CHAPTER 4: RESULTS AND DISCUSSION

4.1. Introduction

The antimicrobial activity of three solvent extracts, petroleum ether, chloroform and methanol (chapter 3.1.1, Diagram 3.4) of *Curcuma zedoaria* in the *in vitro* and the *in vivo* systems was determined against bacterial pathogens by the agar disc diffusion assay (Chapter 3.4.2). Results for both *in vitro* and *in vivo* systems were compared by the method described in chapter 3.6.

The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. After that, the diameter of inhibition zone was measured in millimetres. All tests were repeated three times to minimize test error.

4.2. *In vitro* / *in vivo* systems

4.2.1. Preparing plant extracts

The weight of the solid residue was recorded and taken as yield of crude extract for both systems separately despite the objective of this experiment was to compare the ability for antibacterial activity in two different systems not measuring the concentration of existing phenols or any other chemicals.

Yield = weight of dried sample (mg)/weight of fresh sample (g) × 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% (49mg/g) *in vitro*

Yield percentage for petroleum ether = 0.43% (43 mg/g) *in vivo* and 0.46 % (46mg/g) *in vitro*

Yield percentage for methanol = 0.39% (39 mg/g) 0.39% *in vivo* and 0.44% (44mg/g) *in vitro*

This is considered as an almost acceptable yield percentage comparing to earlier reports (Wilson *et al. 2005*; Chen *et al.* 2008).

As it was only a comparison between two systems, *in vitro* and *in vivo*, and there was no comparison between different species, yield of extract was calculated only as a routine record.

4.2.2. Antimicrobial activity assay

In order to perform the micropropagation test in the most efficient way, different trials were done to obtain the best concentration (diagram 3.12) using IBA and BAP.

Three different concentration based on the previous reports (Miachir *et al, 2000*; Loc *et al, 2005*; Keng and Stanley, 2007) were tested for the IBA as 0.5 mg/l, 1mg/l, 1.5 g/ml and 3 mg/l combined with 3 different concentration for the BAP as 2.5 mg/l, 3 mg/l, 4 mg/l; results are shown in diagram 4.1-4.6 and 4.9-4.14.

After obtaining the best concentrations following several trials (Diagram 3.12) for each PGR and also the PGRs combinations, the stock solutions for the PGRs were prepared.

Diagram.4.1 illustrates the shoot length within four weeks after micropropagation with IBA (0.25 mg/l); Diagram shows a delay for about one week before plants start to grow. After the third week, while a sub culturing was applied, the growth was speeding up but was not higher than two centimetres after twenty eight days.

The result was quite different when 0.5 mg/l IBA was used in the media.

Despite an only 0.25 mg/l increase in the hormone concentration was applied, but the shoots reached three centimetres after twenty eight days with only two days delay (Diagram 4.2).

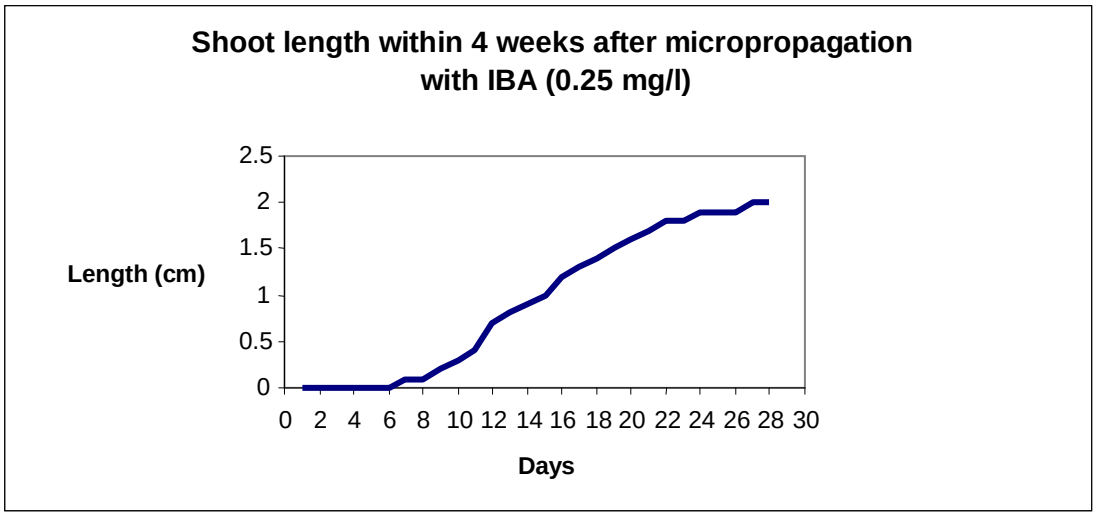


Diagram 4.1 Shoot length within 4 weeks after micropropagation with IBA (0.25 mg/l); diagram shows almost one week pause for the growth and a constant rate of growth during third week.

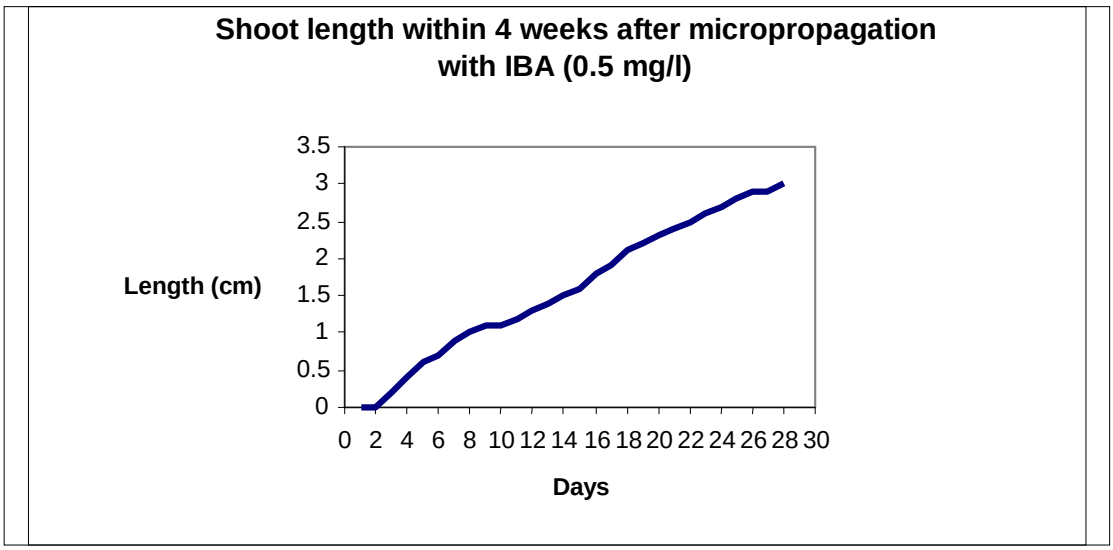


Diagram 4.2 Shoot length within 4 weeks after micropropagation with IBA (0.5 mg/l); diagram shows an almost immediate start for the growth after second day and a constant rate of growth in following days.

When the concentration was increased to 1 mg/l the growth level decreased to maximum two centimetres after twenty eight days and the growth rate also seems to be decreased (Diagram 4.3).

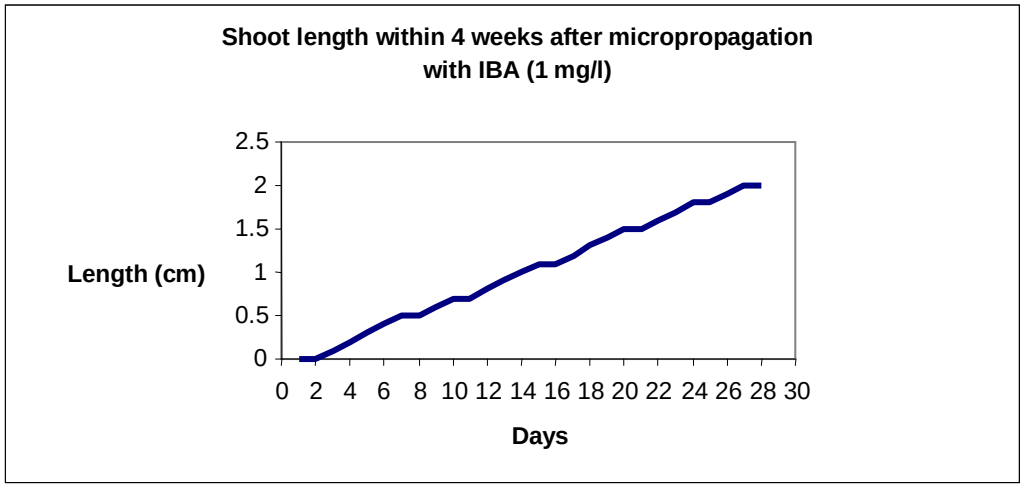


Diagram 4.3 Shoot length within 4 weeks after micropropagation with IBA (1 mg/l); diagram shows the growth rate is pitching up.

The growth rate was again decreased when 1.5 mg/L as shoots grew up only until 1.5 centimetres while the growth rate was decreased. As illustrated in diagram 4.3, in the 16th day when applying 1 mg/l, the shoot length was about 1 cm but the rate of the growth was almost constant but when applying 1.5 mg/l, the rate was decreased after 16th day and did not speed up.

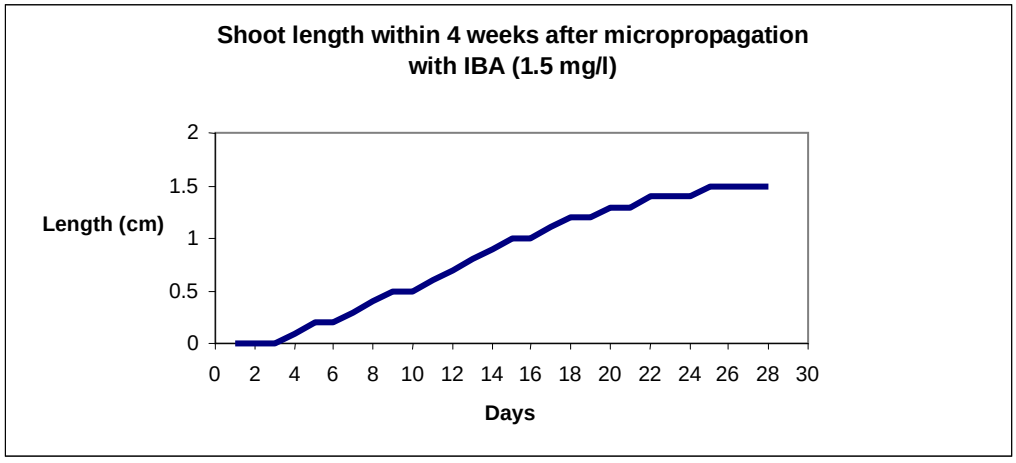


Diagram 4.4 Shoot length within 4 weeks after micropropagation with IBA (1.5 mg/l); diagram shows the growth rate is almost constant, reaching maximum length of 1.5 centimeters.

After increasing the concentration for 0.5 mg/l more IBA in the media, there were no serious changes in the result. As shown in diagram 4.5, a sudden jump form day 17 to day 22 in the growth rate was observed since the shoots were sub cultured on the 14th day.

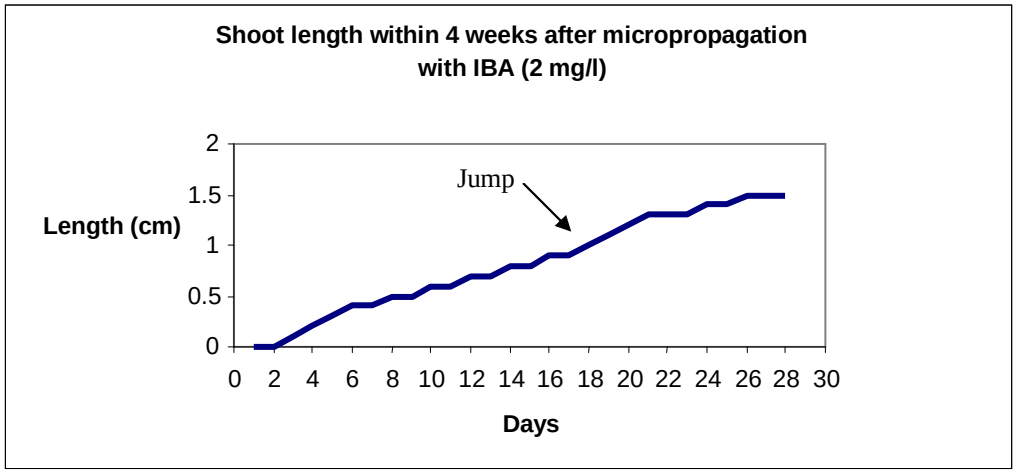


Diagram 4.5 Shoot length within 4 weeks after micropropagation with IBA (2 mg/l); diagram shows the growth rate is faster from day 2 to day 6 and 17 to 22.

The results got worse when 2.5 mg/l was added in the media (diagram 4.6)

Though a high rate of growth was observed form day 12 untill 18 but the growth suddenly stopped on the 28th day. This can be considered as a higher rate of IBA that can decrease the growth rate.

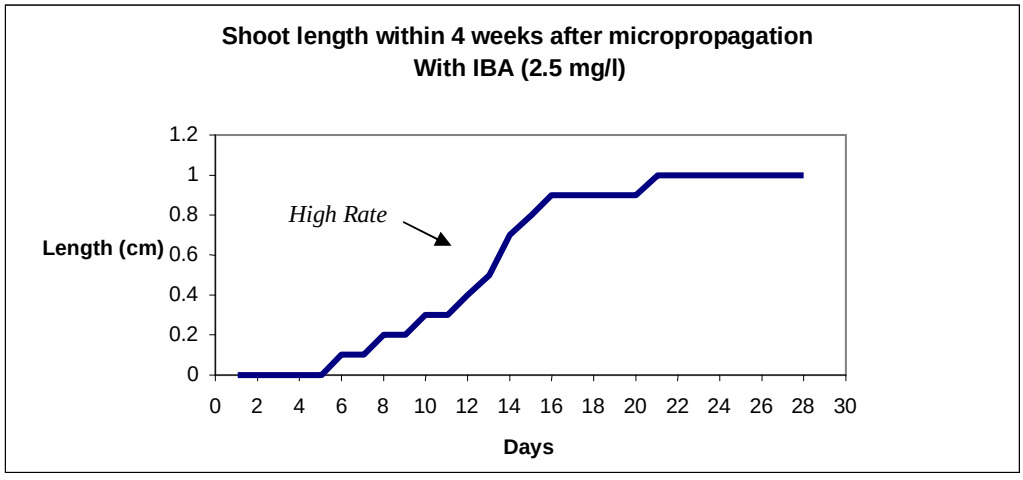


Diagram 4.6 Shoot length within 4 weeks after micropropagation with IBA (2.5 mg/l); diagram shows no growth within first 6 days and a very fast rate of growth from day 11 to 17 and a sudden stop after it .

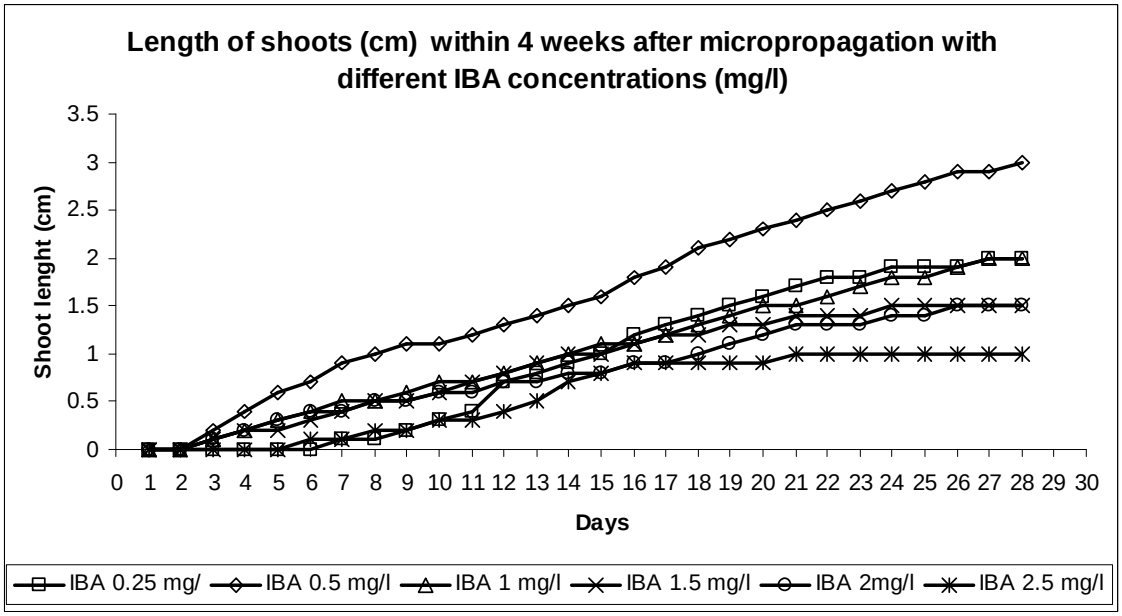


Diagram 4.7 Shoot length within 4 weeks after micropropagation with different concentrations of IBA; diagram shows that using 0.5 mg/l of IBA has provided a higher shoot length in comparison with other concentrations .

Diagram 4.7 illustrates the results together when different concentrations (mg/l) of IBA were applied in the media.

0.5 mg/l IBA is seemed to be the best concentration alone in the media as it has a higher rate of growth of all.

The results, when higher rates than 2.5 mg/l IBA were tested, were interesting as the media was always contaminated; even the test was repeated for three times.

This shows that the higher IBA rates might destroy the media and/or the plant cells or might provide a better environment for microorganisms to grow.

Diagram 4.8 shows the maximum shoot lengths that were gained after applying different concentrations of IBA.

Obviously, 0.5 mg/l IBA alone in the media has made the shoots grow higher than those with other IBA concentrations.

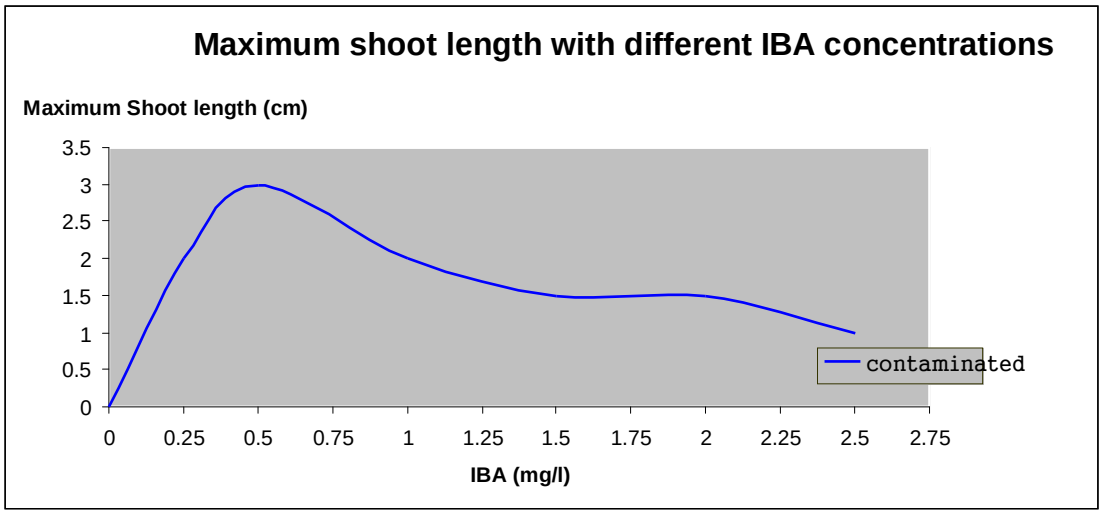


Diagram 4.8 Maximum shoot length with different IBA concentrations; Diagram shows that 0.5 mg/l IBA alone in the media has the best result among all the concentrations.

Diagram 4.9, 4.11 and 4.12 shows that explants with BAP alone, had a better start rather than those with IBA; it seems that shoots had an almost immediate growth after the second day.

Explants had zero growth rates until the second week when 4 mg/l BAP was used alone in the media as illustrated in diagram 4.14. Explants reached maximum 2 centimeters after 4 weeks after one week delay with two centimetres length.

The lower concentrations also didn't show an acceptable result (diagram 4.9-4.10).

The sudden increase in the growth rate is all cause because of the boost effect of Sub- culturing.

Diagrams 4.11 and 4.12 show that using not more than 3 and less than 2.5 mg/l BAP alone in the media can give a better growth rate.

The results for concentrations lower than 1.5 mg/l show almost no growth or very slight growth or even contaminations.

Those are omitted in this discussion. Table 4.1 shows the results briefly.

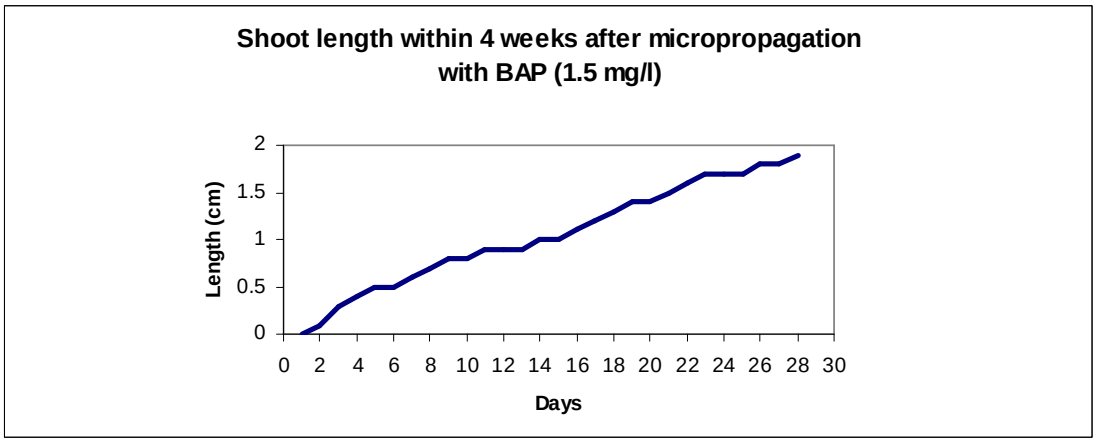


Diagram 4.9 Shoot length within 4 weeks after micropropagation with BAP (1.5 mg/l); Diagram shows an almost immediate start and almost a constant rate till reaching 2 centimeters of length.

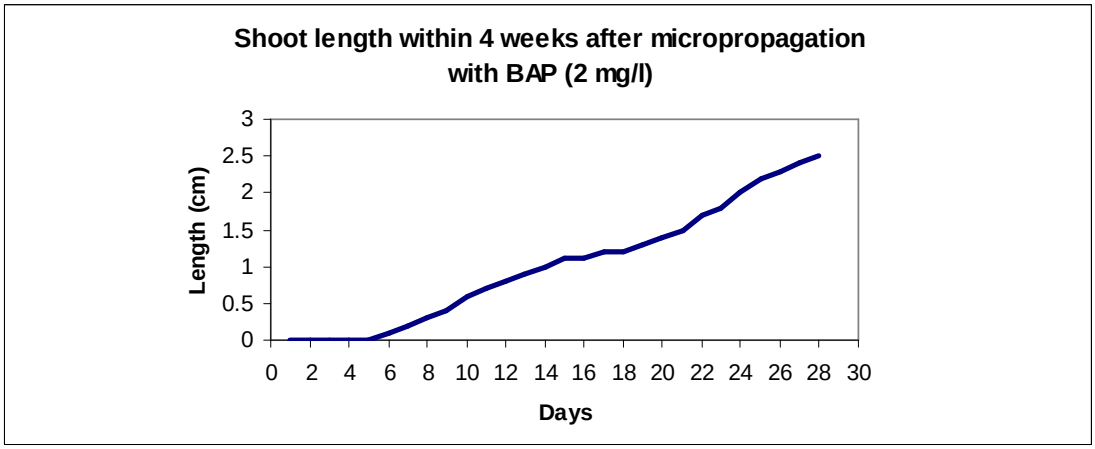


Diagram 4.10 Shoot length within 4 weeks after micropropagation with BAP (2 mg/l); Diagram shows zero growth until five days and shoots gradually growing up until reaching 2.5 centimeters of length .

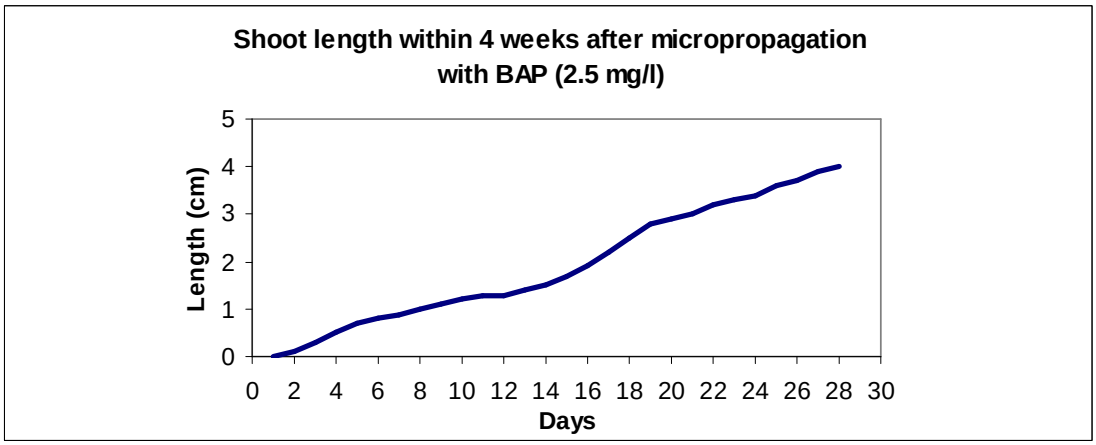


Diagram 4.11 Shoot length within 4 weeks after micropropagation with BAP (2.5 mg/l); Diagram shows a constant rate for growth with a high level of growth as high as 4 centimeters after 28 days

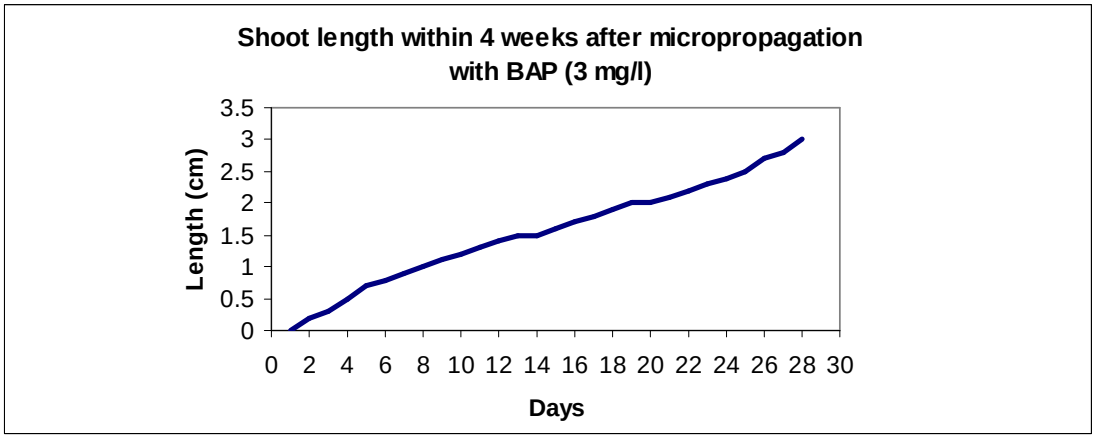


Diagram 4.12 Shoot length within 4 weeks after micropropagation with BAP (3 mg/l); Diagram shows the shoots length has reached 3 centimeters with a constant rate of growth after 28 days

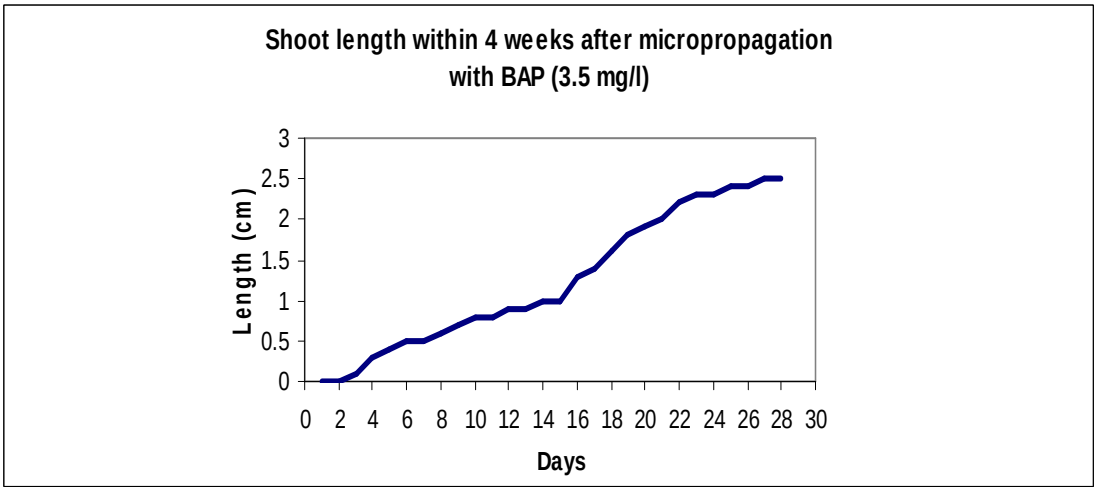


Diagram 4.13 Shoot length within 4 weeks after micropropagation with BAP (3.5 mg/l); Diagram shows that the shoots started to growth after third day for only 5 millimeters after 8 days and 1 millimeter after 16 days and a sudden growth after day 18 until reaching 2.5 centimeters.

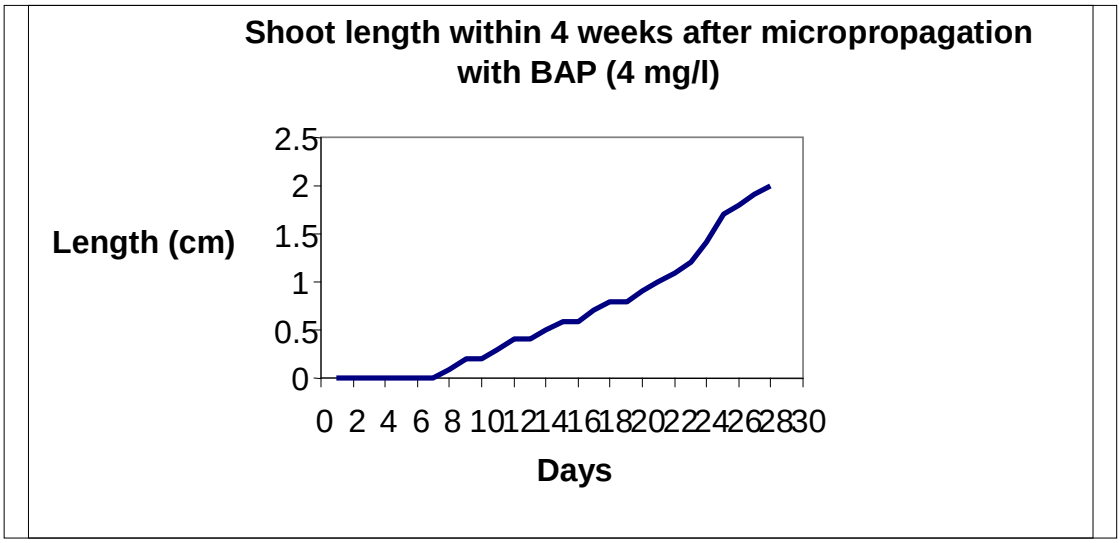


Diagram 4.14 Shoot length within 4 weeks after micropropagation with BAP (4 mg/l); diagram shows zero growth until the second week and gradually slow growth rate reaching maximum 2 centimeters after 4 weeks.

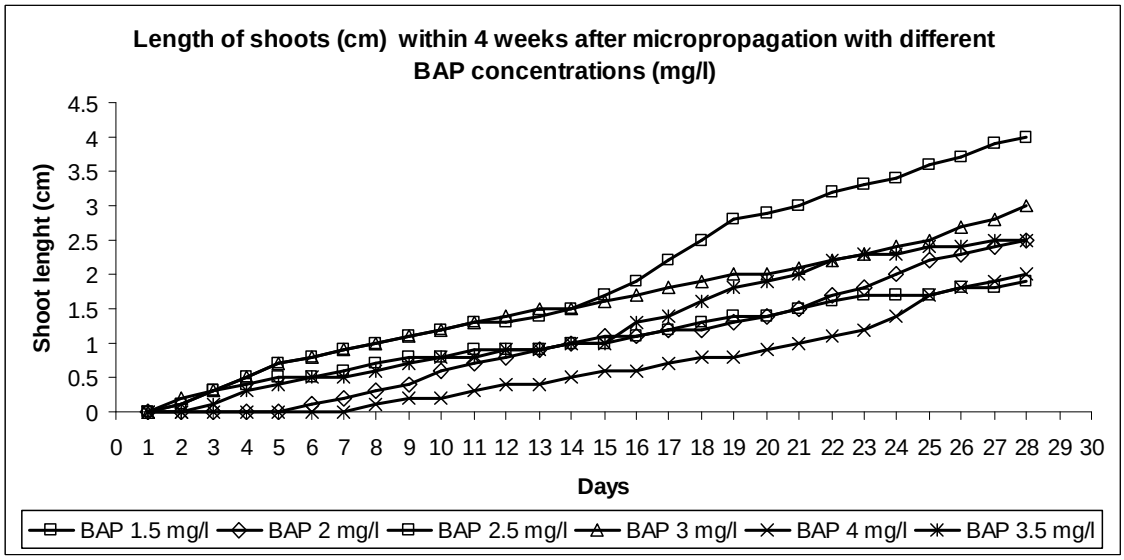


Diagram 4.15 Shoot length within 4 weeks after micropropagation with different concentrations of BAP; Diagram shows that using 2.5 mg/l of BAP has provided a higher shoot length in comparison with other concentrations.

Diagram 4.15 illustrates the results together when different concentrations (mg/l) of BAP that was applied in the media. Table 4.15 shows that 2.5 mg/l IBA is the best concentration alone in the media as it has a higher growth rate and shoots growing with BAP alone, have reached a higher length than those with IBA inside the media.

After collecting all the data and comparing them, the best resulting concentrations were among the PGRs alone in media that were chosen and used together and again tested as shown in table 4.2.

The best results for the IBA was 0.5 mg/l, 1 mg/l and 1.5 mg/l and for the BAP it was 2.5 mg/l, 3mg/l and 4 mg/l which were applied together (table 4.2)

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.

PGR ▶	IBA (mg/l)								BAP (mg/l)							
Shoot length Vs time ▼	0.25	0.5	1	1.5	2	2.5	3	3.5	0.5	1	1.5	2	2.5	3	3.5	4
1 week (cm)	N-G	≤1	0.5	0.5	0.5	N-G	contaminated	N-G	N-G	N-G	1≤	1≤	1≤	1	0.5	N-G
2 weeks (cm)	N-G	1.5	1	1	≤1	≤1	contaminated	contaminated	N-G	1≤	1	1	1.5	1.5	1	0.5
3 weeks (cm)	≤1	2.5	1.5	1.5	1.5	1	contaminated	contaminated	N-G	N-G	1.5	1.5	3	2	2	1
4 weeks (cm)	2	3	2	1.5	1.5	1	contaminated	contaminated	N-G	N-G	2≤	2.5	4	3	2.5	2

Table 4.1 Length of shoots after within 4 weeks of growth after micropropagation with different concentrations of IBA and BAP.

PGR Combinations / Shoot Length (cm) After 4 weeks		IBA (mg/l)		
		0.5	1	1.5
BAP (mg/l)	2.5	4.5	3.5	3
	3	3	2.5	2
	4	N-G	1	N-G

Table 4.2 Length of shoots after 4 weeks of growth after micropropagation with chosen concentrations of IBA and BAP.

As shown in table 4.2, using 0.5 mg/l IBA with 4 mg/l will stop the growth completely as well as the combination of 1.5 mg/l IBA with same amount of BAP.

Though combination of 1mg/l and 1.5 mg/l IBA with 2.5 mg/l BAP gave a good result, but the 1.5 cm difference for the shoot growth when using 0.5 mg/l IBA with the 2.5 mg/l BAP, made it to be chosen as desired concentration and the best among all.

4.3 Comparison

4.3.1. Screening antimicrobial activity

All solvent extracts of *Curcuma zedoaria*, showed acceptable inhibitory activity especially for *Bacillus cereus* and *Staphylococcus aureus*. Inhibition zones were observed against all bacterial strains except *Escherichia coli* with petroleum ether extracts and chloroform extracts.

Petroleum ether and methanol extracts showed a higher activity than chloroform extracts and produced inhibition zones ranging from 1 mm to 9 mm for *in vitro* extracts and 1 to 6 mm for *in vivo* system extracts (table 4.3).

Table 4.3 shows final results as antibacterial activity *in vitro* & *in vivo* of *Curcuma zedoaria* over the three previously described (chapter 3) solvents (petroleum ether / methanol / chloroform) Using 2.5 mg/l BAP mixed with 0.5 mg/l IBA based on diagram 3.14 and 3.15.Results are shown as inhibitory zone in millimeter length.

To avoid space congestions few photos of the disc diffusion tests have been chosen to be presented in this study (picture 4.1-6).

Solvent ▶		Petroleum ether					Chloroform					Methanol				
System ▶		*In Vivo*	IBA	BAP	IBA+BAP	IBA+ BAP+ AC	*In Vivo*	IBA	BAP	IBA+BAP	IBA+ BAP+ AC	*In Vivo*	IBA	BAP	IBA+BAP	IBA+ BAP+ AC
Bacteria ▼																
B. cereus	12h	2	2	1±0.5	1	-	1±0.5	1±0.5	1±0.5	-	-	1±0.5	2	1±0.5	1±0.5	-
	24h	6	7	5	4	1±0.5	2	3	2	1±0.5	1±0.5	4	6	4	3	1±0.5
	48h	6	9	7	4	1±0.5	2	3	2	1±0.5	1±0.5	4	6	4	3	1±0.5
	72h	6	9	7	4	1±0.5	2	3	2	1±0.5	1±0.5	4	6	4	3	1±0.5
E. coli	12h	-	-	-	-	-	-	-	-	-	-	-	1±0.5	-	1±0.5	-
	24h	-	-	-	-	-	-	-	-	-	-	-	2	-	1±0.5	-
	48h	-	-	-	-	-	-	-	-	-	-	-	2	-	1±0.5	-
	72h	-	-	-	-	-	-	-	-	-	-	-	2	-	1±0.5	-
S. aureus	12h	1±0.5	1±0.5		1±0.5	-	-	1±0.5	-	1±0.5	-	-	1±0.5	-	1±0.5	-
	24h	3	4	3	4	1±0.5	1±0.5	2	1	2±0.5	1±0.5	1±0.5	4	1±0.5	2	-
	48h	3	4	4	5	1±0.5	1±0.5	4	1	2±0.5	1±0.5	1±0.5	5	1±0.5	2	1±0.5
	72h	3	4	4	5	1±0.5	1±0.5	4	1	2±0.5	1±0.5	1±0.5	5	1±0.5	2	1
P. aeruginosa	12h	-	1±0.5	-	-	-	-	1±0.5	-	-	-	-	1±0.5	-	-	-
	24h	-	2	-	1±0.5	-	-	2	-	-	-	-	2	1±0.5	-	-
	48h	-	2	-	1±0.5	-	-	2	-	-	-	-	3	-	-	-
	72h	-	2	-	1±0.5	-	-	2	-	-	-	-	3	-	-	-

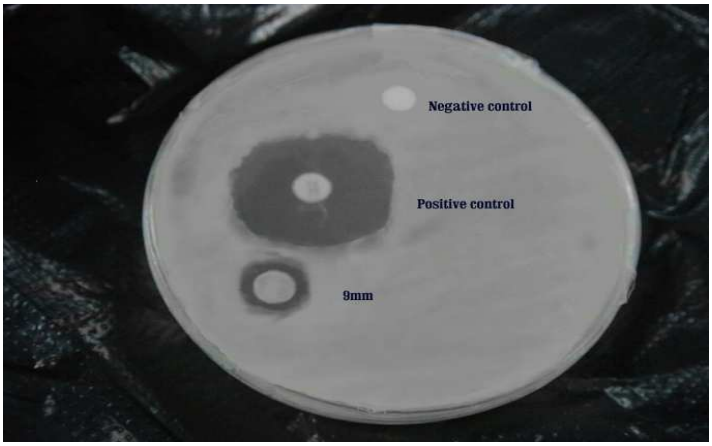
Table 4.3 Antibacterial Activity *of Curcuma zedoaria* in the *in vitro* & *in vivo* systems over different extract solvents (Petroleum ether / Methanol / Chloroform) presented as inhibitory zone in millimeter length (Using 2.5 mg/l BAP mixed with 0.5 mg/l IBA).

A total of 240 plates where observed during the comparison and 107 plates out of 240 plates had a negative result.

For each bacterial strain on different solvent extracts, a distinct table is considered.

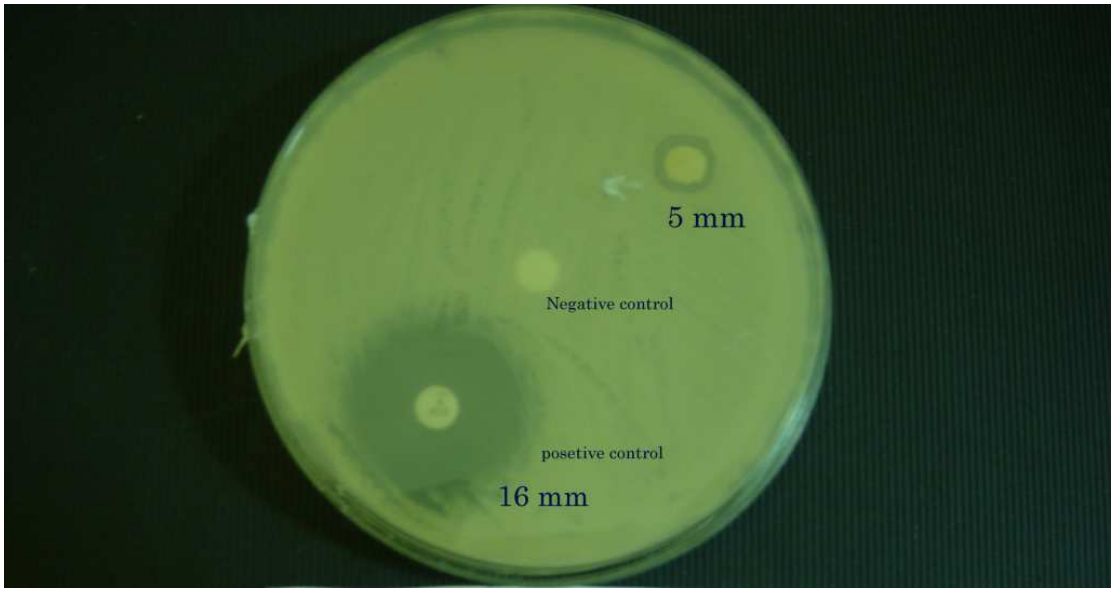
4.3.1.1. *Bacillus cereus*

The *in vivo* system extracts had a great result, inhibiting *Bacillus cereus* with maximum 6 mm diameter of inhibition zone and on the other side, petroleum extracts form *in vitro* system using IBA alone, showed even greater results as inhibiting *Bacillus cereus* for about 9 mm diameter of inhibition zone on the plate. The second place for the inhibition goes to the *in vitro* system using BAP alone by showing maximum 7 mm diameter of inhibition zone after seventy two hours (Table 4.3).



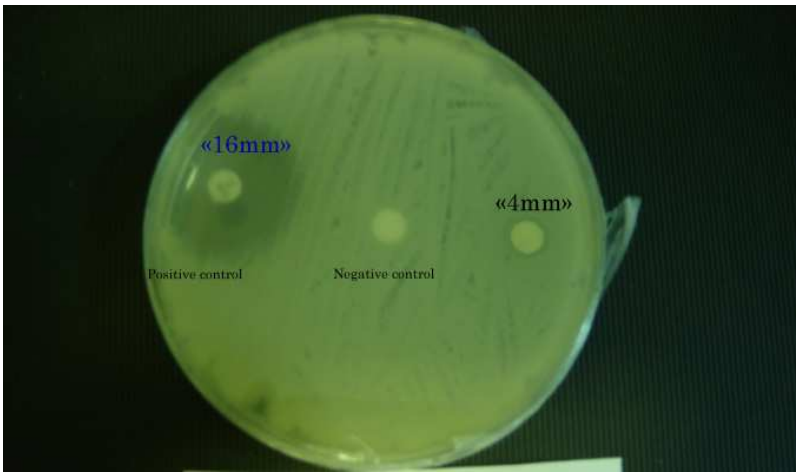
Picture 4.1 Disc diffusion test result on the plate; applying petroleum ether extracts produced from the *in vitro* system on *B.cereus*

Results show that adding the AC to the media with combination of IBA and BAP has decreased the antibacterial activity.



Picture 4.2 Disc diffusion test result on the plate; applying petroleum ether extracts produced from the *in vivo* system on *Bacillus cereus.*

The test was repeated with the IBA alone in the media and ha a better result. Petroleum ether extracts had much more effect than chloroform extracts. Adding AC to the media with IBA and BAP was not effective; nevertheless, chloroform extracts have shown a better effect than the *in vivo* system (Table 4.3).



Picture 4.3 Disc diffusion test result on the plate; applying methanol extracts produced from *in vivo* system on *B.cereus.*

The results for methanol extracts inhibiting *B.cereus* is quite similar to the chloroform results except the zones that are wider than those with chloroform.

Adding AC to the media with IBA and BAP is has reduced the antibacterial power to almost half just like the results with the petroleum ether extracts (Table 4.3).

4.3.1.2. *Escherichia coli*

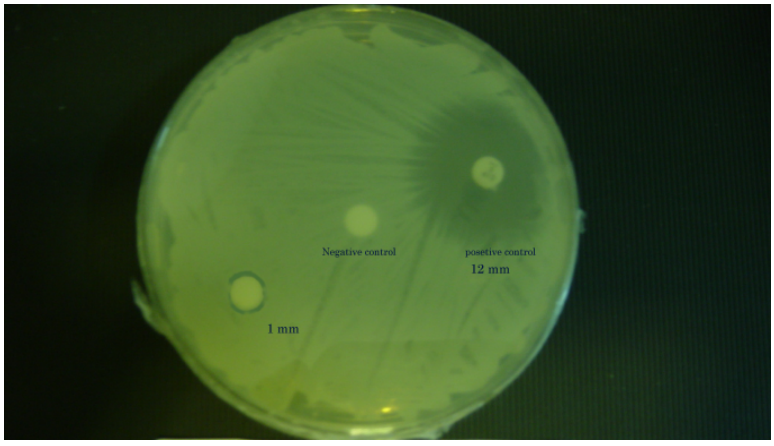
Results (table 4.3) show that none of the solvent extracts inhibited the growth of *E.coli* except methanol extracts and that is only for the extracts made from the shoots which were produced in the *in vitro* system (with IBA alone or mixed with BAP).

Adding AC had no effects at all when adding to the media with the combination of IBA and BAP (Table 4.3).

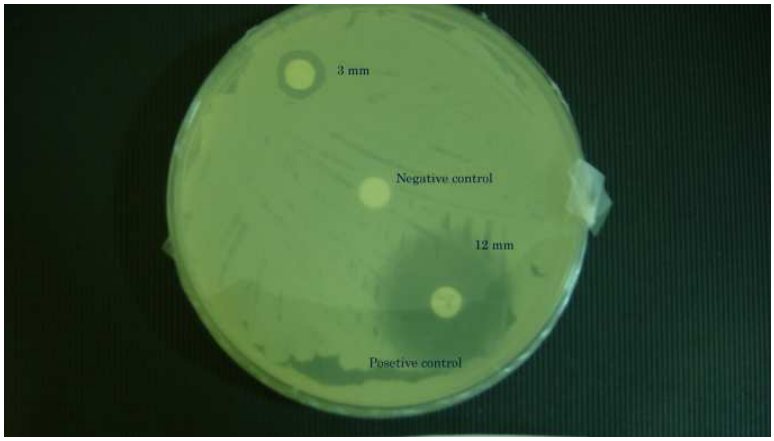
4.3.1.3. *Staphylococcus aureus*

All three solvent extracts were effective on the activity of the *Staphylococcus aureus* and inhibited its growth on the culture plate (table 4.3).

Petroleum extracts were effective on both *in vitro* and *in vivo* systems; the higher rate belongs to the *in vitro* system in which IBA was used with BAP in the media with no AC (Table 4.3).



Picture 4.4
Disc diffusion result on the plate; applying methanol extracts produced from *in vivo* system on *S.aureus.*



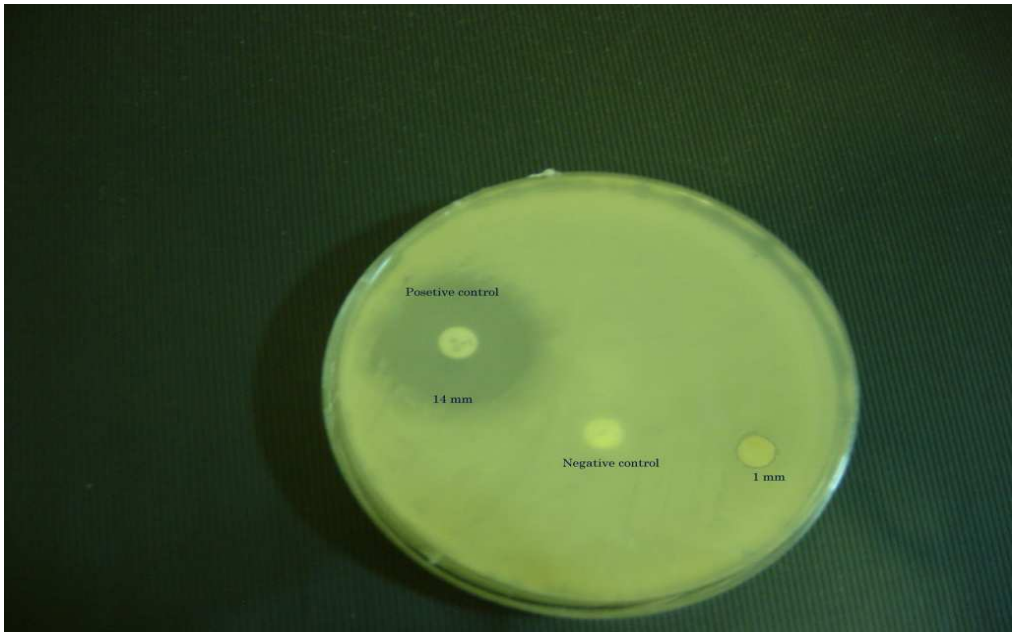
Picture 4.5
Disc diffusion result on the plate; applying petroleum ether extracts produced from *in vivo* system on *S.aureus.*

The interesting result is that the extracts obtained form media with IBA or BAP alone had similar effect on the bacterial activity of *Staphylococcus aureus* as both had produced 4 mm of inhibition zone on the culture plate.

Chloroform extracts were less effective than petroleum ether extracts as they produced an inhibition zone with a maximum of 1 mm in diameter in the *in vivo* system and 4 mm in the *in vitro* system (Table 4.3). Extracts which were obtained from media with IBA alone were more effective than those with BAP alone.

4.3.1.4. *Pseudomonas aeruginosa*

None of the *in vitro* extracts were effective on the activity of *Pseudomonas aeruginosa* and a closer look also shows that adding AC has totally no effect in any types of solvent extracts.



Picture 4.6 Disc diffusion result on the plate; applying petroleum ether extracts produced from *in vitro* system on *P.aeruginosa.*

The best result belongs to the extracts by methanol with a maximum diameter of 4 mm inhibition zone (Table 4.3).

4.3.1.5. AC

More focus on the table 4.3 can confirm the negative effect of adding AC into the media on activity of most of the tested bacteria strains. In a few cases, adding AC has produced inhibition zones with one millimeter of maximum diameter with the decreasing effect on the antibacterial activity of extracts using IBA mixed with BAP (Table 4.3).

The 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 systems too (Boggetti *et al*. 2001). The addition of 0.5 g/l AC to the medium completely inhibited the shoot initiation in some reports (Tivarekar and Eapen, 2001; Wei *et al*. 2006).

4.4. Summary

The results show that *Curcuma 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 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 comparing to the petroleum ether extracts *against Bacillus cereus* and *Staphylococcus aureus* in the *in vitro* system.

Chloroform extracts from the in the *in vitro* system also had similar effect to the *in vivo* system on *Bacillus cereus* and *Staphylococcus aureus* growth.

The interesting part is the positive effect of all three *in vitro* extracts on *Pseudomonas aeruginosa* growth comparing the non- effective extracts of *in vitro* system (Table 4.3). *In vivo* and *in vitro* extracts had no effect on *E.coli* growth when using chloroform and petroleum ether but methanol extracts in the *in vitro* system when using 0.5 mg/l IBA showed considerable results. Table 4.3 indicates that there might been a hormone interaction between IBA and BAP as the inhibition zone has always been smaller when the combination of PGRs had been applied. Some difficulties and problems occurred while performing this experiment. The explants of *Curcuma zedoaria* with high potentials to get contaminated during micropropagation, were always the main problem during this study, therefore, this always imposed delays to the work.