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Full Length Research Paper

Comparison between *in vitro* and *in vivo* antibacterial activity of *Curcuma zedoaria* from Malaysia

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The antimicrobial activity of the extracts of *Curcuma zedoaria* from Malaysia was compared using *in vitro* and *in vivo* systems. The comparison was performed against four bacterial strains including two gram negative strains (*Escherichia coli* and *Pseudomonas aeruginosa*) and two gram positive strains (*Bacillus cereus* and *Staphylococcus aureus*) using the agar well diffusion method. Petroleum ether, chloroform and methanol were used as solvents for preparing the plant extracts. Growth hormones namely indole-3-butyric acid (IBA) and 6-Benzylaminopurine (BAP) were used in the *in vitro* system separately between the range of 0.25 and 3.5 mg/l for IBA and 0.5 and 4 mg/l for BAP and a mix formula containing both hormones using the best ranges after rapid tests. The results showed that the *in vitro* system was more capable of inhibiting *S. aureus* growth compared with *in vivo* system on the agar plate. *P. aeruginosa* and *E. coli* growth were only inhibited by the *in vitro* system on the agar plate, while *B. cereus* was the only strain in the *in vivo* system which produced higher inhibition zone on the agar plate against its growth compared with the *in vitro* system.

Key words: *Curcuma zedoaria,* antibacterial activity.

INTRODUCTION

Curcuma zedoaria is a rhizomatous species from the Zingiberaceae family commonly known as ginger family. “Ginger” is a general term for members or species of the ginger families. “Curcuma” is the generic name for the rhizome herb of Zingiberaceae. *C. zedoaria* is locally known as “kunyit putih” or “temu putih”. *C. zedoaria* grows mainly in East-Asian countries including China, Vietnam, India, Bangladesh, Indonesia, Malaysia (can be found at Kuala Selangor, Teluk Intan; Perak, Labis; Johor, and Pahang) and Japan (Islam et al., 2005; Tipthara et al., 2007). Curcuma spp. such as *C. zedoaria* (Aggarwal et al., 2007) contains curcuminoids including Curcumin with the formula (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-Dione) which are phenolic compounds derived from the roots of Curcuma spp. (Sharma et al., 2005). Curcumin is a low molecular weight polyphenol (Aggarwal et al., 2007) which is generally regarded as the most active compound and comprises 2 to 8% of most extract preparations (Sharma et al., 2005).

C. zedoaria has been traditionally used in many countries especially in South-East Asia as a valuable medicinal plant for many centuries (Wilson et al, 2005 ;Maua et al. 2003) to treat stomach diseases, blood stagnation, diarrhea, coryza, skin disorders, rheumatism, and also is used as a hepato protection and for promoting menstruation (Chen et al., 2008). Antimicrobial activity (Loc et al., 2005), anti-inflammatory, antihepatotoxic, neuroprotective activities and cytotoxic effects against human ovarian cancer cells are all regarded as the abilities of Curcumin productions from *C. zedoaria*. Furthermore, natural products from *C. zedoaria* are used as spices, tonics (Islam et al., 2005) and also, in perfumery (Islam et al., 2005).

The propagation of this plant species has been only performed through rhizomes (Loc et al., 2005). Basically, *C. zedoaria* is not improved by breeding since it seldom produces flowers and the seeds do not form. It is propagated in the vegetative way through underground rhizomes at a very slow rate. Thus, tissue culture techniques can play an important role to speed up the studies of this plant and are relevant for industrial commercialization for medicinal and pharmaceutical objectives.

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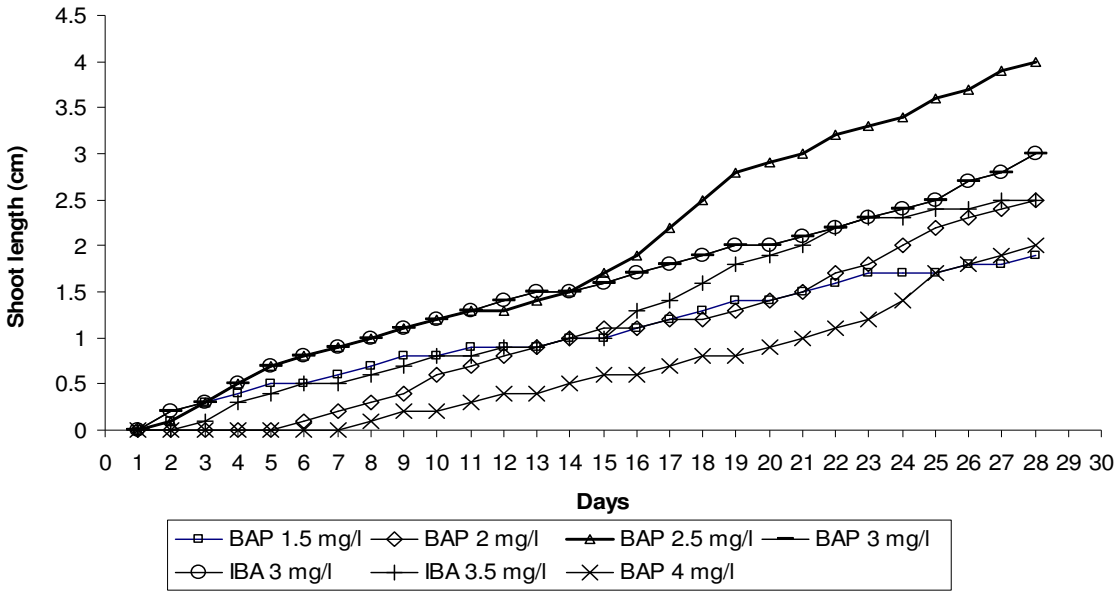

Figure 1. *C. zedoaria* shoot length within 4 weeks after micropropagation with different concentrations of BAP; the figure shows that using 2.5 mg/l of BAP provided a higher shoot length in comparison with other concentrations.

Micropropagation techniques have been used widely in recent years as an effective method for the rapid propagation and production of several high quality and disease free commercially important varieties of plant species (Kapoor et al., 2008). Micropropagation (plant tissue culture) techniques allow rapid and uniform planting material in relatively short periods of time which is not possible with the conventional propagation techniques. Plant tissue culture relies on growing plants based on rich nutrient growth substrates devoid of microbes (Kapoor et al., 2008).

Zingiberaceae family has been reported for their antimicrobial potentials (Aggarwal et al., 2007; Niamsa and Sittiwet, 2009; Wilson et al., 2005; Chen et al., 2008). There are many biological assay techniques that have been developed (Toit and Rautenbach, 2000; Smith et al., 2008) and refined (Smith et al., 2008) to monitor and measure the antimicrobial activity of natural compounds (José et al., *2006*). Agar diffusion tests were used to determine the activity of the antimicrobial agents used in this study. Agar diffusion tests are simple and cost-saving (Volk, 2008). The use of tissue culture in the *in vitro* system (micropropagation) can increase the amount of essential compounds that have antibacterial characteristics in a shorter time compared with the *in vivo* system and consequently, save time and are economical for industrial applications.

MATERIALS AND METHODS

This study is comprised of two major parts: (1) micropropagation and (2) antibacterial activity test. In the first part of the project, a tissue culture test (micropropagation) was performed in order to produce several plants. This was followed by a comparison of the antibacterial activity of *C. zedoaria* extracts between *in vitro* and *in vivo* systems to determine if there was any difference between the two.

Basically, *in vitro* system needs micropropagation techniques in advance before carrying out the comparison. *In vitro* system was followed by establishment of aseptic explants, making the appropriate media culture, making the stock solutions for hormones and establishing the best concentration for the growth of the explants in the *in vitro* system and finally, antibacterial activity test. *In vivo* system included extract preparations for antibacterial activity test until the comparison test was carried out.

Mature rhizome buds of *C. zedoaria* were cut from healthy, disease-free plants collected from the Pahang State, Malaysia. The rhizomes were washed thoroughly under running tap water for one hour and then, was treated with sodium hypochlorite NaOCl (20% solution) for 5 min. Subsequently, the explants were washed three times with distilled water and again treated with sodium hypochlorite NaOCl (20% solution) along with a few drops of Tween 20 for 15 min. The explants were then, rinsed three times with sterilized distilled water again. Further surface sterilization was made with 70% (v/v) ethanol. Finally, the explants were washed and rinsed three times with sterilized distilled water. Sterilized explants were used for both *in vitro* and *in vivo* test.

In order to produce *in vitro* plants, small segments of innermost tissue (1.0 cm) were excised with a sterilized scalpel. Three different concentrations based on previous reports (Miachir et al., 2004; Loc et al., 2005; Keng and Stanley, 2007) were tested for IBA at the rate of 0.5, 1, and 1.5 mg/l combined with 3 different concentrations of the BAP (2.5, 3 and 4 mg/l).

Figures 1 and 2 show the trials to obtain the best concentrations to apply in the main media for the *in vitro* production. The length of the shoots were measured after four weeks of growth and the results showed that 0.5 mg/l of IBA and 2.5 mg/l of BAP provided a higher shoot length in comparison with other concentrations. Finally, the stock solutions for the PGRs were prepared as 3 mg/l BAP, 0.5 mg/l IBA + 3 mg/l BAP and 1.5 mg/l IBA; the test was

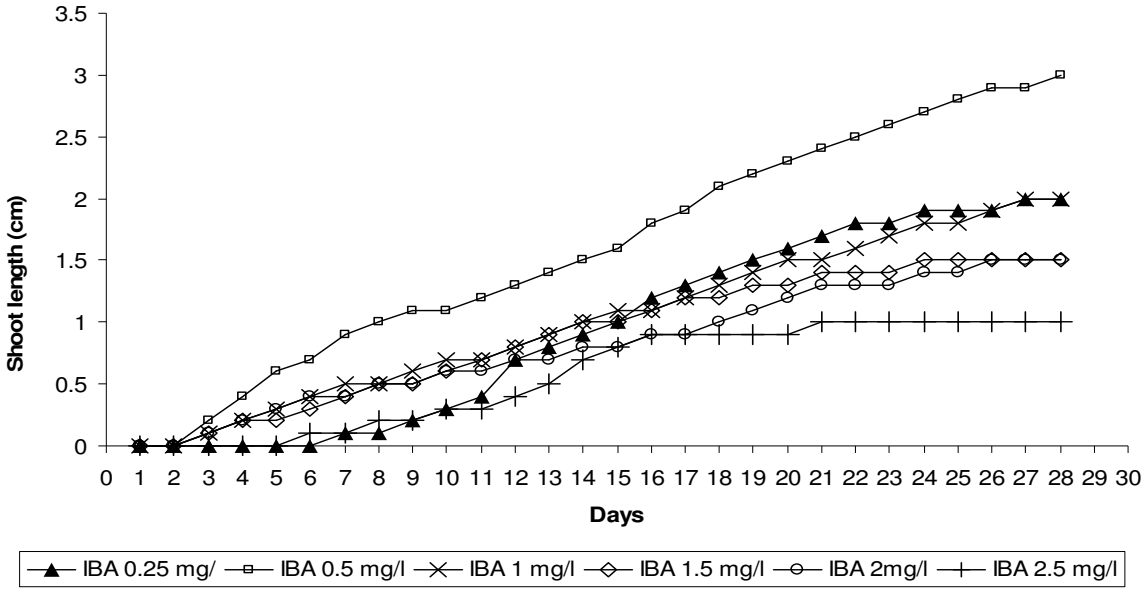

Figure 2. Shoot length within four weeks after micropropagation with different concentrations of IBA; the figure shows that using 0.5 mg/l of IBA provided a higher shoot length in comparison with other concentrations .

repeated with 1 mg/l activated charcoal (AC). Explants were inoculated in small glass culture jars containing 30 ml sterilized agar gel of MS medium using the said combinations of hormones (Murashige and Skoog, 1962).

The pH was adjusted to 5.7 and 5.8, autoclaved at 121°C and maintained at 22°C in a culture room with continuous lighting provided with cool white fluorescent lamps. Sterilized explants were directly used for antibacterial activity test. For the *in vitro* system, an extra step of micropropagation was performed. Antibacterial activity assay and disk diffusion test were carried out using the same procedure as *in vivo* system.

Preparation of plant extracts

After four months, rhizomes of the fresh *in vitro* plants were collected, sliced into small pieces and dried at a temperature not exceeding 40°C and ground into fine powder using a powdering mill; the powders were soaked in three different solvents (petroleum ether, chloroform and methanol) for three weeks each. Dried powdered samples were filtered and evaporated to dryness under reduced pressure at 40°C in a rotary evaporator (BUCHI, Switzerland) and then weighed to determine the total extractable compounds. Same method was used for the *in vivo* system. The weight of the solid residue was recorded and taken as yield of crude extract for both systems separately following this calculation:

Yield = weight of dried sample (mg)/weight of fresh sample (g) × 100%

The crude extracts were then transferred to vials and kept at -4°C and were freshly dissolved in related solvents just prior to screening for antimicrobial activity.

Antibacterial activity

The microbial strains used for testing antimicrobial activity included the gram positive bacteria *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* RF 122 and gram negative bacteria *Escherichia coli* UTI89 and *Pseudomonas aeruginosa* PA7. The test microorganisms used in this study were obtained from the Microbiology Department, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

Screening for antimicrobial activity

Antimicrobial activity was tested using the agar-well diffusion method (Volk, 2008). The bacteria were cultured on Mueller Hinton agar medium. The concentration of the inoculum suspension was adjusted between 0.08 till 0.1 optical densities at the wavelength of 625 nm. The agar plate was streaked with inoculum suspension using sterile cotton swab over the entire surface 3 times for an even distribution of the inoculum. Sterile 6 mm filter paper disks were used to dip into the fraction and placed onto the agar plate with inoculum. Within 15 min, all the agar plates were sealed with parafilm and incubated at 37°C for 14 h. The positive controls used were tetracycline (30 µg) from Oxoid England. Water was used as the negative control. The plates were checked after 16 h. Zone of inhibition were measured and the results were recorded.

The method applied was based on the "British Society for Antimicrobial Chemotherapy" and "National committee for clinical Laboratory Standards" (NCCLS, 2004). The diameter of the inhibition zones around each of the wells was taken as a measure for the antimicrobial activity. Each experiment was carried out in triplicates and the mean diameter of the inhibition zones was recorded as the final result.

RESULTS

Micropropagation

As described in the materials and method section, the weight of the solid residue was recorded and taken as yield of crude extract for both systems separately. Yield = weight of dried sample (mg)/weight of fresh sample (g) ×

BAP (mg/l)	Shoot length (cm) after 4 weeks	IBA (mg/l)		
		0.5 ↓	1 ↓	1.5 ↓
2.5	→	**4.5***	3.5	3
3	→	3	2.5	2
4	→	N-G	1	N-G

Figure 3. Length of shoots after four weeks of growth after micropropagation with chosen concentration of IBA and BAP. The best results for the IBA were 0.5, 1 and 1.5 mg/l and for the BAP they were 2.5, 3 and 4 mg/l which were applied together. The result showed that using 0.5 mg/l IBA with 2.5 mg/l BAP (after 3 times testing), could be the best combination for the test. * All values in this table represent the mean.

100%; weight of dried sample is the weight of evaporated samples which is left in the machine; yield percentage for chloroform = 0.68% (68 mg/g) *in vivo* and 0.49% (49 mg/g) *in vitro;* Yield percentage for petroleum ether = 0.43% (43 mg/g) *in vivo* and 0.46% (46 mg/g) *in vitro;* Yield percentage for methanol = 0.39% (39 mg/g) 0.39% *in vivo* and 0.44% (44 mg/g) *in vitro.*

Figure 1, shows the shoot lengths within four weeks after micropropagation with different concentrations of BAP. 2.5 mg/l of BAP provided a higher shoot length (4 cm) in comparison with other concentrations of BAP. Figure 2, illustrates the results together when different concentrations (mg/l) of IBA were applied in the media. Figure 2 shows that 0.5 mg/l IBA was the best concentration alone in the media as it has a higher rate of growth (three centimetres) comparing with other concentrations.

The best results were recorded when 0.5, 1 and 1.5 mg/l IBA and 2.5, 3 and 4 mg/l BAP were used. In the next step, length of shoots was measured during the four weeks of growth after micropropagation with the said concentrations of IBA and BAP.

The results showed that using 0.5 mg/l IBA with 2.5 mg/l BAP (after three times testing), could be the best combination for the test since it produced higher length shoots (Figure 3).

Methanolic extracts *in vitro* system showed less inhibitory effect on *B. cereus* growth but more on *S. aureus* growth compared with the *in vivo* system. *P. aeruginosa* growth was not affected by any of the two systems.

Antibacterial assay

The results showed that *C. zedoaria* rhizomes explants which were produced through the *in vitro* system had a similar ability with those which were applied from the *in vivo* system to act as an antibacterial agent. In general, most of the extracts evaluated for antimicrobial activity were active against bacteria strains which were employed for the test. Moreover, the antimicrobial activity of most of the petroleum ether extracts seemed to be stronger than the methanol and chloroform extracts. Moreover, the antimicrobial activity for most of the petroleum ether extracts seemed to be stronger than the methanol and chloroform extracts. Nevertheless, methanolic extracts from the *in vivo* system also showed stronger antimicrobial activity compared to the petroleum ether extracts against *B. cereus* and *S. aureus* in the *in vitro* system. Chloroform extracts from the *in vitro* system also had similar effect to the *in vivo* system on *B. cereus* and *S. aureus* growth.

All the three *in vitro* extracts had a positive effect on *P. aeruginosa* growth compared to the non-effective extracts of the *in vitro* system. *In vivo* and *in vitro* extracts had no effect on *E.coli* growth when chloroform and petroleum ether were used but methanol extracts in the *in vitro* system using 0.5 mg/l IBA showed considerable results (Table 1). Table 1 shows the final results after 72 h of bacteria growth on MH agar with placed disks. Finally, as a conclusion, it can be confirmed that petroleum ether extracts in the *in vitro* system had similar antimicrobial strength with those of *in vivo* system on *B. cereus* and *S. aureus*. Petroleum ether extracts in the *in vitro* system had greater strength to inhibit *P. aeruginosa* growth than those of the *in vivo* system as well. Chloroform extracts had less effect on *B. cereus,* similar effect on *E. coli* and *P. aeruginosa* and stronger effect on *S. aureus* growth inhibition *in vitro* system compared with the *in vivo* system (Table1).

DISCUSSION

The yield percentage for chloroform, methanol and petroleum ether is considered as an almost acceptable yield percentage compared with earlier reports (Wilson et al., 2005; Chen et al., 2008). Figure 1 show a sudden increase in the growth rate after day 14 which is caused by the boost effect of sub-culturing. Figure 2 shows a delay in growth of shoots for about one week before plants begin

Table 1. Antibacterial activity *of Curcuma zedoaria* in the *in vitro* and *in vivo* systems over different extract solvents (petroleum ether / methanol / chloroform) presented as inhibitory zone in millimetre length (2.5 mg/l BAP mixed with 0.5 mg/l IBA).

Time (%)	*In vivo*	IBA	BAP	IBA+BAP	IBA+BAP+AC
Chloroform extracts effect on *S. aureus*					
48	1 ± 0.5[a]	4	1	2 ± 0.5	1 ± 0.5
72	1 ± 0.5	4	1	2 ± 0.5	1 ± 0.5
Chloroform extracts effect on *B. cereus*					
48	2	3	2	1 ± 0.5	1 ± 0.5
72	2	3	2	1 ± 0.5	1 ± 0.5
Methanol extracts effect on *B. cereus*					
48	4	6	4	3	1 ± 0.5
72	4	6	4	3	1 ± 0.5
Methanol extracts effect on *E. coli*					
48	N/A	2	N/A	1 ± 0.5	N/A
72	N/A	2	N/A	1 ± 0.5	N/A
Methanol extracts effect on *S. aureus*					
48	1 ± 0.5	5	1 ± 0.5	2	1 ± 0.5
72	1 ± 0.5	5	1 ± 0.5	2	1
Petroleum ether extracts effect on *P. aeruginosa*					
48	N/A	2	N/A	1 ± 0.5	N/A
72	N/A	2	N/A	1 ± 0.5	N/A
Petroleum ether extracts effect on *B. cereus*					
48	6	9	7	4	1 ± 0.5
72	6	9	7	4	1 ± 0.5
Petroleum ether extract effect on *S. aureus*					
48	3	4	4	5	1 ± 0.5
72	3	4	4	5	1 ± 0.5

[a] All values in this table represent the mean ± SD (n=3).

to grow when using 0.25 mg/l IBA. After the third week, we sub-cultured the shoots and the growth was speeded up but was not higher than 2 cm after 28 days. The result was quite different when 0.5 mg/l IBA was used in the media. Despite only 0.25 mg/l increase in the hormone concentration was applied but the shoots reached 3 cm after 28 days with only two days delay compared with when it was only 0.25 mg/l IBA in the media. After increasing the concentration to 2 mg/l IBA in the media, there was a sudden jump from day 17 to day 22 in the growth rate since the shoots were sub cultured on day 14. Although, we had a high rate of growth from day 12 to 18, but the growth suddenly stopped on day 28 when 2.5 mg/l IBA was used. The lower growth rate may be attributed to a higher rate of IBA application. We could not get any results when higher rates of IBA exceeding 2.5 mg/l were used, since the media became contaminated (Figure 4).

After comparing Figures 1 and 2, we realized that explants with BAP alone had a better and faster growth rather than those with IBA. Explants in the media with 2.5 and 3 mg/l BAP started to grow after only three days and reached more than 0.5 cm, while it took more than a week for explants in the media with 1 mg/l IBA to reach that length. It seemed that shoots had an almost immediate growth after the second day. Table 1 shows the negative effect of adding AC into the media on the activity of most of the tested bacteria strains. Adding 1 mg/l AC to the media with certain amounts of hormones (2.5 mg/l BAP mixed with 0.5 mg/l IBA) produced smaller inhibition zones compared with when same hormones (with same amount) were applied without AC in the media. The

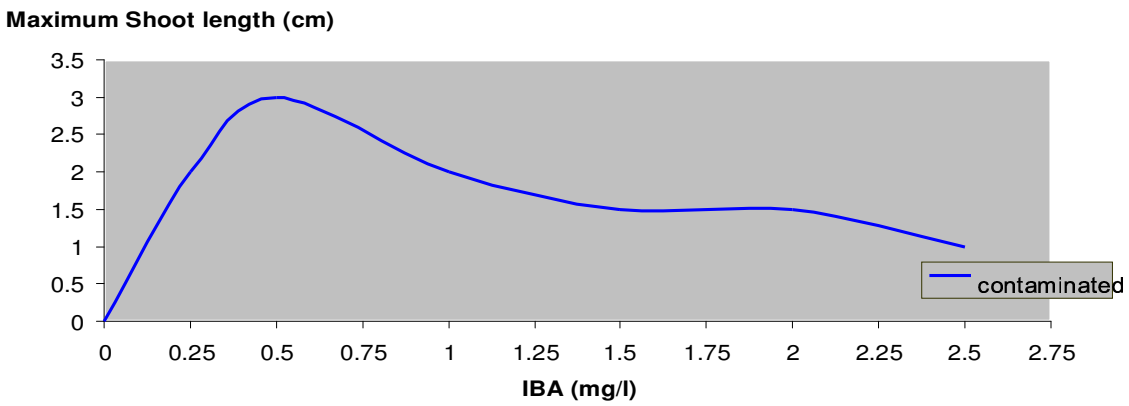

Figure 4. Maximum shoot length with different IBA concentrations; the figure shows that 0.5 mg/l IBA alone in the media had the best result among all the concentrations.

negative effect of AC in micropropagation is possibly due to the adsorption of essential factors required for tissue growth in the plant.

This issue has been reported earlier in some reports too (Tivarekar and Eapen, 2001; Boggetti et al., 1999). One report mentioned that the addition of 0.5 g/l AC to the medium completely inhibited the shoot initiation in some reports (Tivarekar and Eapen, 2001).

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