

# ANTIMICROBIAL ACTIVITY OF GYNOSTEMMA PENTAPHYLLUM EXTRACTS AGAINST FUNGI PRODUCING AFLATOXIN AND FUMONISIN AND BACTERIA CAUSING DIARRHEAL DISEASE

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**Abstract.** *Gynostemma pentaphyllum* was investigated to determine its antimicrobial activities against human and animal pathogens that produce aflatoxin, fumonisin, and diarrheal disease. The fungi were *Aspergillus flavus*, *Aspergillus parasiticus* and *Fusarium verticillioides*. The bacteria were *Vibrio*, *Salmonella*, *Shigella*, *Escherichia coli* and *Staphylococcus aureus*. *G. pentaphyllum* was extracted by five different methods. The obtained extracts were designated Extracts A, B, C, D and E. The results of the antifungal assay against *A. flavus* and *A. parasiticus* showed Extracts A and B at 10,000 ppm inhibited growth at 8-28%. Extracts A and B at 10,000 ppm also showed activity against *F. verticillioides* at 41-43%. Extract A, B and C were able to inhibit the tested strains better than the Extracts D and E. The MIC values of the extracts against gram-negative bacteria ranged from  $\leq 0.98$  to 31.25 mg/ml and MIC values against *S. aureus*, a gram-positive bacteria, was 3.9-15.62 mg/ml. *G. pentaphyllum* extracts had activity against bacterial and fungal infections and could be used to control these organisms.

**Keywords:** *Gynostemma pentaphyllum*, antimicrobial activity, aflatoxin, fumonisin, diarrheal disease

## INTRODUCTION

*Gynostemma pentaphyllum* belongs to the family Cucurbitaceae. It is an edible plant used as a medicine in oriental countries. Recently, saponins isolated from *G. pentaphyllum* have attracted attention due to their purported health benefits in preventing cardiovascular disease (Kawpinit, 1993; Li *et al*, 1993; Tan *et al*, 1993;

Tanner *et al*, 1999), cancer (Han *et al*, 1995; Zhou *et al*, 2000, 2004) and gastric ulcers (Rujjanawate *et al*, 2004) and for their lipid lowering properties (La Cour *et al*, 1995), inhibition of inflammation (Lin *et al*, 1993) and antidiabetic properties (Poomecome, 1999). No reports regarding the effect of *G. pentaphyllum* extracts against fungi producing aflatoxin and fumonisin and bacteria that caused diarrheal disease have been made.

*Aspergillus flavus* and *Aspergillus parasiticus* can be pathogenic to plants and animals, including humans and domestic animals. The fungi grow on a broad variety

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of foods and produce aflatoxin, a toxic and carcinogenic compound (CAST, 2003). Exposure of livestock to aflatoxin has been found to reduce food intake, lower daily weight gain and reduce feeding efficiency, which leads to economic loss for the producer (Pier, 1992). Residues of aflatoxin in animal products also pose a threat to human health (Orriss, 1997). *Fusarium verticillioides* (formerly *F. moniliforme*) is often found in corn and produces a toxic substance, fumonisin. Fumonisin toxicosis causes equine leukoencephalomalacia in horses, pulmonary edema in swine and esophageal cancer in humans (Voss *et al*, 2007).

*Vibrio*, *Salmonella*, *Shigella*, *Escherichia coli*, including enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and *Staphylococcus aureus* are etiologic agents for diarrheal disease. Dramatic increases in multiresistant strains in many countries have been observed (Threlfall *et al*, 2000; Kondo *et al*, 2001; Nogrady *et al*, 2007). The emergence of resistant isolates leads to severe problems in treatment. Surveillance and control is necessary to prevent spread.

This study aimed to examine the antimicrobial properties of *G. pentaphyllum* against fungi and bacteria in order to develop this plant as an alternative use for the treatment of some bacterial and fungal infections.

## MATERIALS AND METHODS

### Plant extraction

The dried plant of *G. pentaphyllum* was purchased from Phuchiangta Community Enterprise, Chaiyaphum Province, Thailand. The plant was extracted as described below.

**Ethanolic extraction.** Dried leaves and stems of *G. pentaphyllum* were macerated with 95% ethanol (1:5 w/v) for 72 hours at room temperature. The procedure of maceration was repeated three times. The obtained mixture was filtered and evaporated at 55°C. The extract was subsequently freeze dried. Extract A was designated for the obtained extract.

**Butanolic extraction with prior wash by hexane and chloroform.** Extract A was dissolved in water and washed with hexane and chloroform. The aqueous fraction was partitioned using n-butanol. The butanol phase was evaporated then freeze dried. Extract B was designated for the obtained extract.

**Butanolic extraction.** Extract A was dissolved in water. The butanol phase and water phase were fractionated and subsequently evaporated and freeze dried. The obtained extracts were designated Extracts C and D, respectively.

**Crude gypenoside extraction** (Techadumrongsin *et al*, 2005). Extract B was dissolved in water and applied to a Sep-Pak C-18 cartridge. The column was washed with 50% methanol and then 60% methanol, respectively. The crude gypenoside was next eluted with 100% methanol and dried in a rotary evaporator, then freeze dried. The obtained extract designated Extract E.

### Sources of fungi and bacteria

**Fungal strains.** *A. flavus* TISTR 3366 and *E. verticillioides* TISTR 3175 were obtained from the Thailand Institute of Scientific and Technological Research. *A. parasiticus* BCC 5773 was obtained from the National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand).

**Bacterial strains.** The clinical isolates were collected from Songklanakarind Hospital, Thammasat Hospital and the Enteric

Diseases Department, USAMC-AFRIMS, Thailand. The bacterial strains were *V. cholerae*, *V. vulnificus* and *E. coli*, including enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC); *S. Typhi*, *S. Typhimurium*; *S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei* and *Staphylococcus aureus*.

#### Antifungal assay and statistic analysis

Extracts A and B were diluted with 1% DMSO. The extract solution (1 ml) was incorporated into 20 ml of PDA medium (approximately 45°C) to obtain concentrations of 100, 1,000, 5,000 and 10,000 ppm in the medium. 1% DMSO (1 ml) was also added to the medium as a negative control. Circular blocks of mycelia from stock culture of *F. verticillioides*, *A. flavus* and *A. parasiticus* (5 mm in diameter) were punched using sterile cork-borers and centrally placed on the medium with the concentrations of extract on the plate. The tests were performed in triplicate. The growth diameter (in mm) of each fungus was measured on Day 7. The data were subjected to analysis of variance and least square means were analyzed using SAS version 9.1 (SAS, 2004). Using the mean values, the percentage of inhibition was calculated using the formula described by Srichana *et al* (2009a). Extracts C, D and E were not tested for antifungal efficacy due to inadequate extraction.

#### Antibacterial assay

The disc diffusion method (NCCLS, 2004) and modified microtiter plate-based antibacterial assay (Sarker *et al*, 2007) were performed. The assay was modified by adding resazurin after incubating at 35-37°C for 16-18 hours, then further incubated for 2 hours. The inoculum prepared was equivalent to 0.5 McFarland standard.

Ampicillin and 1% DMSO were used as positive and negative controls, respectively. Viability of a bacterial control was also included. The tests were performed in triplicate.

## RESULTS

#### Antifungal assay

Antifungal growth of Extracts A and B were quantitatively assessed by percentage of inhibition as shown in Tables 1 and 2.

#### Growth inhibition of *A. flavus*

The inhibitory effects of Extract A at 100, 1,000, 5,000 and 10,000 ppm on the growth of *A. flavus* were 0.01, 15.5, 18.4 and 20.0%, respectively. The inhibitory effects at 5,000 and 10,000 ppm were not significantly different ( $p>0.05$ ; Table 1). Extract B at 100, 1,000, 5,000 and 10,000 ppm inhibited growth of *A. flavus* at 1.3, 2.5, 26.3 and 28.3%, respectively. The inhibitory effects of 100 and 1,000 ppm were not significantly different ( $p>0.05$ ) as shown in Table 2.

#### Growth inhibition of *A. parasiticus*

*A. parasiticus* was inhibited at 0, 2.2, 7.4 and 7.8% by Extract A at 100, 1,000, 5,000 and 10,000 ppm, respectively. The inhibitory effects at 5,000 and 10,000 ppm were not significantly different ( $p>0.05$ ; Table 1). Extract B at 100, 1,000, 5,000 and 10,000 ppm inhibited growth of *A. parasiticus* at 0, 0, 4.1 and 7.7%, respectively, as shown in Table 2.

#### Growth inhibition of *F. verticillioides*

*F. verticillioides* growth was significantly inhibited by Extract A at 6.4, 31.7, 37.8 and 43.4% at 100, 1,000, 5,000 and 10,000 ppm, respectively, ( $p<0.05$ ) as shown in Table 1. Extract B at 100, 1,000, 5,000 and 10,000 ppm inhibited the growth

Table 1  
Growth inhibition of *A. flavus*, *A. parasiticus* and *F. verticillioides* on PDA incorporated with ethanolic extract of *Gynostemma pentaphyllum* (Extract A) (%).

| Extract A (ppm) | <i>A. flavus</i>  | <i>A. parasiticus</i> | <i>F. verticillioides</i> |
|-----------------|-------------------|-----------------------|---------------------------|
| 100             | 0.01 <sup>a</sup> | 0 <sup>a</sup>        | 6.4 <sup>a</sup>          |
| 1,000           | 15.5 <sup>b</sup> | 2.2 <sup>b</sup>      | 31.7 <sup>b</sup>         |
| 5,000           | 18.4 <sup>c</sup> | 7.4 <sup>c</sup>      | 37.8 <sup>c</sup>         |
| 10,000          | 20.0 <sup>c</sup> | 7.8 <sup>c</sup>      | 43.4 <sup>d</sup>         |
| SE              | 0.55              | 0.27                  | 1.06                      |

<sup>abcd</sup>Columns that do not have a common superscript are significantly different ( $p < 0.05$ ); SE, standard error

Table 2  
Growth inhibition of *A. flavus*, *A. parasiticus* and *F. verticillioides* on PDA incorporated with butanolic extract of *Gynostemma pentaphyllum* (Extract B) (%).

| Extract A (ppm) | <i>A. flavus</i>  | <i>A. parasiticus</i> | <i>F. verticillioides</i> |
|-----------------|-------------------|-----------------------|---------------------------|
| 100             | 1.3 <sup>a</sup>  | 0 <sup>a</sup>        | 25.2 <sup>a</sup>         |
| 1,000           | 2.5 <sup>a</sup>  | 0 <sup>a</sup>        | 30.5 <sup>b</sup>         |
| 5,000           | 26.3 <sup>b</sup> | 4.1 <sup>b</sup>      | 39.5 <sup>c</sup>         |
| 10,000          | 28.3 <sup>c</sup> | 7.7 <sup>c</sup>      | 41.2 <sup>c</sup>         |
| SE              | 0.39              | 0.11                  | 1.27                      |

<sup>abcd</sup>Columns that do not have a common superscript are significantly different ( $p < 0.05$ ); SE, standard error

of *F. verticillioides* at 25.2, 30.5, 39.5 and 41.2%, respectively (Table 2).

#### Antibacterial assay

Extracts A, B and C were able to inhibit the test strains better than Extracts D and E. The MIC values for extracts against gram-negative bacteria ranged from  $\leq 0.98$  to 31.25 mg/ml, while the range of MIC values against *S. aureus*, a gram-positive bacteria, was 3.9 - 15.62 mg/ml. *V. cholerae* had a MIC in a range of 1.95-31.25 mg/ml while *V. vulnificus* had MIC values of extracts from  $\leq 0.98$  to 7.812 mg/ml. All bacterial strains of *Salmonella* and *E. coli* and some strains of *Shigella*, including *S. flexneri* and *S. sonnei* and *V. cholerae* O1

Ogawa had no differences in MIC values (15.62 mg/ml) (Table 3).

#### DISCUSSION

Growth inhibition of Extracts A and B at 10,000 ppm on the growth of *A. flavus* ranged from 20 to 28% indicating a low inhibitory effect compared to a study by Soliman and Badeaa (2002). They found 100% inhibition of growth of *A. flavus* by the essential oils, caraway, fennel, anis, thyme, spearmint and basil at 2,000, 3,000, 500, 250, 3,000 and 3,000 ppm, respectively. Srichana *et al* (2009a, b) found the ethanolic extracts of mangosteen fruit hull and betel leaf at 10,000 ppm inhibited growth

Table 3  
Antibacterial activities of extracts of *Gynostemma pentaphyllum* against bacteria that cause diarrheal disease.

| Strains                                    | MIC (mg/ml) |           |           |           |           |
|--|-------------|-----------|-----------|-----------|-----------|
|  | Extract A   | Extract B | Extract C | Extract D | Extract E |
| <i>Salmonella</i>                          |             |           |           |           |           |
| H67 ( <i>Salmonella</i> gr B)              | 31.25       | 31.25     | 31.25     | 31.25     | 15.62     |
| H464 ( <i>Salmonella</i> gr B)             | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| CG02-3005 ( <i>S. Typhimurium</i> )        | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| NPHL-385/059 ( <i>S. Typhi</i> )           | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| <i>E. coli</i>                             |             |           |           |           |           |
| CG98-K-71-1 (ETEC)                         | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| EDL 933 (EHEC)                             | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| NP-04-065-1 (EAEC)                         | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| C04-201-1 (EPEC)                           | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| VN 1306-4 (EIEC)                           | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| <i>S. aureus</i>                           |             |           |           |           |           |
| 070/07                                     | 7.81        | 7.81      | 3.9       | 15.62     | 15.62     |
| <i>Shigella</i>                            |             |           |           |           |           |
| 68-03/sh ( <i>S. flexneri 2a</i> )         | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| 17-04/sh ( <i>S. flexneri 6</i> )          | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| Kenya-64 ( <i>S. dysenteriae</i> )         | 7.812       | 7.812     | 7.812     | 15.62     | 15.62     |
| 4354 ( <i>S. boydii</i> )                  | 7.812       | 7.812     | 7.812     | 15.62     | 15.62     |
| VN-538 ( <i>S. sonnei</i> )                | 15.62       | 15.62     | 15.62     | 15.62     | 15.62     |
| <i>Vibrio</i>                              |             |           |           |           |           |
| N18 ( <i>V. cholerae</i> O1 Inaba)         | 7.812       | 7.812     | 1.95      | 15.62     | 7.812     |
| N28 ( <i>V. cholerae</i> O1 Inaba)         | 3.9         | 3.9       | 3.9       | 15.62     | 7.812     |
| N6 ( <i>V. cholerae</i> O1 Inaba)          | 1.95        | 3.9       | 3.9       | 15.62     | 7.812     |
| VC 203 ( <i>V. cholerae</i> O1 Ogawa)      | 15.62       | 15.62     | 15.62     | 15.62     | 31.25     |
| VN1 ( <i>V. cholerae</i> non- O1/non-O139) | 3.9         | 3.9       | 3.9       | 7.812     | 7.812     |
| V5 ( <i>V. cholerae</i> non- O1/non-O139)  | 7.812       | 7.812     | 3.9       | 15.62     | 15.62     |
| N49 ( <i>V. cholerae</i> O139)             | 7.81        | 15.62     | 7.81      | 31.25     | 15.62     |
| WSF-112 ( <i>V. vulnificus</i> )           | <0.976      | <0.976    | 1.95      | 3.9       | 7.812     |

of the fungus at 58 and 100%, respectively. The inhibitory effect of Extracts A and B at 10,000 ppm on *A. parasiticus* growth (8%) was low compared to essential oils which generated total inhibition (100%) (Soliman and Badeaa, 2002).

The present study showed the inhibitory effects of Extracts A and B at 10,000 ppm against *F. verticillioides*, similar to a

previous report showing 43% inhibition by the ethanolic extract of mangosteen fruit hull (Srichana *et al*, 2009b). However, the potential inhibitory effect of both extracts at 10,000 ppm on growth of this fungus was lower than that of betel leaf extract (Srichana *et al*, 2009a) and essential oils (Soliman and Badeaa, 2002) at the same concentration.



The results obtained in this present study suggest Extracts A and B have a good inhibitory effect against only *F. verticillioides* while the inhibition effect against *A. flavus* and *A. parasiticus* was low. These results reveal Extracts A and B could be useful as an antifungal medicinal plant.

The MIC of *Gynostemma pentaphyllum* extracts against gram-negative bacteria tested in this study gave MIC values from  $\leq 0.98$  to 31.25 mg/ml while the MIC values for *S. aureus* were between 3.9-15.62 mg/ml. Interestingly, the water-insoluble extracts designated Extracts A, B, C and E showed no significant activity compared to the water-soluble Extract D. These results suggest the active compounds in all extracts were similar.

Tzung-Hsun *et al* (2008) reported the antibacterial activity of twelve extracts of tea against *Streptococcus sanguinis* and *S. mutans*. MIC values of 1 mg/ml and 4 mg/ml were detected in eight different tea extracts. These results indicate extracts of *Gynostemma pentaphyllum* may be effective for inhibiting gram-positive bacteria at high concentrations. It is necessary to adjust the concentration to be the most effective in treating infections.

MIC values of all extracts against *E. coli* were higher than MIC values of Extracts A, B and C against *S. aureus* and some strains of *Shigella* and *Vibrio*. These results correspond to a previous report indicating *Gynostemma pentaphyllum* extract is not able to inhibit growth of *E. coli* (Tzung-Hsun *et al*, 2008).

The results of antimicrobial activity of *Gynostemma pentaphyllum* extracts compared with a previous report of Pitkutbenjakul extracts against the same set of bacteria causing diarrhea showed no differences (Kondo *et al*, 2010). The strains tested in this study were resistant to many

antibiotics (data not shown). Effective medicinal plant extracts could be useful as alternative drugs. Prophylactic use of antimicrobials in food animals to prevent emergence of drug resistant strains in the food chain was suggested in a previous report (Threlfal *et al*, 2000). Medicinal plants could be used similarly. Extraction of the active ingredients of *Gynostemma pentaphyllum* should be studied further to develop effective antimicrobial agents.

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