>	32 ^f	33 ^f	45 ^g	50 ^e	68 ^d
<i>Cymbopogon citratus</i>	56 ^d	60 ^d	69 ^d	76 ^c	79 ^c
<i>Cymbopogon nardus</i>	44 ^e	48 ^e	56 ^e	65 ^d	100 ^a
<i>Leptochloa chinensis</i>	30 ^f	38 ^f	50 ^f	53 ^e	63 ^d
<i>Eupatorium odoratum</i>	28 ^f	46 ^e	47 ^f	57 ^e	74 ^c
<i>Piper betle</i>	58 ^d	100 ^a	100 ^a	100 ^a	100 ^a
<i>Synedrella nodiflora</i>	33 ^f	37 ^f	37 ^g	39 ^f	42 ^f
<i>Cassia siamea</i>	45 ^e	49 ^e	59 ^e	70 ^c	84 ^b
<i>Sorghum bicolor</i>	11 ^g	23 ^g	25 ^h	30 ^g	32 ^g
<i>Rosmarinus officinalis</i>	71 ^b	73 ^c	75 ^c	79 ^b	100 ^a
<i>Origanum vulgare</i>	100 ^a	100 ^a	100 ^a	100 ^a	100 ^a
C. V. (%)	15.64	12.85	13.26	10.25	11.26

In each column, mean followed by a common letter are not significantly different at the 5% level by DMRT.

panicle disease of rice) and the ginger crude extracts (*Z. officinale*) inhibited mycelial growth of *T. padwickii* in rice (Shetty et al., 1989). On the other hand, Ali et al. (2010) who reported the extract of *P. betle* against four pathogens namely *A. flavus*, *A. niger*, *A. fumigatus* and *A. paraciticus* found that the extract showed the inhibition of the mycelial growth of the pathogens.

The crude extracts of *Z. cassumunar* and *C. aromatica* showed 100% inhibition on mycelial growth *Fusarium* sp. at 5,000-10,000 ppm. These results are in agreement with that the researches of Sawatdikarn (2011) and Saleem et al. (2011).

The four crude extracts, namely *B. pandurata*, *C. longa*, *A. xanthioides* and *K. galanga* showed 100% inhibition of mycelial growth at 7,500-10,000 ppm. These results have been confirmed by some researches, for examples Sawatdikarn (2011) noted that three plant crude extracts in Zingiberaceae species namely *B. pandurata*, *A. xanthioides* and *K. Galanga* at 10,000 ppm concentrations showed the highest inhibition on mycelial growth of

Curvularia sp. for 100 100 and 95%, respectively. In another study, Saleem et al. (2011) who studied essential oil of *C. longa* against three pathogens, including *E. coli*, *P. aeruginosa* and *S. aureus* found that the essential oil of *C. longa* showed the highest 100% inhibition of the pathogens.

For the three plant crude extracts namely *C. xanthorrhiza*, *C. nardus* and *R. officinalis* had 100% inhibition on mycelial growth *Fusarium* sp. at 10,000 ppm. These results are in agreement with the researches on antimicrobial activity including Husein et al. (2009) showing the antimicrobial activity of crude extracts from *C. xanthorrhiza* against *E. coli*, *S. aureus* and *B. cereus* and that the ethanol extract inhibited *S. aureus* and *B. cereus* and ethyl acetate extract inhibited *E. coli*. On the other hand, Prasad et al. (2010) found *C. nardus* essential oil as highly effective in controlling *Phomopsis azadirachtae* (the causative agent of die-back disease of neem) showed 100% inhibition of mycelial growth at 2,500 ppm. The crude extracts from citronella grass (*C. nardus*)

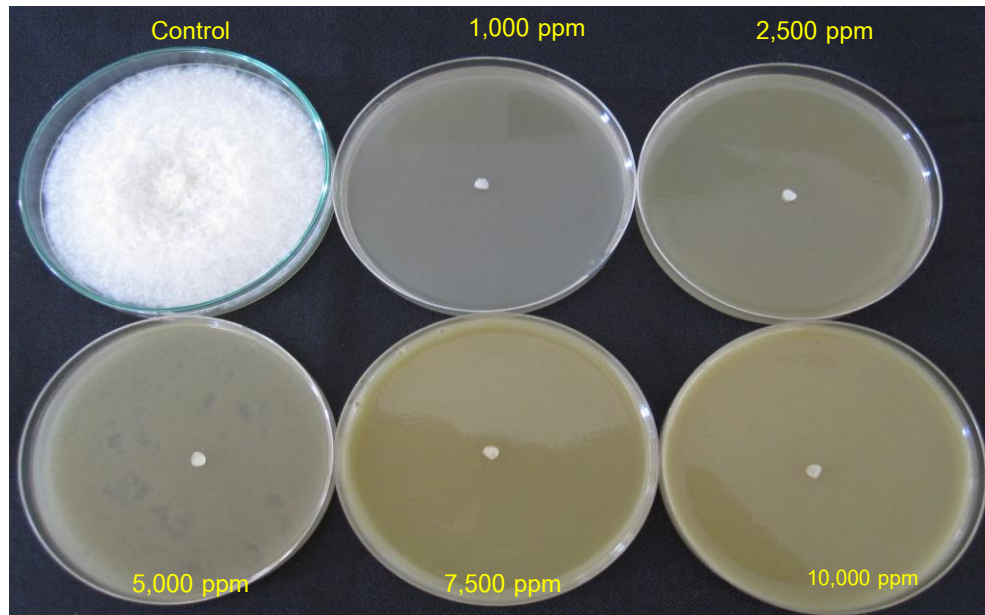


Figure 1. Effect of *Syzygium aromaticum* crude extract on the mycelial growth of *Fusarium* sp. at different concentrations (control treatment, 1000, 2000, 2500, 5000, 7500 and 10,000 ppm).

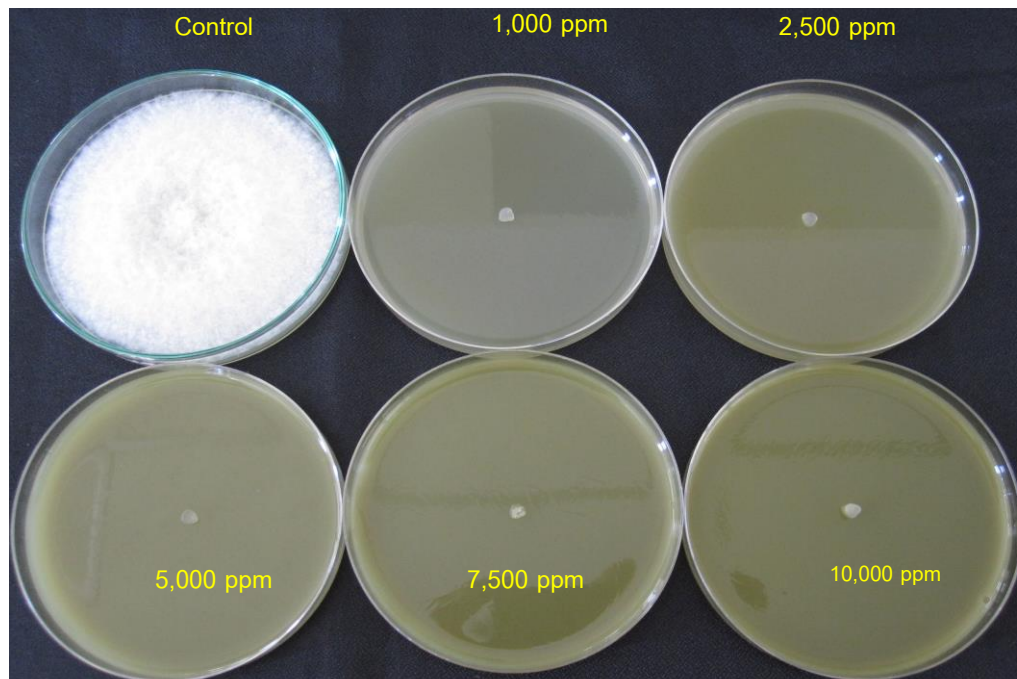


Figure 2. Effect of *Origanum vulgare* crude extract on the mycelial growth of *Fusarium* sp. at different concentrations (control treatment, 1000, 2000, 2500, 5000, 7500 and 10,000 ppm).

showed inhibition on mycelial growth of *Fusarium* sp. because of decreased wall synthesis, plasma membrane

disruption, microcondrial structure disorganization and inhibitor of biodegradation and storage contaminating fungi

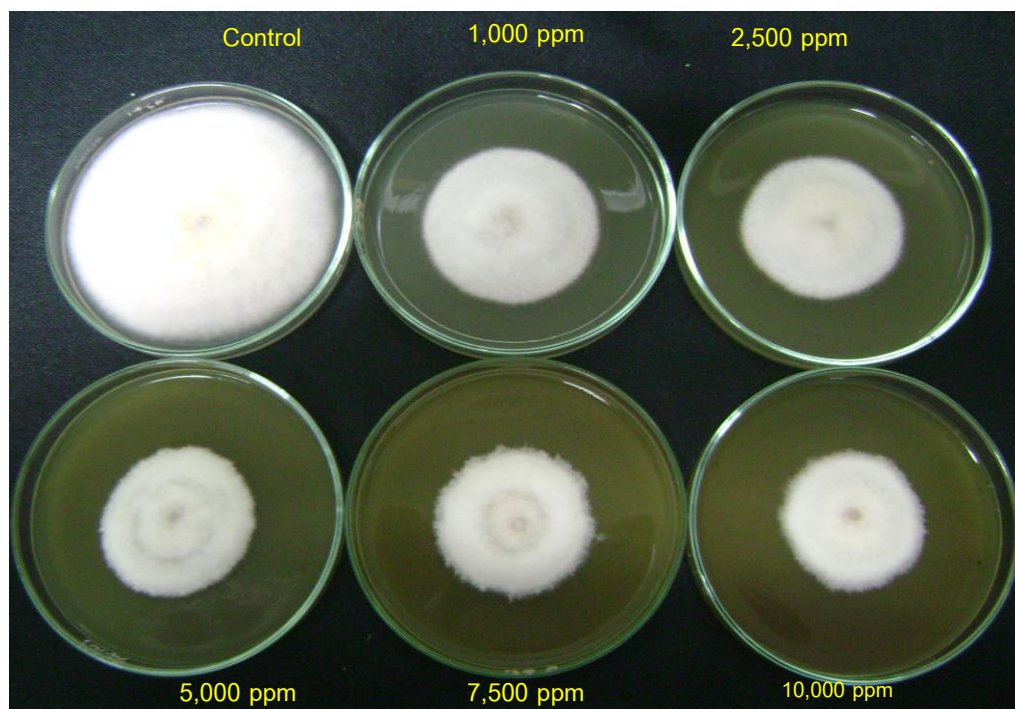


Figure 3. Effect of *Synedrella nodiflora* crude extract on the mycelial growth of *Fusarium* sp. at different concentrations (control treatment, 1000, 2000, 2500, 5000, 7500 and 10,000 ppm).

(Billerbeck et al., 2009). In another study, although, Sawatdikarn (2011) reported *R. officinalis* crude extract against *Curvularia* sp. (the pathogen of dirty panicle disease of rice) at different concentrations and showed *R. Officinalis* crude extract at 7,500-10,000 ppm, it can completely inhibit the mycelial growth of *Curvularia* sp., and showed agreement with the antibacterial activity of *R. officinalis* essential oil against *B. cereus* (Valero and Salmeron, 2003).

The remaining crude extracts such as *A. galanga*, *K. parviflora*, *A. krervanh*, *A. sativum*, *A. ascolonicum*, *C. citratus*, *L. chinensis*, *E. odoratum*, *S. nodiflora*, *C. siamea* and *S. bicolor* showed inhibition at 10,000 ppm concentrations on mycelial growth of *Fusarium* sp. between 32% of *S. bicolor* crude extract to 88% of *A. krervanh* crude extract (Table 1). Among the crude extracts of the tested plants, eleven showed effective potentials against *Fusarium* sp., these results have been confirmed by former several researches, for examples Johnny et al. (2011) tested rhizome extracts of *A. galanga* extracted against *C. capsici* at different concentrations and found that the crude extracts showed the highest inhibition of radial growth at 10.0 µg/ml concentrations. On the other hand, Sawatdikarn (2011) who studied four crude extracts namely *K. parviflora*, *A. krervanh*, *A. ascolonicum* and *L. chinensis* against *Curvularia* sp. (the pathogen of dirty panicle disease of rice) at different concentrations found

that the *K. parviflora*, *A. krervanh*, *A. ascolonicum* and *L. chinensis* crude extracts at 10,000 ppm showed inhibition of mycelial growth for 68, 88, 70, and 63%, respectively. On the other hand, Chohan et al. (2011) tested plant extracts (*Allium sativum*) for their efficacy against *F. oxysporum* f. sp. *gladioli* (the pathogen of corm rot of gladiolus) at different concentrations and found extract of *A. sativum* at 8% showed inhibition of the mycelial growth of 35%.

For lemongrass (*C. citratus*) crude extracts showed the inhibition on mycelial growth of *Fusarium* sp. for 56-79% at 1,000-10,000 ppm concentrations, the research is related to the lemongrass (*C. citratus*) essential oil for the inhibited on mycelial growth of some pathogen; *Collectotrichum gloeosporioides* (Palhano et al., 2004). *F. moniliforme* (Nwachukwu and Umechuruba, 2001) and *F. verticillioides*, *F. proliferatum* and *F. graminearum* (Velluti et al., 2004) and the essential oils of lemongrass showed the inhibition of microbial agent in storage of some seeds; maize and cowpea (Adegoke and Odesola, 1996) and melon (Bankole and Joda, 2004). The phytochemical from lemongrass crude extracts showed the inhibition on mycelial growth *Fusarium* sp. because of decrease of hyphal diameter and hyphal wall, plasma membrane disruption, microcondrial structure disorganization and inhibitor of biodegradation and storage contaminating fungi (Helal et al., 2006).

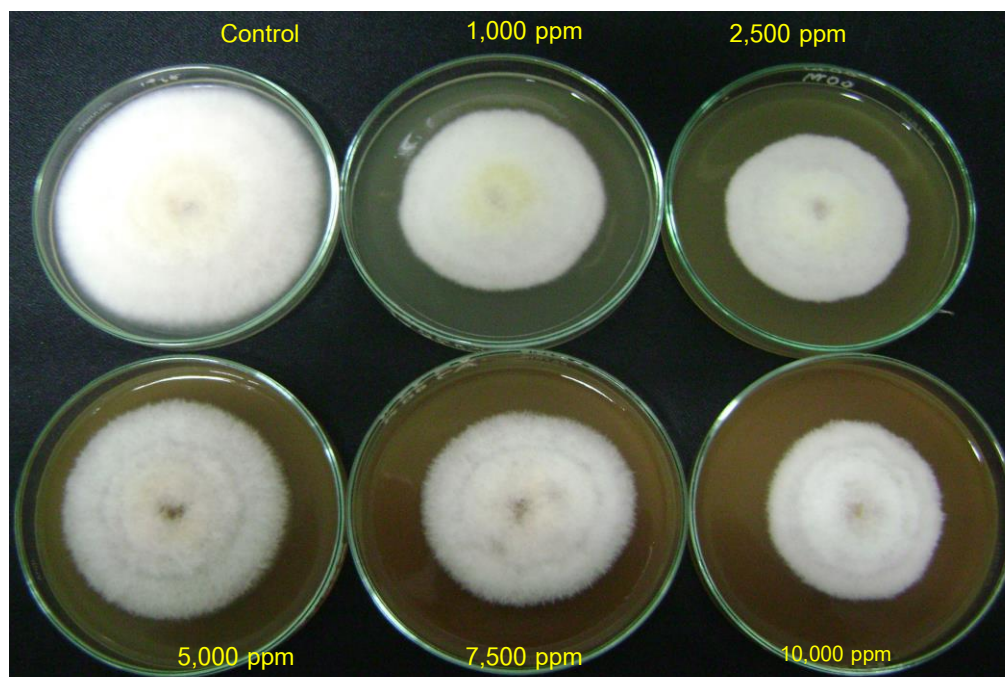


Figure 4. Effect of *Sorghum bicolor* crude extract on the mycelial growth of *Fusarium* sp. at different concentrations (control treatment, 1000, 2000, 2500, 5000, 7500 and 10,000 ppm).

The crude extracts of *E. odoratum* showed inhibition at 10,000 ppm for 74% of mycelial growth of *Fusarium* sp. These results are in agreement with those of Owalabi et al. (2010). On the other hand, the crude extract of *C. siamea* had 84% inhibition at 10,000 ppm (Table 1) which agree with the antifungal activity of *C. siamea* crude extract against fungal growth in grain maize storage (Chatterjee, 1990) and the methanolic extracts of *C. siamea* exhibited strong antibacterial activities against *B. cereus*, *L. monocytogenes* and *S. aureus* (Nanasombat and Teckchuen, 2009).

The objective of this study was to screen the effect of twenty-four crude extracts on the mycelial growth of *Fusarium* sp. The usage of all crude extract was the best for *Fusarium* sp. control due to their harmless effect on environmental condition, to user and to consumer. The study is related to several researches that noted the antifungal activity of crude extracts and essential oils including, the crude extracts in *Zingiberaceae* species namely *B. pandurata*, *A. xanthioides* and *K. Galanga* for *Curvularia* sp. (the pathogen of dirty panicle disease of rice) (Sawatdikarn, 2011), the lemongrass (*C. citratus*) essential oil that inhibited mycelial growth of some pathogen; *Collectotrichum gloeosporioides* (Palhano et al., 2004) *F. moniliforme* (Nwachukwu and Umechuruba, 2001) and *Fusarium verticillioides*, *F. proliferatum* and *F. graminearum* (Velluti et al., 2004) and the essential oils of citronella grass (*C. nardus*) for *Phomopsis azadirachtae*

(the causative agent of die-back disease of neem) control (Prasad et al., 2010).

The phytochemical effect of each crude extracts on inhibiting the mycelial growth of *Fusarium* sp., have been confirmed by several researches, for examples alkaloids and flavanoids from the leaves of *S. nodiflora* (Bhogaonkar et al., 2010), saponins and tannins from the leaves of *S. bicolor* (Soetan et al., 2006), methoxyflavone from the rhizome of *K. parviflora* (Kummee et al., 2008), alpha-pinene and beta-pinene from the leaves of *E. odoratum* (Owalabi et al., 2010), carnosic acid and rosmarinic acid from the leaves of *R. officinalis* (Frankel et al., 1996) and curcumin from the rhizome of *C. xanthorrhiza* (Husein et al., 2009).

This study indicated that the twenty-four crude extracts can be used for *Fusarium* sp. control and two plant crude extracts can be used for dirty panicle control. The crude extracts of *S. aromaticum* and *O. vulgare* showed 100% inhibition on mecelial growth of *Fusarium* sp. at all concentrations.

Conclusion

All the used twenty-four crude extracts showed significant antifungal activity against *Fusarium* sp. The result showed that the *S. aromaticum* and *O. vulgare* crude extracts showed 100% inhibition on mycelial growth at all

concentrations, whereas, the *S. nodiflora* and *S. bicolor* crude extracts at 10,000 ppm gave the lowest inhibition of 42 and 32%, respectively.

Conflict of Interests

The author has not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of terpene from leaves of *Croton macrostachyus* (Bissana)

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***Croton macrostachyus*, one of the *Croton* species found in Ethiopia, is used traditionally for treatment of a number of health ailments including diabetes, malaria, stomachache, ascariasis, abdominal pain, gonorrhoea, wounds, ringworm infestation and hemorrhoids. In the present study, phytochemical screening of ethanol extract from leaves of *C. macrostachyus* revealed the presence of terpenes, flavonoids, alkaloids and saponins. Silica gel column chromatographic separation of the ethanol extract afforded a pentacyclic triterpenoid reported for the first time from the genus. The structure of the compound was determined using combination of spectroscopic techniques via UV-Vis, IR, ¹H NMR, ¹³C NMR and DEPT-135.**

Key words: *Croton macrostachyus*, pentacyclic triterpenoids, anti-malaria, spectroscopic techniques, phytochemical screening.

INTRODUCTION

Medicinal plants have been used to treat various health ailments for a long period of time in different countries. Natural products have been playing dominant role in drug discovery efforts for treatment of human and livestock diseases (Newman and Cragg, 2012). With the upsurge in the use of plants medicines, a thorough scientific investigation of these plants is imperative based on the need to validate their folklore use. *Croton* species are among the most common traditional medicinal plants used in Africa, Asia, and South America for treatment of

diabetes (Moshi et al., 2000), digestive problems (Yirga et al., 2011), malaria (Mohammed et al., 2007; Alshawsh et al., 2009; Mesfin et al., 2009), insomnia and head-ache (Bum et al., 2012), hemorrhoids and ulcers (Antonio, 2007). The genus has been reported to have a number of biological activities for instance anti-hypertensive, anti-inflammatory, antimalarial and anti-viral (Mbiantcha et al., 2013; Habtamu et al., 2012; Prozesky et al., 2001). Triterpenoids, either pentacyclic or steroidal, volatile oils containing mono and sesquiterpenoids, shikimate-derived

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compounds and phenolic compounds are among secondary metabolites reported from the genus *Croton*. Reports on the pharmacological activity of *mactostachyus* species suggested that the genus is a potential source of enormous bioactive compounds (Antonio, 2007). In Ethiopia, people use the stem and roots of the plant for diarrhea treatment and decoction of the leaf to treat malaria (Giday et al., 2007). Recent studies by Sendeku et al. (2015) and Taye et al. (2011) indicated that the methanol and ethanol extracts from leaves of *C. macrostachyus* showed antibacterial activity while the aqueous extracts were comparatively ineffective. Following the potential of the plant in traditional medicine and its efficient pharmacological activity, further analysis is required to determine the possible bioactive components.

In the ongoing study to analyze the chemical constituents of medicinal plants found in Ethiopian flora, we hereby report a comprehensive phytochemical screening, isolation and a complete characterization of one triterpenoid from the leaves of *C. macrostachyus*.

MATERIALS AND METHODS

Instruments

Nuclear Magnetic Resonance (NMR) analysis was recorded on a Bruker Avance 300 MHz spectrometer with deuterated CDCl_3 using tetramethylsilane (TMS) as internal standard. Structural elucidations were done on the basis of NMR spectra both 1D (^1H NMR, ^{13}C NMR and DEPT-135) and IR. All NMR data were obtained in CDCl_3 . Chemical shifts are reported in parts per million (ppm, δ). Spectral splitting patterns are designated as s: singlet, d: doublet, t: triplet, q: quartet, m: complex multiplet (chemically non-equivalent H's), br s: broad signal. ^{13}C NMR spectra were proton decoupled and recorded on a 100 MHz Bruker spectrometer using TMS as the internal standard. Column chromatography (CC) was performed over silica gel (Merck, 230-400 mesh). Analytical thin layer chromatography (TLC) was carried out using pre-coated 0.2 mm silica gel 60 F254 on aluminum foil and compounds on TLC were detected under UV lamp at 254 and 365 nm. UV-Vis spectrum was recorded on UNICAM UV-300 double beam spectrophotometer in the range of 200 to 1000 cm^{-1} using CHCl_3 as internal standard. Infrared (IR) spectrum was recorded on Perkins-Elmer Bx Infrared spectrometer using KBr disc in the range 4000 to 400 cm^{-1} . Melting point was recorded by Mettler Toledo apparatus, Type FP62, and it was uncorrected. All reagents were obtained from Sigma Aldrich and used as received unless noted otherwise.

Preliminary phytochemical screening test was done based on standard procedures according to Sofowora (1982), Trease and Evans (1989) and Harborne (1973).

Plant material

The leaves of *C. macrostachyus* (Bissana in Amharic, local language) were collected from Dawuro area, in Southern Nation and Nationalities of People Region (SNNPR), located at a distance of 512 km from the capital city, Addis Ababa, Ethiopia in July 2009. The plant material was identified by Dr. Ensermu Kelbessa, Department of Biology, Faculty of Natural Sciences, Addis Ababa University (AAU) and a voucher specimen (CM-001) was deposited

at National Herbarium of Ethiopia, Addis Ababa University. The leaves of *C. macrostachyus* samples were collected and spread to enhance drying at room temperature and then grounded into fine powder using mortar and kept in a transparent polyethylene bags until extraction.

Preparation of crude ethanol extract

250 g powdered leaf samples of *C. macrostachyus* was soaked with petroleum ether for 3 days with occasional shaking and filtered to remove the fat contents. After filtration, the air dried marc was further soaked in 650 ml of ethanol for three days at room temperature. The ethanol extract was then filtered and concentrated using Rotary Evaporator at 40°C, air dried and weighted to yield 36.6 g (15.3%) dark green crude extract. The ethanol extract was suspended in water and partitioned with diethylether and ethyl acetate successively and combined to give a total yield of 4.7 g dark green solid. TLC analysis of the organic layer and aqueous extract showed six and eight spots, respectively, using chloroform/ethyl acetate (9:1) and *n*-hexane: chloroform (4:1) as eluent.

Isolation and purification of compound 1

3.5 g Crude organic layer part (3.5 g) was subjected to silica gel column chromatography (silica gel, 70 g) with increasing gradient of ethyl acetate in chloroform followed by methanol in chloroform as an eluent. A total of 31 fractions, each 50 mL, were collected and analyzed by TLC. Fractions from Fr 12 to 18 (5 to 20% ethyl acetate in chloroform) showed two spots on TLC. They were combined and further purified using silica gel column chromatography to give a single clear spot (compound 1) using chloroform/ethyl acetate (1:1) as eluent.

Isolation and purification of compound 2

Greenish ethanol extract (4.7 g) was dissolved in 15 ml chloroform, subjected to silica gel column chromatographic separation (80 g silica gel) and eluted with increasing gradient of chloroform in *n*-hexane and then ethyl acetate in chloroform successively. A total of 17 fractions (each 50 ml) were collected. The constituent profile of each fraction was monitored by TLC (40% ethyl acetate in chloroform) and visualized under UV-Vis light (λ_{max} 254 and 366 nm). Based on their TLC profile, fractions 4 to 8 were combined and purified to give a single spot on TLC analysis (chloroform: ethyl acetate (9:1)). Fraction 9 to 14 were also combined, purified and taken to spectroscopic analysis due to their amount and level of purity. The last two fractions were dark green in color and show cloudy red spots on fluorescent UV-Vis light. Fractionation stopped after observing the dark green part at bottom. The fraction then labeled compound 2 was obtained as a pale yellow precipitate. (M.pt: 244.1°C \pm 2, 44.8 mg).

Preliminary phytochemical screening

Detections of common secondary metabolites were performed for ethanol extract of leaves of *C. macrostachyus* using the preceding analytical procedures (Kebede et al., 2015).

Test for terpenes

250 mg of ethanol extract was mixed with 2 ml of CHCl_3 and 30 ml of concentrated H_2SO_4 was added carefully to form a layer

Table 1. Phytochemical screening tests of the ethanol extract.

Plant constituent	Observation
Alkaloids	+
Flavonoids	+
Terpenes	+
Tannins	-
Saponins	+
Anthraquinones	-

(-) Not Detected, (+) Detected

(Debjyoti, 1995). Reddish-brown coloration of the interface was inspected.

Test for flavonoids

250 mg of ethanol extract was dissolved in small amount of dilute NaOH and concentrated HCl (3 ml) was added (Farnsworth, 1996). A yellow solution that turns to colorless was inspected.

Test for tannins

Small quantity of the ethanol extract was mixed with water and heated on water bath. The mixture was filtered and small amount of solid FeCl₃ was added to the filtrate (Sofowora, 1982). Dark-green solution was inspected.

Test for alkaloids

250 mg of the crude extract was mixed with 2 ml of concentrated hydrochloric acid. The mixture was then filtered and mixed with small amount of amyl alcohol at room temperature (Ganjewala et al., 2009). The mixture was kept for observation of the color resulted from the alcoholic layer.

Test for saponins

250 mg of the ethanol extract was shaken with 5 ml of distilled water for 30 min and then heated to boil. Appearance of creaming mix of small bubbles (frothing) was inspected (Farnsworth, 1996).

Test for anthraquinones

500 mg of the ethanol extract was boiled with concentrated hydrochloric acid for few minutes in water bath and filtered. The filtrate was allowed to cool and equal volume of CHCl₃ was added to it. Few drops of ammonia were added to the mixture and heated in water bath. Formation of rose-pink color was inspected (Sofowora, 1982).

RESULTS AND DISCUSSION

Preliminary phytochemical screening of the crude ethanol extract of the leaf revealed the presence of various

metabolites such as terpenoids, flavonoids, saponins and alkaloids whereas tannins and anthraquinones were not detected (Table 1).

Silica gel column chromatographic separation of the crude ethanol extract afforded one pentacyclic triterpenoid and complete characterization of the compound is presented subsequently. All the NMR chemical shift values, δ , are presented in ppm. The IR spectrum of compound 1 indicated absorption peaks at 2926.56 and 2856.24 cm⁻¹ (sp³ C-H methyl stretching and sp² C-H methylene, respectively), 1738.71 cm⁻¹ (carbonyl group of ketone moiety), 1468.91 cm⁻¹ (olefinic carbon), 1377.08 and 1365.51 cm⁻¹ (C-O of ether functionality). The UV-Vis spectrum revealed absorption peak λ_{max} at 380 nm attributed to n- π^* transition.

The ¹H NMR spectrum showed peaks at δ 5.40 (*t*, 2H, H-7) and 5.13 (*s*, 2H, H-3) attributed to olefinic protons and a methine hydrogen next to a ketone and an ether group. An isopropyl group attached to heteroatom oxygen and a methylene attached to carbonyl group were clearly evident at δ 4.64 (*m*, 1H, H-27) and 2.43 (H-9), respectively. Furthermore, methyl groups were observed at δ (ppm) 1.83 (H-16), 1.74 (H-30, 31), 1.32 (H-33, 34) and 1.21 (H-25, 26).

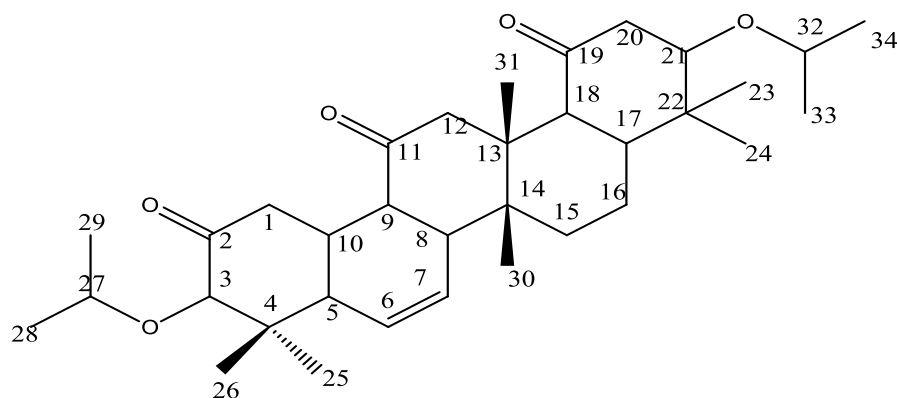
The ¹³C-NMR spectrum showed a total of 34 well-resolved carbon signals attributed to three ketone carbonyls [δ 184.1 (C-19), 179.80 (C-11) and 175.5 (C-2)], four quaternary carbons, twelve methines, five methylenes and ten methyls, also supported by DEPT-135. Of these, four of the methines were observed to be linked to heteroatom, possibly oxygen [δ 82.3 (C-3), 78.3 (C-21), 75.6 (C-32) and 74.5 (C-27)] and two olefinic carbons δ 131.8 (C-6) and 131.6 (C-7)], also confirmed from DEPT-135 spectrum. Seven peaks observed in ¹³C NMR spectrum [δ 184.1 (C-19), 179.8 (C-11), 175.5 (C-2), 65.7 (C-22), 65.5 (C-4), 65.2 (C-14) and 65.1 (C-13)] were not detected in DEPT-135 spectrum suggesting that these carbons belong to quaternary carbons. The down field chemical shift of methyl group (C-31) compared to methyl (C-30) is attributed to the anisotropic effect of the ketone group (C-19), in agreement with the position of the carbonyl group at C-19 (Table 2). Based on the aforementioned spectroscopic data and comparison with literature the compound was found to be a pentacyclic triterpenoid (Figure 1) reported for the first time from the genus.

Compound 2 was found as pale yellow crystalline powder with R_f value of 0.58 (40% ethyl acetate in chloroform as eluent). The ¹H NMR spectrum of the compound showed OH protons aromatic phenyl protons. The ¹³C NMR spectrum of the compound 2 showed a total of around sixty carbon atoms peaks of which one carbonyl carbon multiple aromatic and aliphatic carbons. The idea was supported by the IR spectrum showing broad region for OH with methylene and characteristic aromatic C=C peaks with C=O peaks. However, we are not able to elucidate the structure from

Table 2. Spectral data of Compound 1 ^1H NMR (300 MHz; CDCl_3), ^{13}C NMR (100 MHz; CDCl_3).

Position	^1H NMR (δ ppm)	^{13}C NMR (δ ppm)	DEPT-135
1	2.81 (<i>d</i>)	74.1	$\text{CH}_2\text{-(C=O)}$
2	-	175.5	C=O
3	5.13(<i>s</i>)	82.3	CH-O
4	-	71.9	C
5	2.81 (<i>t</i>)	65.4	CH-(C=C)
6	5.40 (<i>t</i>)	131.6	CH=CH
7	5.40 (<i>t</i>)	131.8	CH=CH
8	2.81 (<i>m</i>)	65.2	CH-(C=C)
9	2.43 (<i>m</i>)	71.6	CH-(C=O)
10	2.28-2.31 (<i>m</i>)	64.9	CH-(CH-(C=O))
11	-	179.8	C=O
12	2.81 (<i>s</i>)	72.5	$\text{CH}_2\text{-(C=O)}$
13	-	65.7	C
14	-	65.1	C
15	2.28-2.31 (<i>m</i>)	65.5	$\text{CH}_2\text{-(C)}$
16	1.83 (<i>q</i>)	65.2	$\text{CH}_2\text{-(CH)}$
17	2.28-2.31 (<i>m</i>)	40.73	CH
18	2.28-2.31(<i>m</i>)	73.4	CH-(C=O)
19	-	184.1	C=O
20	2.81 (<i>d</i>)	74.5	$\text{CH}_2\text{-(C=O)}$
21	5.13 (<i>s</i>)	78.3	CH-O
22	-	72.7	C
23	1.21(<i>s</i>)	30.8	CH_3
24	1.21(<i>s</i>)	30.8	CH_3
25	1.21 (<i>s</i>)	30.8	CH_3
26	1.2(<i>s</i>)	30.8	CH_3
27	4.64 (<i>m</i>)	74.5	CH-O
28	1.32 (<i>m</i>)	23.8	CH_3
29	1.32 (<i>m</i>)	23.8	CH_3
30	1.74 (<i>s</i>)	18.6	CH_3
31	1.74 (<i>s</i>)	45.6	CH_3
32	4.64 (<i>m</i>)	75.6	CH-O
33	1.32 (<i>m</i>)	23.7	CH_3
34	1.32 (<i>m</i>)	23.7	CH_3

Values may be interchangeable within the same compound (CDCl_3 , 100 MHz).

**Figure 1.** Proposed structure of compound 1.

the spectroscopic data and further advanced spectroscopic studies are needed to elucidate the structure of compound 2.

Conclusion

Preliminary phytochemical screening test on the ethanol extract of leaves of *C. macrostachyus* revealed the presence of alkaloids, terpenes, flavonoids and saponins. Anthraquinones and tannins were not detected in the present work. The pentacyclic triterpenoid identified in this study was reported for the first time from *C. macrostachyus*. The traditional use of the plant may be attributed to its high content of polar bioactive constituents. Apart from the phytochemical screening of the ethanol leaf extract, isolation and NMR characterization of one triterpene was successfully achieved. This work will give background record to use *C. macrostachyus* as a potential drug source. Further work on isolation of the detected bioactive molecules in concert with their biological activities including antiviral and antibacterial effects is going on.

Conflict of Interests

The authors have not declared any conflict of interest.

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