

CHAPTER 3: MATERIALS AND METHODS

3.1. Introduction

In this study, work and tasks are divided to two main sections in order to make a comparison between *in vitro* and *in vivo* system. 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 establish 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.

Curcuma zedoaria rhizomes needed special attention when treated with sterilants since they are very much sensitive toward the sterilizers and decontaminants.

Different test methods and conditions were used in earlier reports and this made it difficult to establish a certain method to perform all the tests but several attempts was done to perform a good treatment, micropropagation and finally a comparison in an acceptable level for this dissertation.

The first step was plant material preparation, the collection of the source rhizomes.

This initial task was carried out for both *in vitro* and *in vivo* system, for next steps, micropropagation (for *in vitro*) and preparing the extracts for antibacterial activity (for both systems) test were followed, each separately and at different time but parallel.

An overall view of the work is illustrated in diagram 3.1.

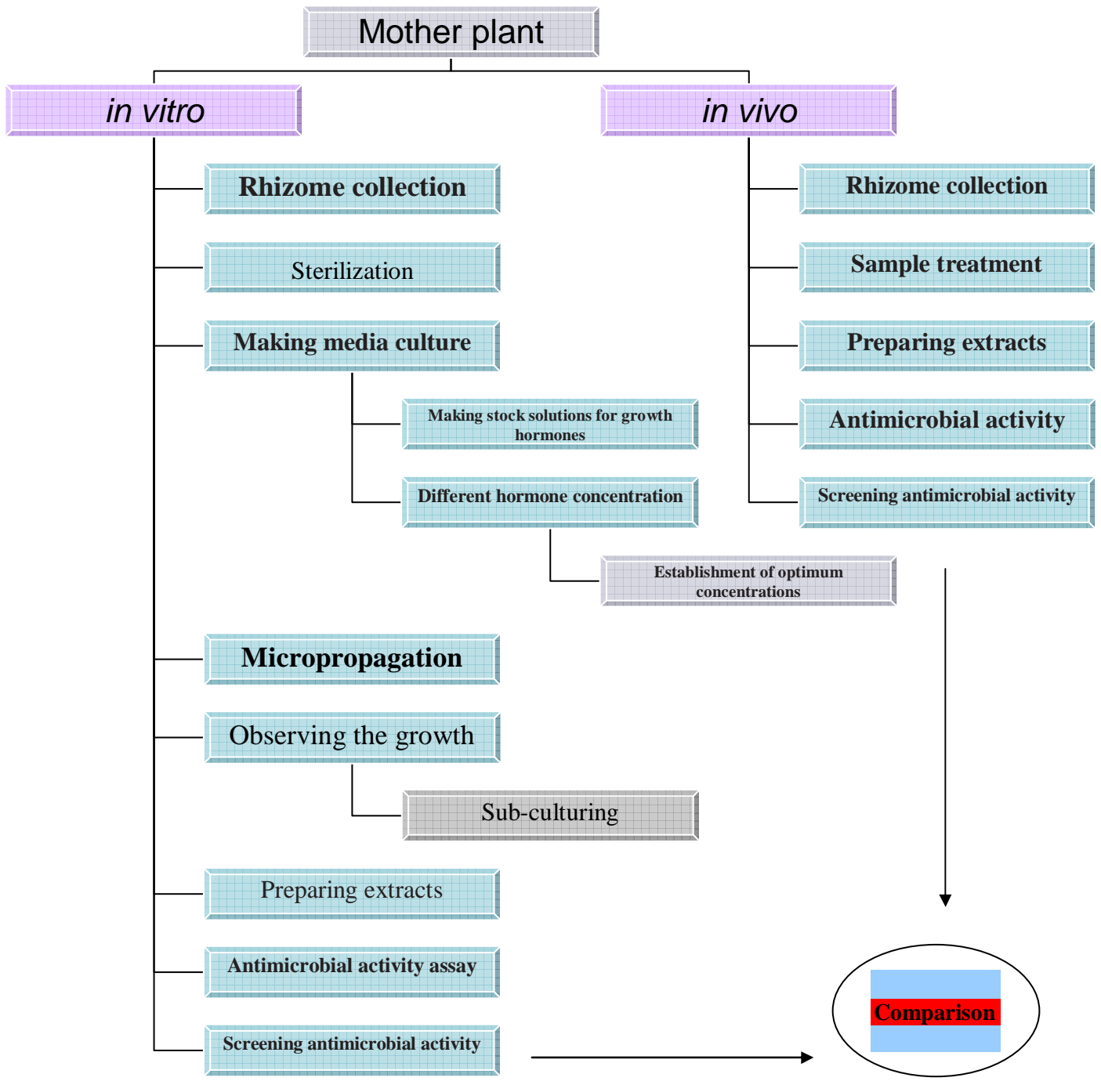


Diagram 3.1 the overall view of the work; this study is basically made of two major sections; *in vitro* and *in vivo*



3.2. Plant material

The rhizome source of *Curcuma zedoaria* is from Pahang state in Malaysia

The rhizomes were first transferred into the soil at garden at the Institute of Biological Sciences at University of Malaya to increase the number of rhizomes and multiply the plants in order to have more samples for the experiment and acclimatization (acclimation) as well for adjusting to change in the new environment. This took about three months for the rhizomes to grow up in the garden.

Tough even the smallest patio or porch could boast the rhizomes in containers, But containers with narrow openings and cheap plastic were not used since small pots restrict the root area and dry out very quickly; therefore ceramic pots were chosen. Several drainage holes were made (holes were about 1-2 inches across and the base of the pot was lined with newspaper to prevent soil loss). Containers had between forty to sixty liters of capacity.

The containers were set on bricks to allow free drainage.



Picture 3.1
Ceramic pot



Picture 3.2
Holes were about 1-2 inch across and the base of the pot



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Fertilizer with a mixture of K, N and P (Potassium, Nitrogen and Phosphor) were added rapidly to the soils every two weeks.

After three months the desired level of the plants growth/ numbers were obtained and the rhizomes were already matured and enough to perform the *in vivo* / *in vitro* experiment (Diagram 3.2).

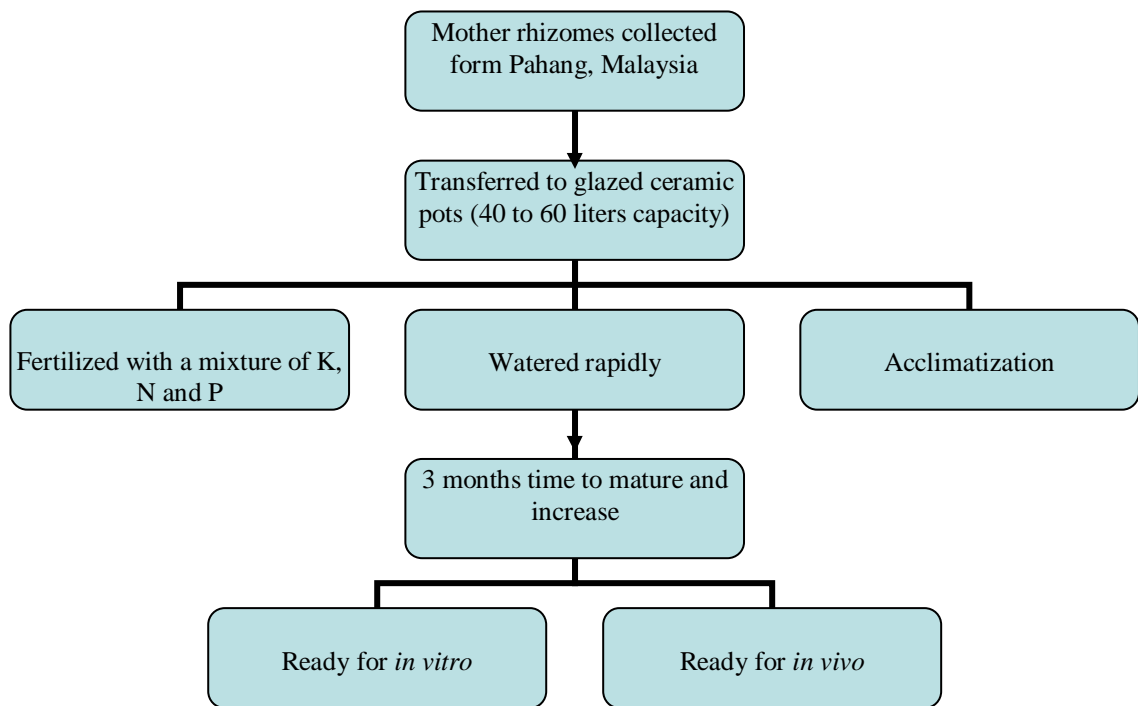


Diagram 3.2 Plant material preparations for both *in vitro* and *in vivo*

3.3. *In vivo* system

3.3.1. Preparing plant extracts

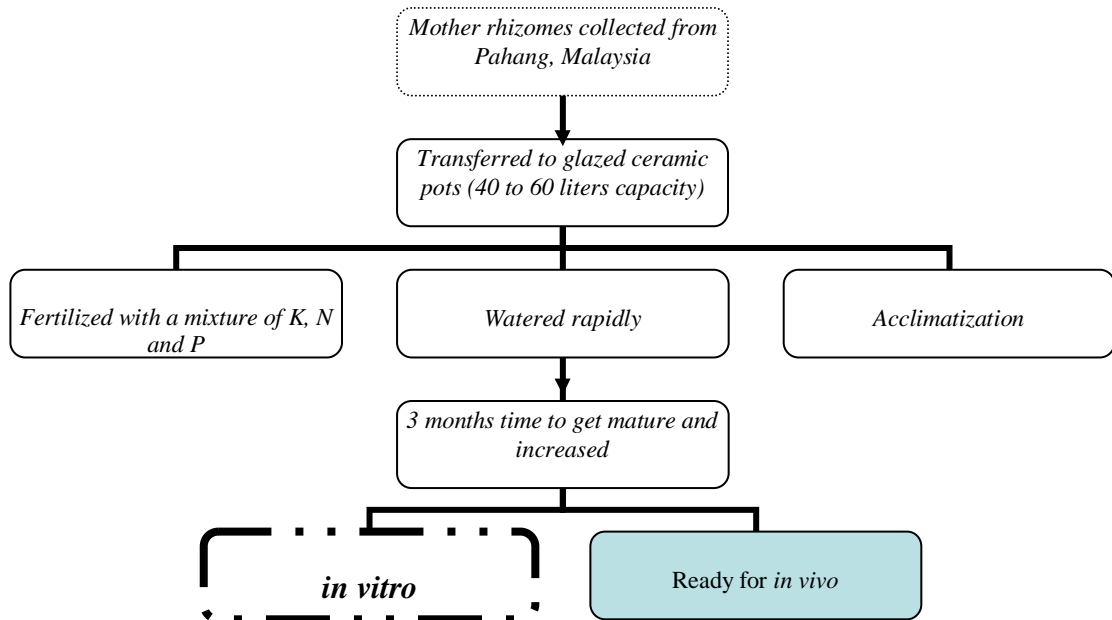


Diagram 3.3 Stages of plant extracts preparations prior to *in vivo* test

a) After collection, freshly harvested plant samples were cleaned, washed with tap water, sliced into small pieces and kept at a temperature not exceeding 40°C for about twelve days in an incubator to completely dry.



Picture 3.3 *Curcuma zedoaria* rhizomes ready for preparing extracts.



Picture 3.4 *Curcuma zedoaria* rhizomes, sliced before drying.

In the next step the dried samples were ground in a mini commercial mill (mixer) and pulverized to powder form. The powder was soaked in petroleum ether, chloroform, and methanol respectively (by increasing the solvent polarity in order to have better efficiency of the extracted yield). Duration of six days was regarded for each solvent with the powder soaked in to completely isolate the desired compounds.



Picture 3.5 Rhizomes after they were dried in the incubator.



Picture 3.6 Dried rhizomes after being ground in the miller, ready to soak in the solvent.

The weight for the powder was measured before applying the solvents.

b) The solvents were then distilled under reduced pressure at 40 °C in a rotary evaporator (BUCHI , Switzerland) until it became completely dry and weighed to determine the total extractable compounds. The weight of the solid residue was recorded and taken as yield of crude extract.

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 the antimicrobial activity.



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The yield of methanol, petroleum ether and chloroform extracts was determined as milligram of extract per gram of fresh rhizomes weight, calculated as follows:

$$\text{Yield} = \text{weight of dried sample (mg)} / \text{weight of fresh sample (g)} \times 100\%$$

The process is illustrated in diagram 3.4 and 3.5, from preparation the plant material until it is ready for the antibacterial activity assay.

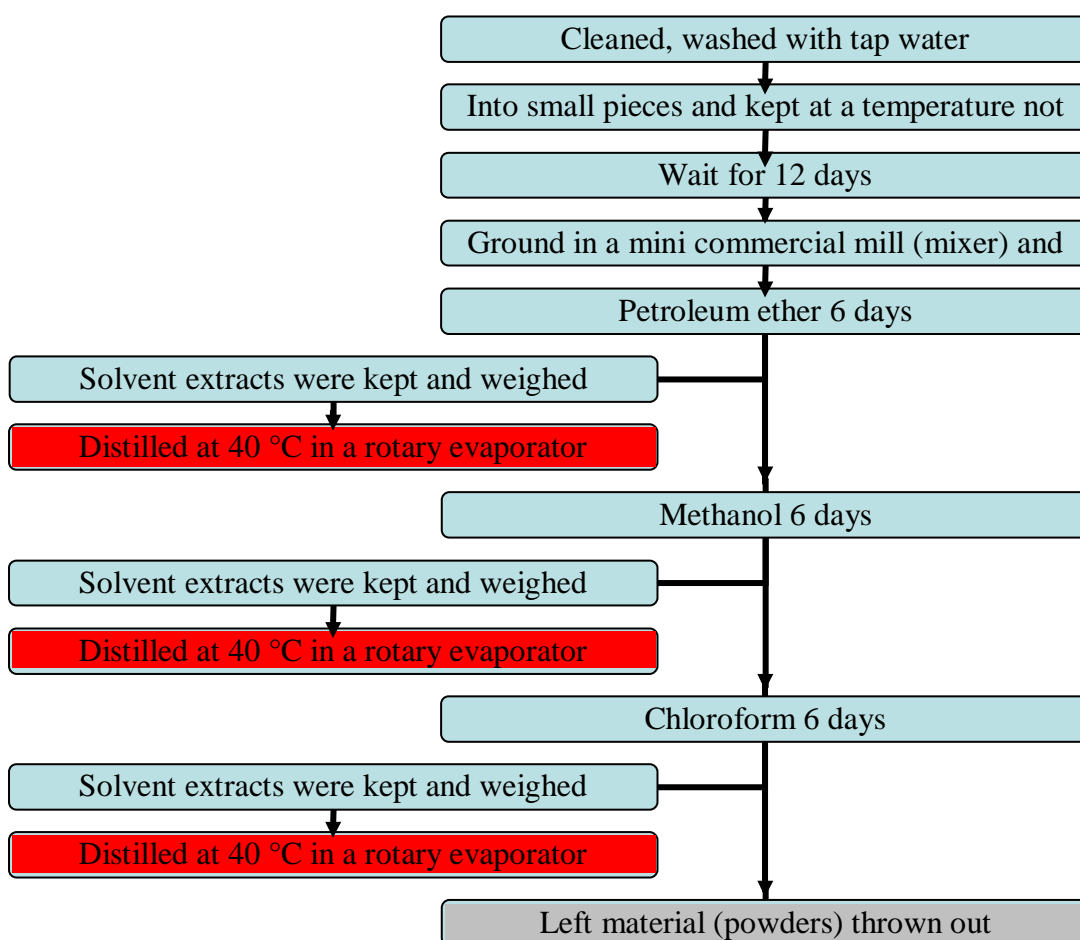
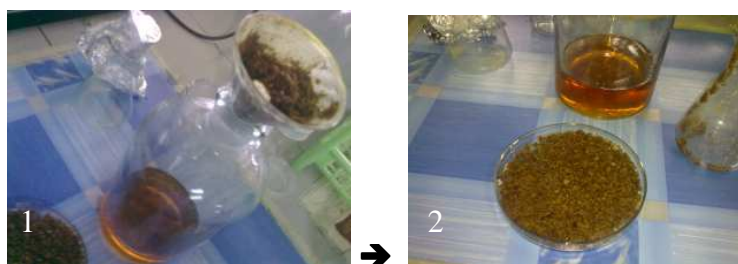


Diagram 3.4 Preparation of plant extracts before antibacterial activity assay.



Picture 3.7
Powders being soaked in the solvent.



Picture 3.8.1-2
Powders soaked in the solvents are filtered in order to distill the solvent in the evaporator.



Picture 3.9
Extracts ready for distillation.



Picture 3.10
Rotary evaporator used to distill the solvents.



Picture 3.11
Solvents were distilled under reduced pressure at 40 °C in the rotary evaporator.

3.4. Antimicrobial activity assay

3.4.1. Preparing the extract material for disc diffusion test

Diagram 3.5 explains the process before applying the agar well diffusion disc test.

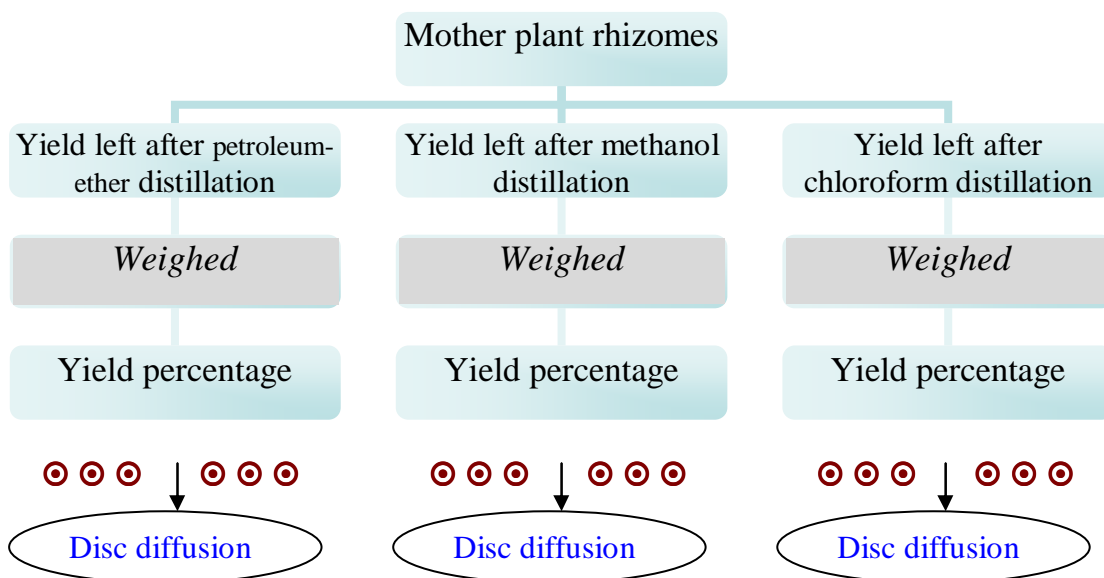


Diagram 3.5 Preparation of plant extracts before antibacterial activity assay.

In this step the related solvents were again employed but in a little amount in order to just solve extract which is now fully dried after distillation of the solvents.

What is left in the flasks after the solvents were distilled contains all the desired compounds which are capable and mighty to have antibacterial activity.

The routine calculation was used to obtain the standard concentration needed for a disc diffusion test.

Discs needed to get soaked in the extracts but all had to be exactly at same level of concentration.

The standard **100mg/ml concentration** was calculated for the weight of three groups of extracted yields. The stock was ready to put the discs inside.



3.4.2. Preparing the Bacterial material for disc diffusion test

3.4.2.1 Microbial strains

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.

3.4.2.2 Bacteria preparation

Before starting the agar-well disc diffusion test, the bacteria group chosen for this study needs to grow in a broth media in order to get prepared to be cultured on the agar plates for applying the agar well diffusion test (Volk, 2008).

Muller-Hinton media was used as the media. The media was autoclaved at 121°C for 10 minutes. Media tubes were prepared and the media was poured in the tubes and kept in the incubator at 37°C for twenty four hours. This step was done in order to confirm that the test tubes containing the broth are all sterile and ready for inoculation. Microorganisms were inoculated in the MH broth media using a sterile cotton swab in a laminar flow cabinet, fully sterilized with UV light and then allowed to grow over night.

One tube was kept free of inoculated bacteria as a negative control though the contamination was already checked the previous night.

For over night growth, a standard microbiology lab incubator was used and the temperature was adjusted to 37°C.

The next day, after checking the negative control, the test tubes were diluted each with sterile Saline buffer containing 0.9% NaCl for ten times in order to reach about 10^8 colony forming unit /ml.



Picture 3.12

Microorganisms were inoculated in MH broth media using sterile cotton swab in a Laminar flow cabinet.



Picture 3.13

Tubes ready after spectrophotometry and dilution stage .



Picture 3.14

Disposable spectrophotometer cuvettes for spectroscopy

The concentration of the inoculum suspension was adjusted between 0.08 - 0.1 optical densities at the wavelength of 625 nm using a spectrophotometer device.

The whole progress is briefly illustrated in diagram 3.6.

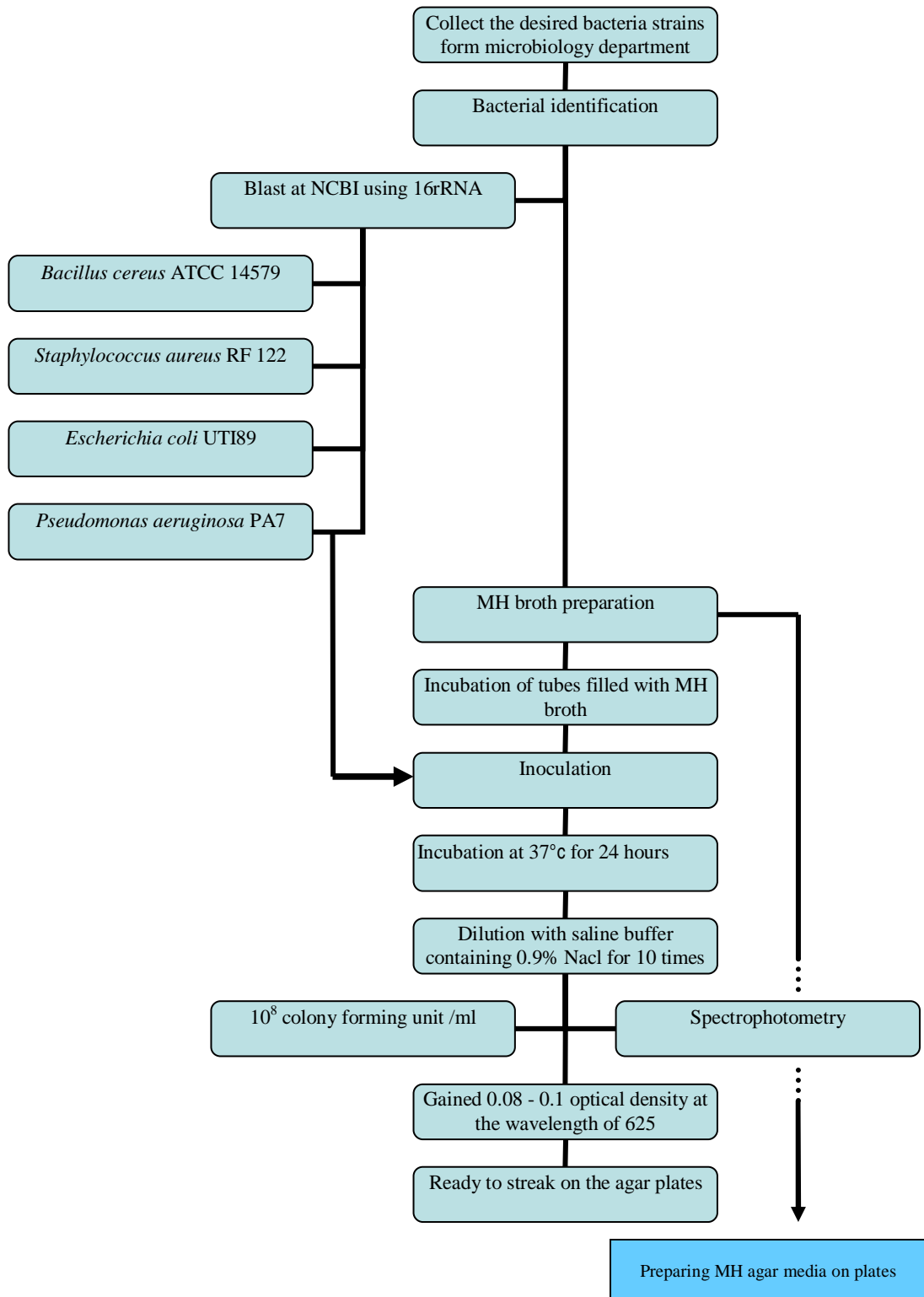


Diagram 3.6 Progress of preparing bacteria strains for the disc diffusion method



Similar concentration for each bacteria group was available in the tubes after obtaining the desired optical density using the spectrophotometer, ready to streak on the Muller-Hinton agar. As illustrated in diagram 3.6, the same material was used to prepare the MH agar. To prepare the agar, 17gr/l agar was used to make the Muller-Hinton agar media in the plates, following by autoclaving.

(MH powder + Water + Agar → Autoclave → keep in incubator to confirm sterility)

For one liter media 22g Muller-Hinton powder + 17g Agar was used.

3.4.3. Agar-well disc diffusion test

At this stage, everything was ready to culture the bacteria and place the discs on the plates. For each selected bacteria strain, three discs were considered as negative control (water), positive control (antibiotic) and the disc which was soaked on the extract.

The agar plate was streaked with inoculum suspension using a sterile cotton swab over the entire surface three times for an even distribution of the inoculum. Sterile six millimeter disc filter paper was used to dip into the fraction and placed onto the agar plate with inoculum. Within fifteen minutes all the agar plates were sealed using parafilm and incubated at 37 °C for fourteen hours. The positive controls used were tetracycline 30µg from Oxoid England. The plates were checked after sixteen hours. Zone of inhibition were measured and the result were recorded (diagram 3.7).

The method applied was according to the “British society for antimicrobial chemotherapy” and “National committee for clinical Laboratory Standards” (NCCLS).

The diameter of the zones of inhibition around each of the wells or discs (well/disc diameter included) was taken as a measure for the antimicrobial activity.

Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

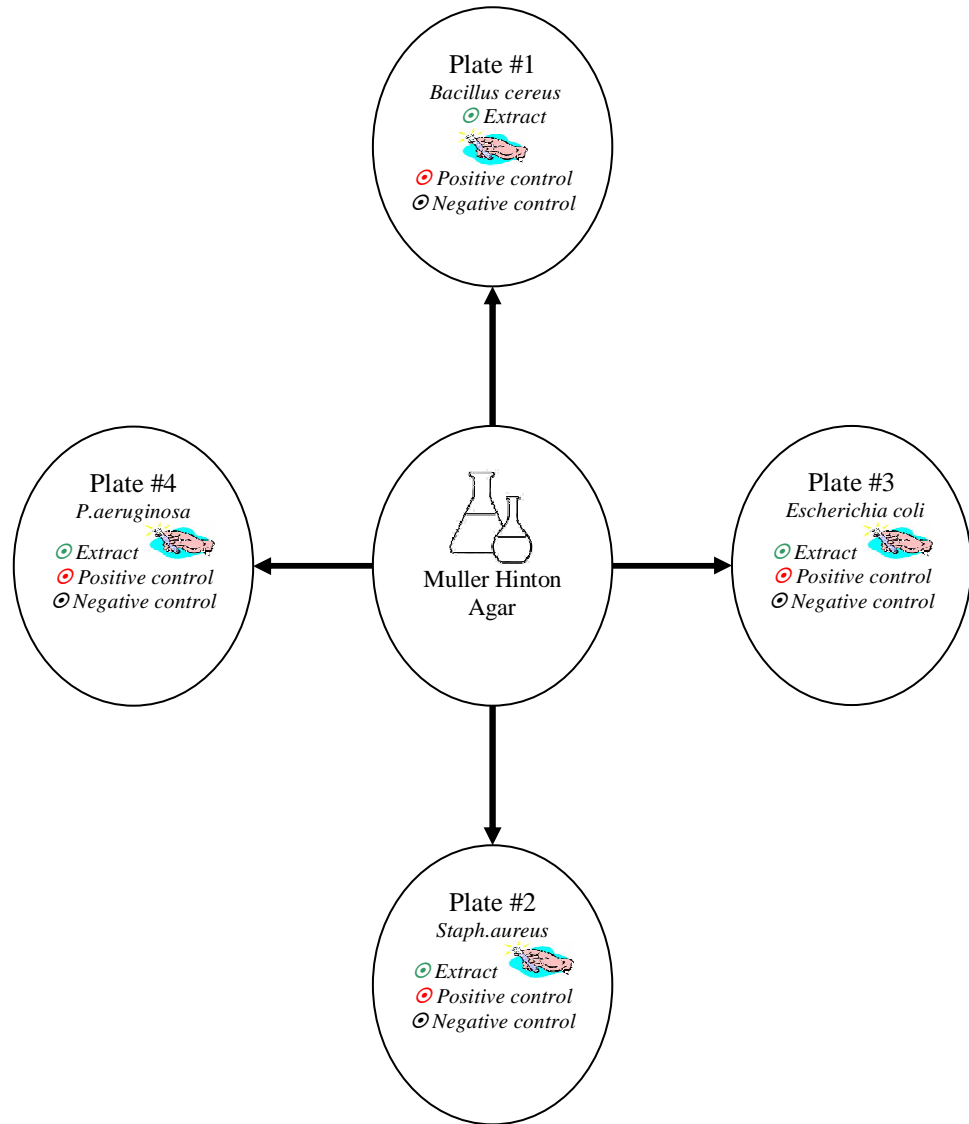


Diagram 3.7

Agar well disc diffusion method (Volk, 2008). For each selected bacteria strain, three discs were considered as Negative control (Water), Positive control (Antibiotic) and the disc which was soaked on the extract.

3.5. *In vitro* system

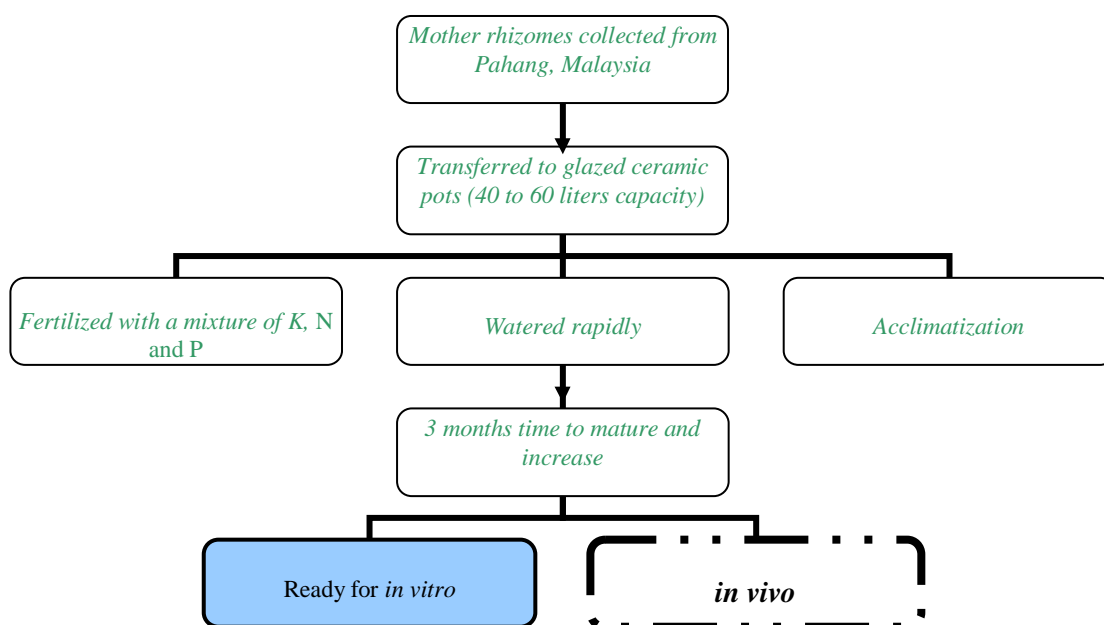


Diagram 3.8 Stages of plant extracts preparations prior to the *in vitro* test.

Diagram 3.8 clarifies what has been done before this step.

For the *in vitro* system, (unlike the *in vivo*) an extra step was performed and that was micropropagation. Antibacterial activity assay and disc diffusion test were all carried out using the same procedure in the *in vivo* system.

When this dissertation was carried out, both *in vivo* and *in vitro* systems were supposed to be processed at the same time; but testing different concentrations for growth hormones, applying various methods for decontamination, office works and chemical delivery difficulties almost made the processes not done at the same time.



3.5.1. Micropropagation

3.5.1.1 Equipments and chemicals

In order to perform the micropropagation method, certain equipments, chemicals were prepaid as follows:

- ✓ Laminar Flow with UV light
- ✓ Stirrer
- ✓ Digital laboratory Scale
- ✓ Sodium hypochloride NaClO (20% solution)
- ✓ Sterile distilled water
- ✓ MS Media + Vitamins
- ✓ PhytoGel Agar
- ✓ HCL 1M
- ✓ Safety laboratory Masks
- ✓ Forceps
- ✓ Conical flasks and bottles
- ✓ Autoclave machine
- ✓ pH meter
- ✓ ethanol
- ✓ Small jars with cap
- ✓ Tween 20
- ✓ Sucrose
- ✓ AC (activated charcoal)
- ✓ NaOH 1M
- ✓ Scalpel
- ✓ Measuring cylinder
- ✓ Magnetic bead

3.5.1.2. Surface sterilization

Before starting the tissue culture (micropropagate) the rhizomes, they needed to be decontaminated, sterilized and ready to inoculate in the media.

The rhizomes were washed thoroughly under running tap water for one hour to remove loose contaminants/soil and other particles. Afterwards, they were put in conical flasks which were half-filled with sodium hypochloride NaClO (20% solution) for five minutes. Subsequently, the explants were washed three times with distilled water with strong agitation using a stirrer. Next, the explants were treated by sodium hypochloride NaClO (20% solution) added with a few drops of Tween® 20 and being agitated constantly for fifteen minutes. Following that, they were rinsed three times with sterilized distilled water again with agitation using stirrer. Further surface sterilization was made with 70% (v/v) ethanol. Finally, the explants were washed and rinsed three times with sterilized distilled water. Diagram 3.9 illustrates the progress briefly.

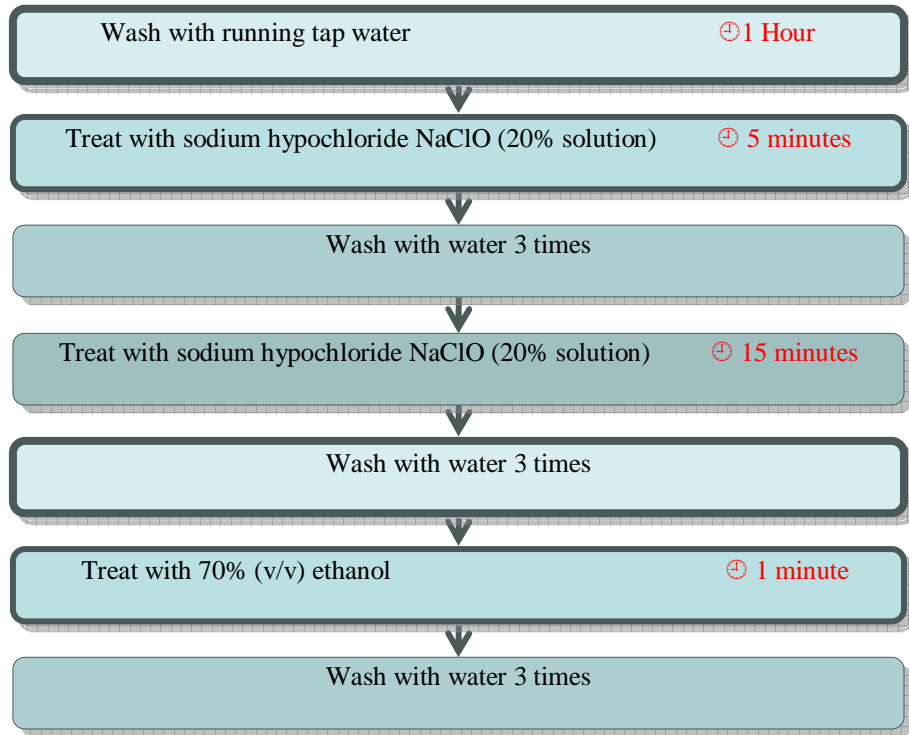
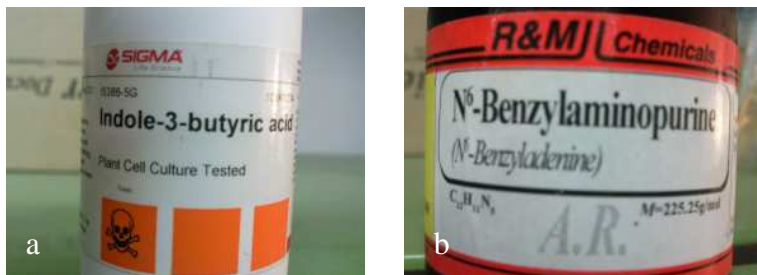


Diagram 3.9 Surface sterilization progress before inoculating the explants to the media.

3.5.1.3. Preparing media

The MS media form Duchefa Netherlands with concentration of 4405.19 mg/l plus vitamins was used for the media preparation; the ingredients are all in table 3.1.



Picture 3.15 a) IBA box
b) BAP box



Picture 3.16 a) MS box
b) Phytigel
Agar box



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Media was made for one liter (L) of water (following the instructions on the manual, 117.9g of powder for fifty liter water). For making a liter of MS medium, 800 ml distilled water was poured in a beaker and placed on the stirrer (using magnetic beads for stirring) and stirred while adding thirty grams sucrose.

The pH level was adjusted to 5.8 with the pH meter. Next, the volume of the medium was increased to one liter (L) by using a measuring cylinder.

For solidifying the media, eight grams phytoagar was added while turned on the heating button in order to melt the agar.

The next step was preparing the stock solutions for the PGRs (plant growth hormones).

Ingredient	mg/L
<i>NH₄NO₃</i>	<i>1650</i>
<i>KNO₃</i>	<i>1900</i>
<i>CaCl₂•2H₂O</i>	<i>440</i>
<i>MgSO₄•7H₂O</i>	<i>370</i>
<i>KH₂PO₄</i>	<i>170</i>
<i>H₃BO₃</i>	<i>6.2</i>
<i>MnSO₄•H₂O</i>	<i>16.9</i>
<i>ZnSO₄•H₂O</i>	<i>8.6</i>
<i>KI</i>	<i>0.83</i>
<i>NaMoO₄•2 H₂O</i>	<i>0.25</i>
<i>CuSO₄•5H₂O</i>	<i>0.025</i>
<i>CoCl₂•6H₂O</i>	<i>0.025</i>
<i>FeSO₄•7H₂O</i>	<i>27.8</i>
<i>Na₂EDTA•2H₂O</i>	<i>37.3</i>
<i>myo-inositol</i>	<i>100.0</i>
<i>nicotinic acid</i>	<i>0.5</i>
<i>thiamine•HCl</i>	<i>0.1</i>
<i>pyridoxine•HCl</i>	<i>0.5</i>
<i>glycine</i>	<i>2.0</i>
<i>sucrose</i>	<i>30000</i>
<i>Phytoagar</i>	<i>6500</i>

Table 3.1 Murashige-Skoog medium composition

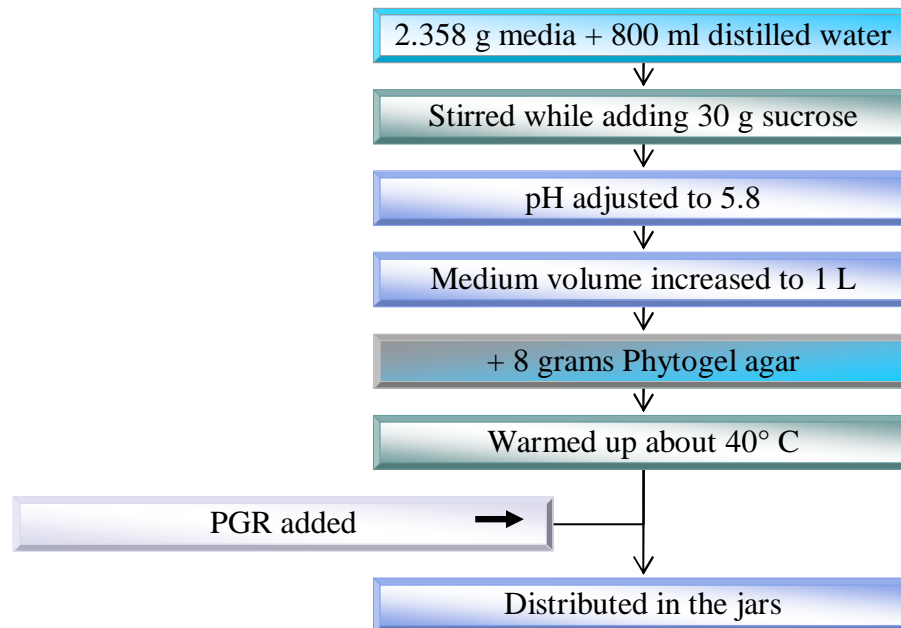


Diagram 3.10 Preparation of the media showing the time when PGRs are added

3.5.1.4. Preparing Growth hormone stock

In order to perform the micropropagation test, two different hormones were engaged in the experiment; IBA (Indole-3-butyric acid) from Auxins and BAP (6-Benzylaminopurine) from Cytokinins.

Different trials were done to obtain the best concentration (diagram 3.12).

three different concentration based on the previous reports (Miachir *et al.* 2004; Loc *et al.* 2005; Keng and Stanley, 2007) were tested for the IBA as 0.5 mg/l, 1mg/l, 1.5 and g/ml and 3 mg/l combined with three different concentration for the BAP as 2.5 mg/l, 3 mg/l, 4 mg/l .

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.



The plan was to make the media with 2 different categories as follows:

Category 1:

MS +

- 3 mg/l **BAP**
- 0.5 mg/l **IBA** + 3mg/l **BAP**
- 1.5 mg/l **IBA**

Category 2:

MS +

- 3 mg/l **BAP** + 1 g/l **AC**
- 0.5 mg/l **IBA** + 3mg/l **BAP** + 1 g/l **AC**
- 1.5 mg/l **IBA** + 1 g/l **AC**

At first, the stock solutions were made for each category.

The routine concentration for PGRs, 1 mg/ml (1000 mg/L), was used to make it in an easy and fast calculating way of using the formula $M_1V_1 = M_2V_2$ (M as concentration and V as volume), e.g., 3 mg/L BAP can easily be made up by dispensing 3 ml of a 1 mg/ml BAP stock.

As plant grows, hormones hardly adopt the aqueous solution and they need to be dissolved in something as solvent. As most PGRs are somehow acidic or basic (cytokinins are weak bases and auxins are weak acids) molar equivalents of the opposing compounds were used to make the PGRs completely dissolve.

1M NaOH for IBA and 1M HCL for BAP was prepared as the solvent.

For making the 1M HCL, 36.5ml of HCl was added in one liter of distilled water and for 1M NaOH, forty grams of NaOH were dissolved in one liter of distilled water.

Solution were stirred until all granules went into solution, it took about an hour for this process (Diagram 3.11)

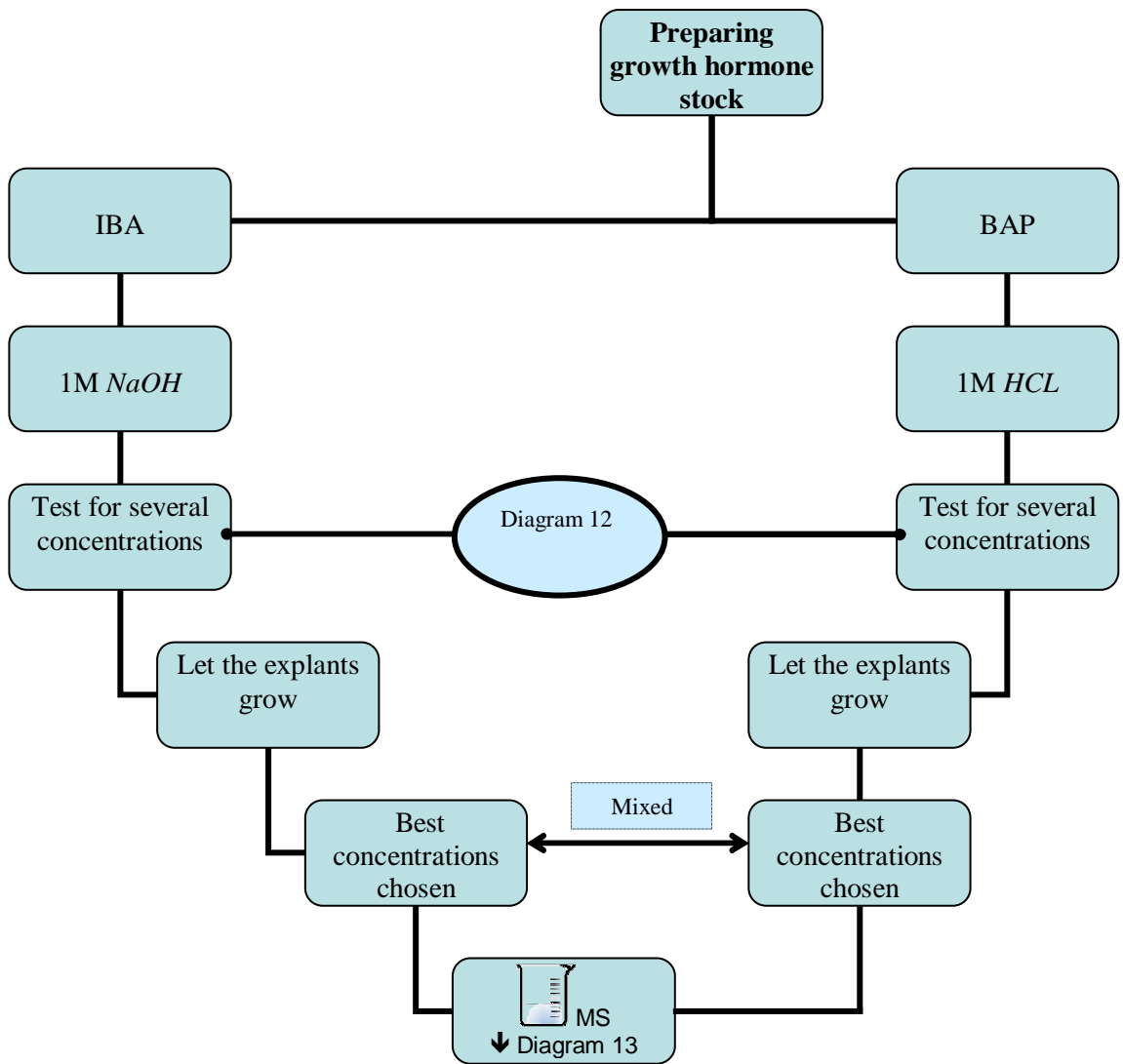


Diagram 3.11 Preparing growth hormone stock

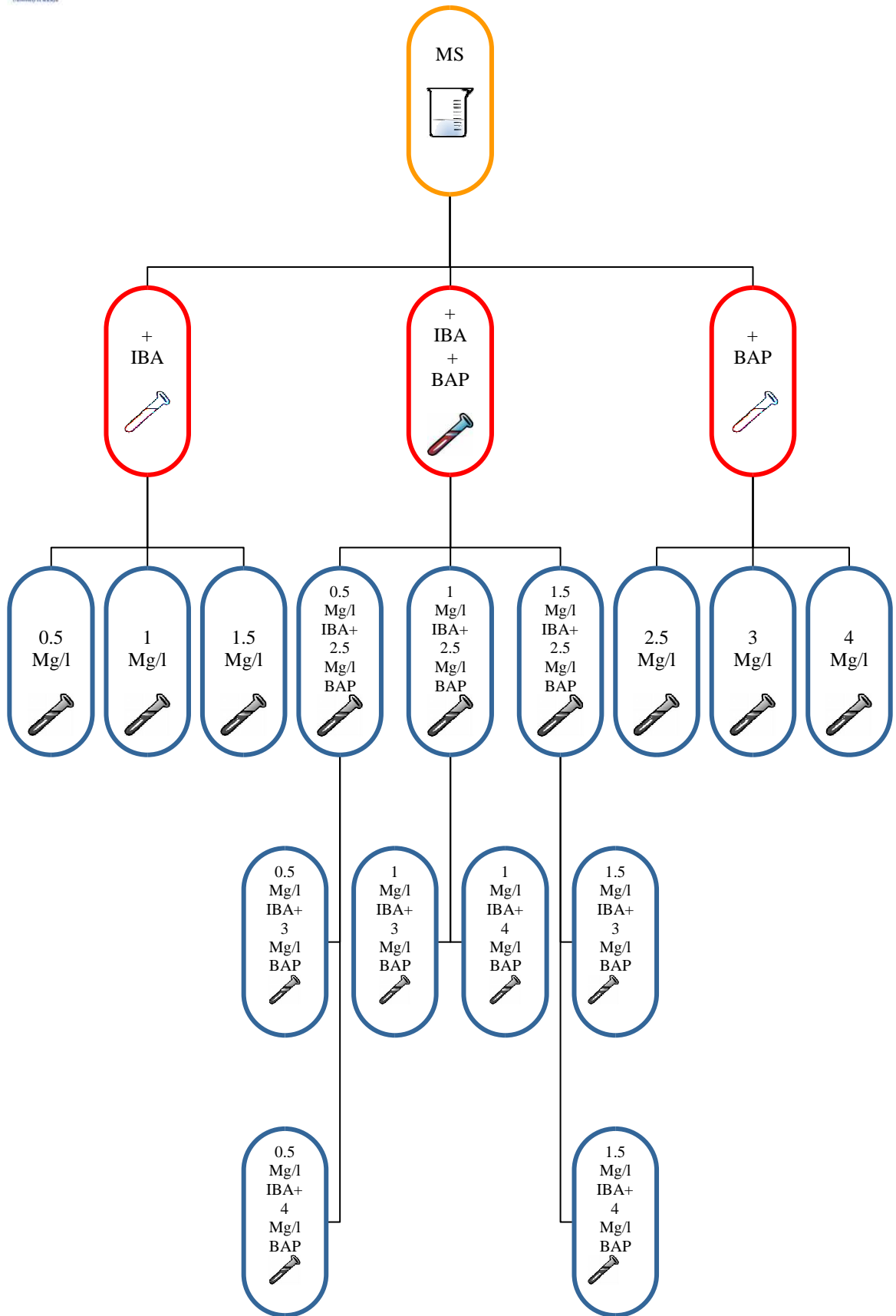


Diagram 3.12 Testing different PGR concentration mixed or single in order to obtain the optimum combinations

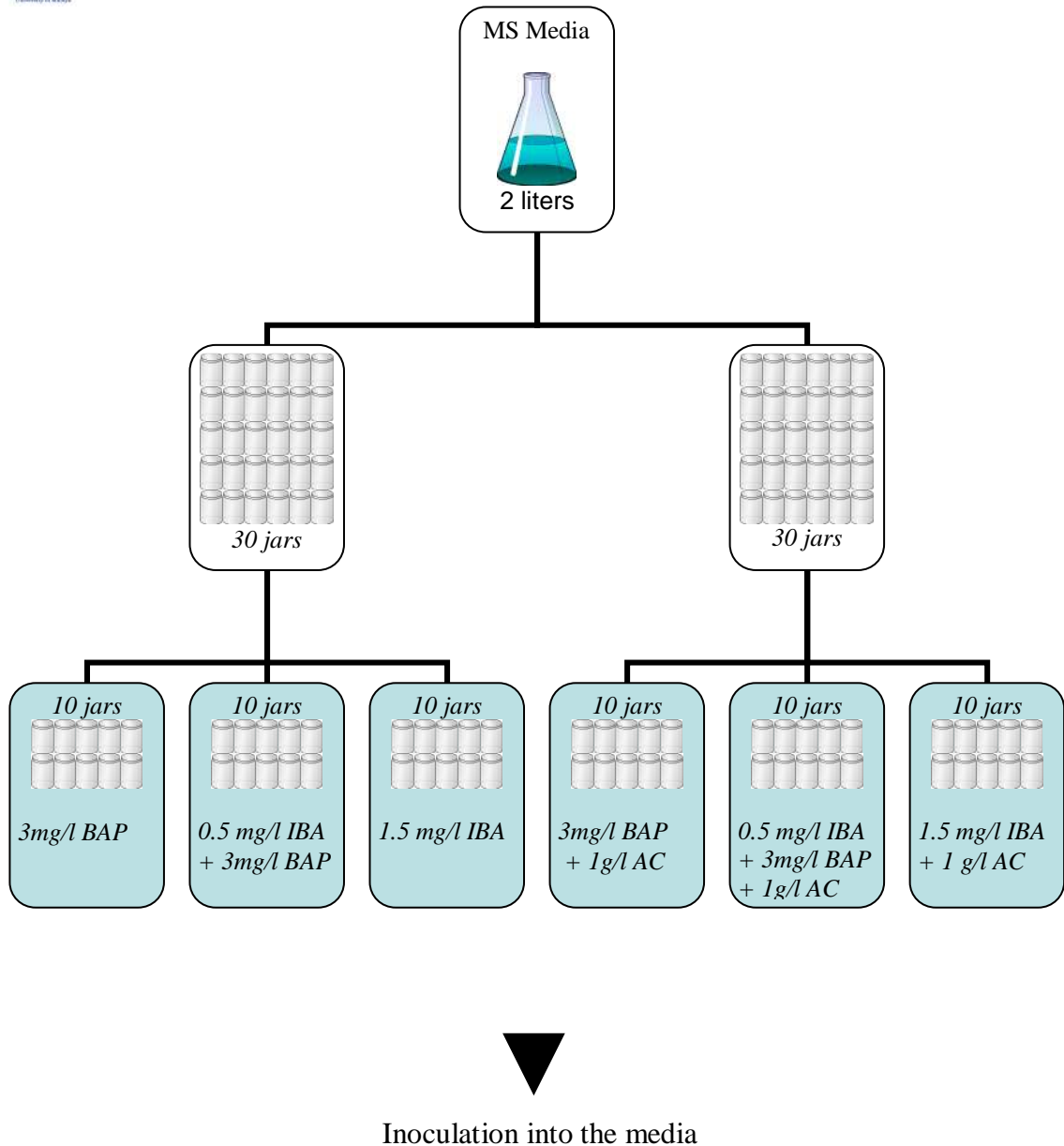


Diagram 3.13 Media distribution after preparing the PGR stocks and chooses the best combination

Jars were closed loosely and autoclaved for fifteen minutes at 121° C.

After the autoclaving, they were immediately moved inside the laminar flow cabinet which was previously sterilized using UV light and ethanol spray.



3.5.1.5. Inoculation of explants into media

After surface sterilization (Diagram 3.9), the explants were prepared under laminar air-flow cabinet. Using rhizome as explants, small segments (1.0 cm) of innermost tissue are excised with a sterilized scalpel.

Prepared explants are carefully inoculated in a baby glass jar culture containing 30 ml sterilized agar gelled of MS (Murashige and Skoog, 1962) medium with different combination of hormones (Diagram 3.11-3.13).

The jars were then closed and sealed with parafilm and kept at 25° C at growth room with the sufficient light.

After three months, the grown plants were taken out from the sub-cultured jars in order to make the extracts for the antibacterial activity test.

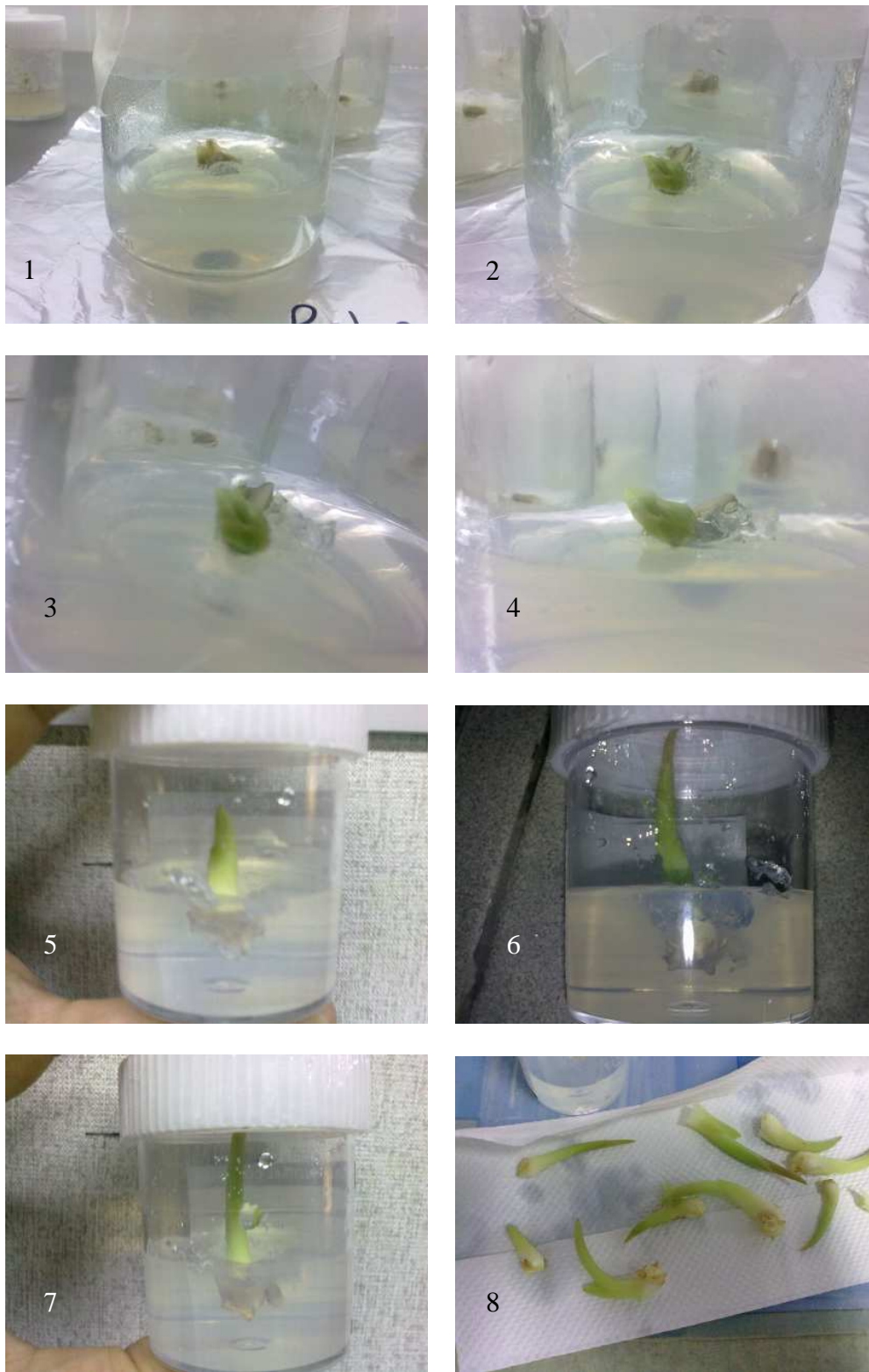
During these three months, every two weeks, Jars were substitute with new ones contain exactly same formula for the media and PGRs concentrations.

3.5.2. Preparing extracts

The procedure for preparing the extracts and using the solvents were carried out exactly in the same method as in the *in vivo* system (refer to *in vivo* system, diagram 3.4 and diagram 3.5)

3.5.3. Antimicrobial activity assay

The procedure for preparing the bacteria , disc diffusion test , and applying the antimicrobial activity test were carried out with exactly same methods as *in vivo* system (refer to *in vivo* system and diagram 3.5-3.7)



Picture 3.17.1-8 Growth progress of *in vitro* explants until desired length and level of growth

3.6. Comparison

After establishing the *in vitro* and *in vivo* system and recording the results, a comparison was made based on the records and data that were collected.

Comparison was between the diameter of the inhibition zone in the bacteria culture plates obtained from the *in vivo* plants and those were propagated in the *in vitro* system.

Diagrams 3.15 and 3.16 show the plan for the comparison.

Results and discussion will be explained in the next chapter, (chapter 4).

Diagram 3.14 shows the progress until the comparison stage.

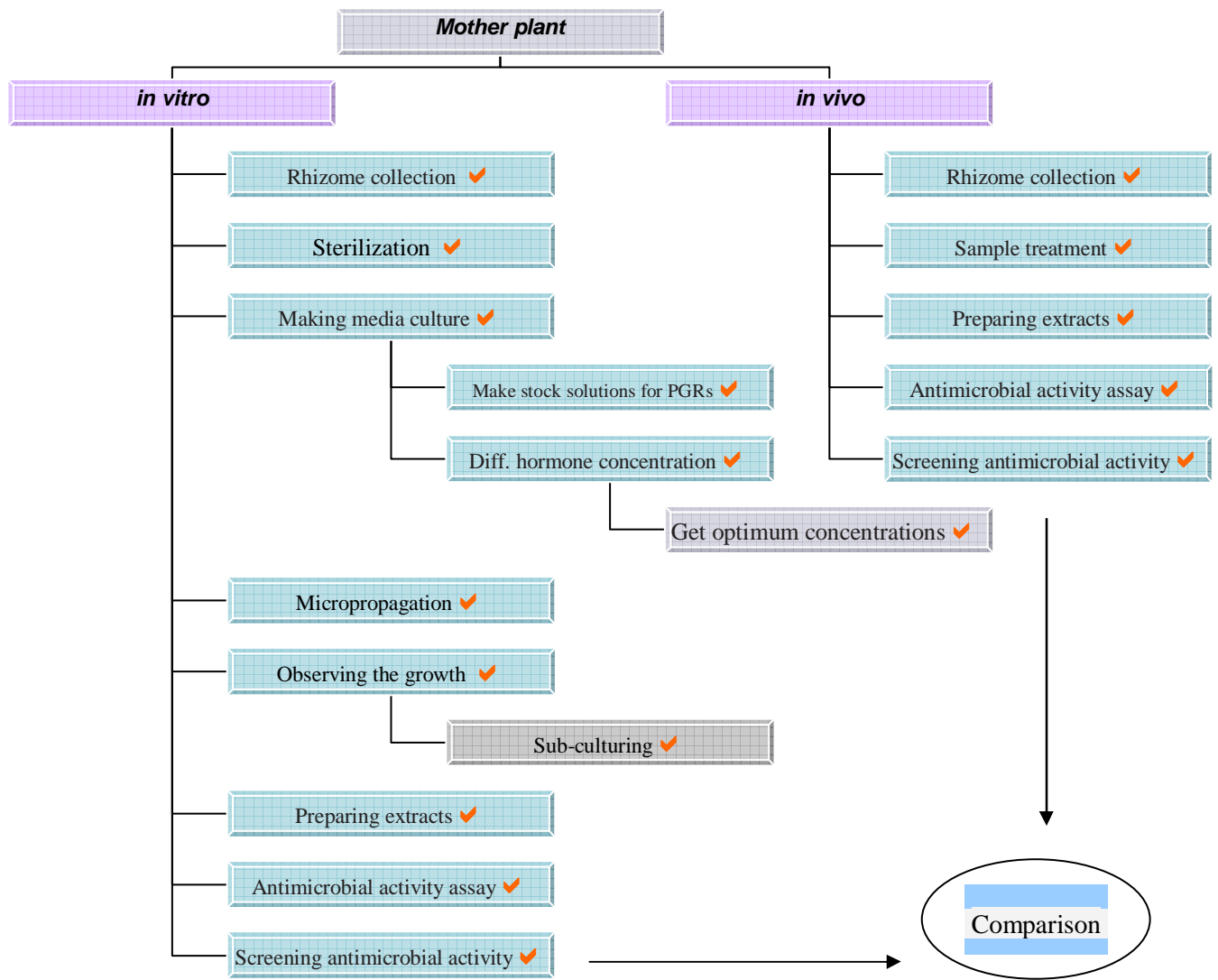


Diagram 3.14 Progress of the work until the comparison stage.



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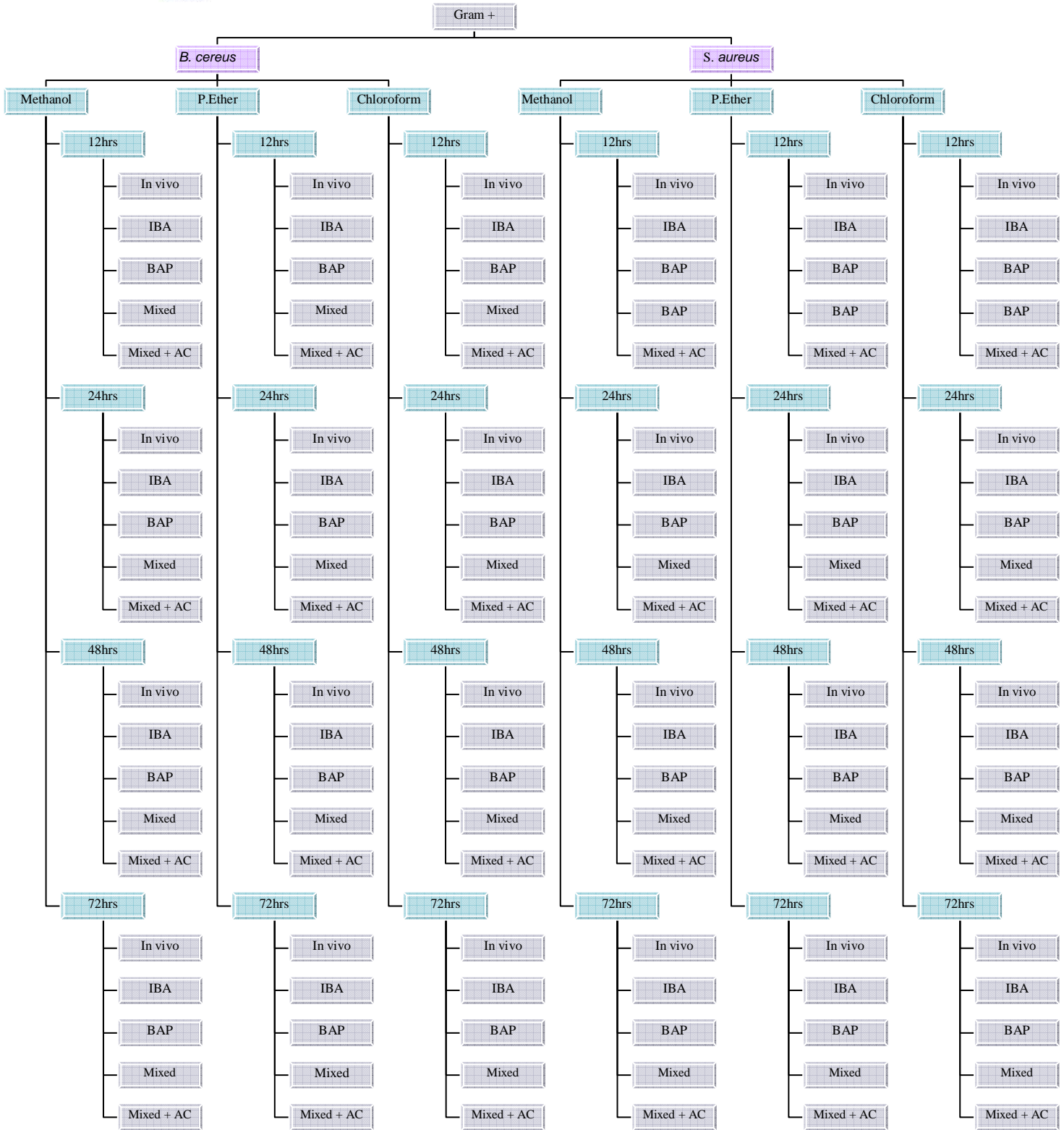


Diagram 3.15 Plan for comparing the results for gram positive bacteria, *Bacillus cereus* and *Staphylococcus aureus* from *in vitro* and *in vivo* system base on measuring the inhibition zones on the bacteria culture plates



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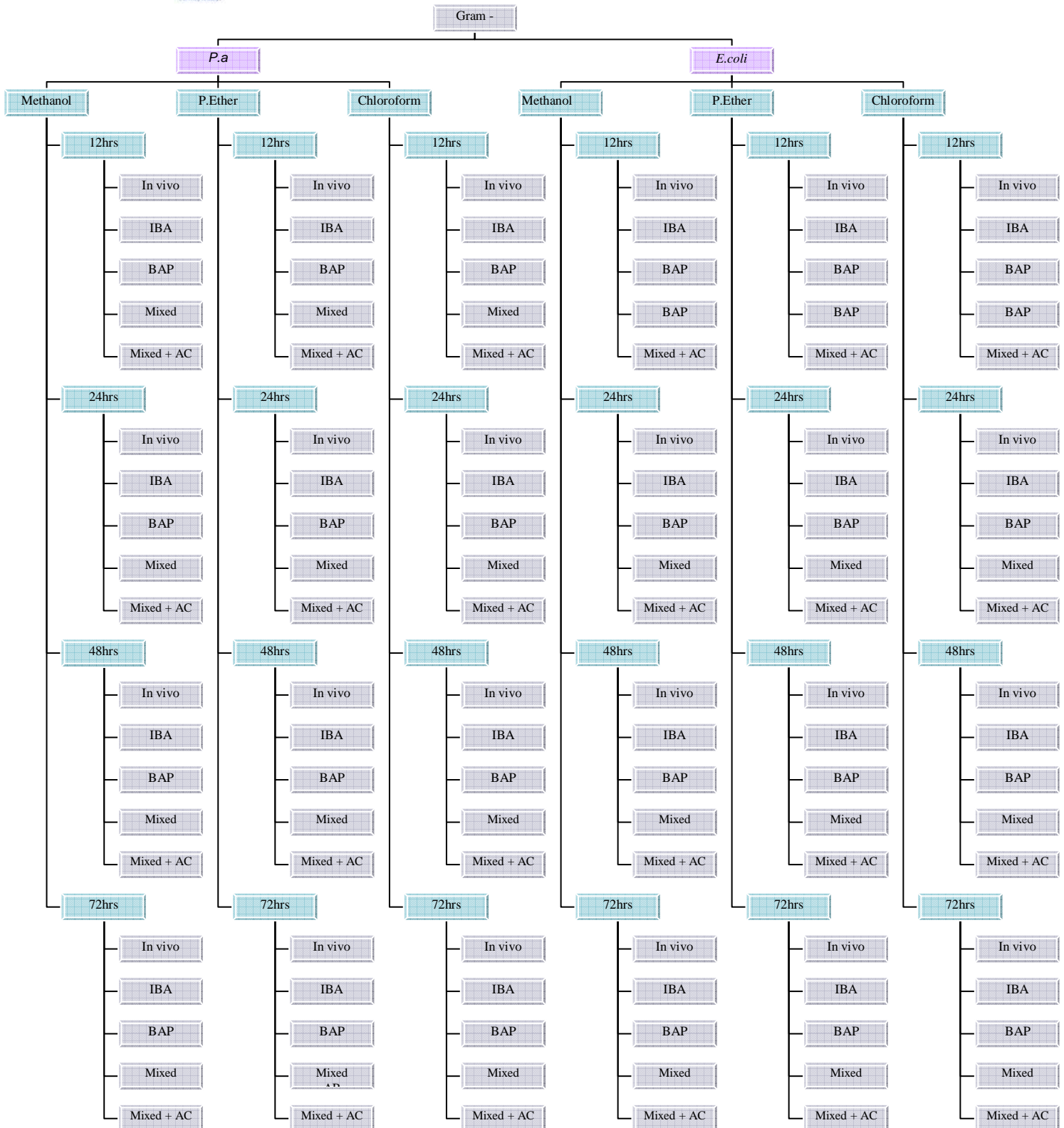


Diagram 3.16 Plan for comparing the results from gram negative bacteria, *Pseudomonas aeruginosa* and *E.coli* from *in vitro* and *in vivo* system base on measuring the inhibition zones on the bacteria culture plates.