PHG 322

Lab#

3

Practical Application of Some

Biological Assay

Used in Drug Discovery From

Natural Products

PHG 322 Practical course

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Background:

Microbiogical screening techniques.

Objectives

- ✓ Evaluate the effects of **Antibacterial** agents in natural products.
- ✓ Evaluate the effects of **Anticancer** agents in natural products.
- ✓ Demonstrate Yeast-Based Bioassay for DNA-Damaging Agents.
- ✓ Evaluate the effects of **Antioxidant** agents in natural products.
- ✓ Understand How antioxidants work.

Antimicrobial Screening of Natural Products Using Agar Dilution Method

The currently available screening methods for the detection of antimicrobial activity of natural products fall into three groups, including bioautographic, diffusion, and dilution methods. The bioautographic and diffusion methods are known as qualitative techniques since these methods will only give an idea of the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered quantitative assays once they determine the minimal inhibitory concentration.

Procedures:

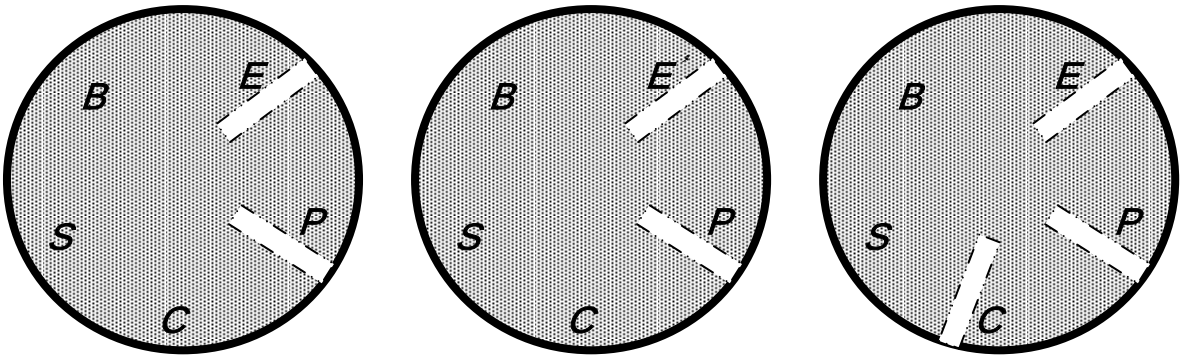
1- The desired concentration of natural products is dissolved in very small amount of DMSO.

2- The solution is added to 10 ml of the melted agar medium and mixed will.

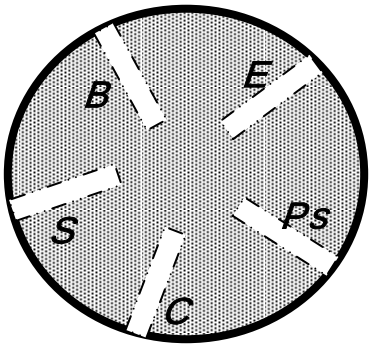
3- The agar is poured into the Petri dish often desired dimensions and cooled.

4- The desired test organism are then applied a lines from the edges to the center of the plate.

5- The plate is then incubated and growth of organisms is recorded..



Compound A **Compound B** **Compound C**



Control

Results:

1- In the above figure five organisms were used to screen natural products A, B and C at 1mg/10 ml agar.

2- The used organisms are:

E: *Escherichia coli*

S: *Staphylococcus aurous*

B: *Bacillus subtillus*

Ps: *Pseudomonas aurogenosa*

C: *Candida albicans*

3- The results revealed that:

a. Natural products A and B are active against:

B: *Bacillus. subtillus*,

S: *Staphylococcus aurous* and

C: *Candida albican*s

b. Natural products C is active against:

B: *Bacillus. subtillus*,

S: *Staphylococcus aurous*

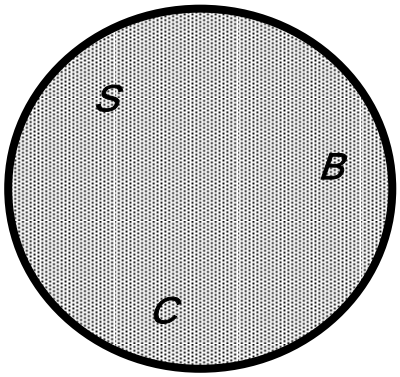
c. The fourth plate is the control showing growth of all organisms

Determination of the MIC for Natural Product [A] using Agar Dilution Methods

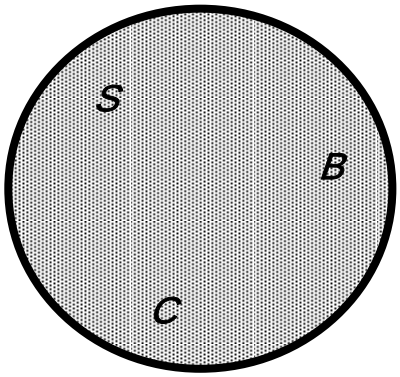
MIC = (Minimum Inhibitory Concentration)

Procedure:

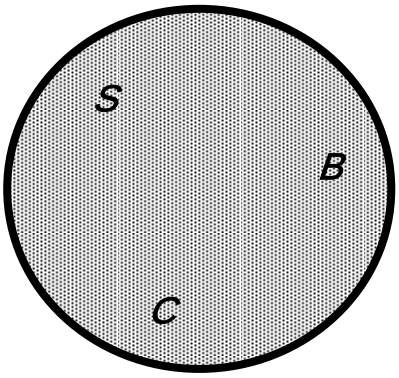
1- The same procedures are applied using different concentrations of the tested natural products A.
2- In this experiment 10, 5, 2.5, 12.5, 0.625 and 0. 3125 µg/ m1 agar.



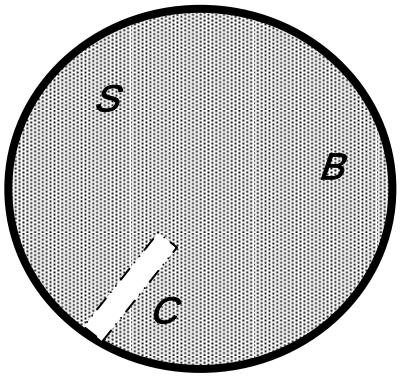
Conc. =10 µg/ ml



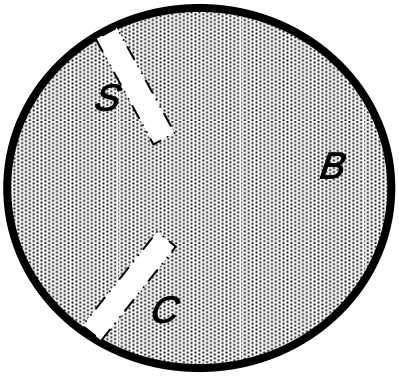
Conc. =5 µg/ ml



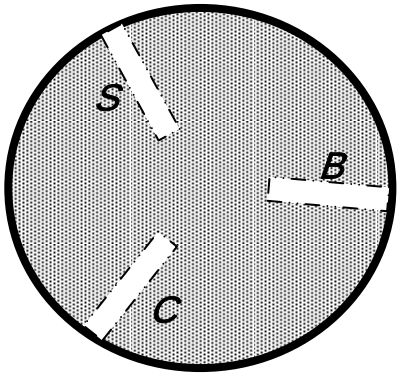
Conc. =2.5 µg/ ml



Conc. =1.25 µg/ ml



Conc. =0.625 µg/ ml



Conc. =0.3125µg/ ml

Results:

MIC of A is:

2.5 µg//ml against **C**: *Candida albicans.*

1.25 µg//ml against **S**: *Staphylococcus aurous.*

0.625 µg//ml against **B**: *Bacillus subtillus.*

Anticancer Screening of Natural Products Using Genetically Engineered Yeast Assay

There are currently four structural classes of plant derived anticancer agents on the market in the U.S.. These are:

1. The vinca alkaloids (vinblastine, vincristine).
2. The epipodophyllotoxins (etoposide and teniposide).
3. The taxanes (paclitaxel and docetaxel).
4. The camptothecin derivatives (topotecan and irinotecan).

Vinblastine and vincristine were isolated from ***Catharanthus roseus*** and are used for the treatment of a wide variety of cancers, including leukemia, bladder cancer and testicular cancer.

Assays suitable for fractionation must be in vitro assays since animal-based in vivo assays are expensive and time consuming. In general in vitro assays can be divided into two groups:

i. cellular and molecular (mechanism-based) assays. While cellular assays use intact cells, molecular assays determine activity using isolated systems such as enzymes, receptors, DNA, etc. The cellular assays can be divided into cytotoxicity assays and other assay types such as morphological assays.

ii. Mechanism-based assays detect effective anticancer agents that are involved in processes of **neoplastic growth** (e.g. inhibitors of protein kinase C and protein tyrosine kinase), or **bind to target receptors** (e.g., camptothecin and topoisomerase I, etoposide and topoisomerase II, etc.). These assays aid in the procurement of unique natural compounds. However, the determination of the exact efficacy of promising anticancer agents needs subsequent evaluation in more advanced testing systems, followed by (pre) clinical tests.

Yeast-Based Bioassay for DNA-Damaging Agents

Many anticancer drugs act by causing DNA damage either directly (such as the nitrogen mustards) or indirectly (such as etoposide and topotecan). The use of an assay that identifies new DNA-damaging agents is thus likely to yield new anticancer drugs.

All eukaryotic cells have DNA repair mechanisms, which are essential for cell survival in the event of DNA damage. One of the important repair mechanisms is known as the *RAD52* pathway, which repairs double-stranded breaks of DNA. Yeast strains that lack the *RAD52* gene are available; a yeast lacking this gene will be sensitive to DNA-damaging agents, since DNA that is damaged in a way that would normally be repaired by the *RAD52* pathway will not be repaired.
One selective bioassay, which is employed in the Kingston research group, thus uses a mutant yeast strain (***Saccharomycese cerivisiae***) that lacks the gene for the *RAD52* DNA recombinatorial repair pathway. This yeast is sensitive to drugs that target the enzymes **topoisomerase I** and **topoisomerase II**, as well as to DNA-damaging agents, since inhibition of either of the topoisomerases also leads to DNA damage.

EXPEREMENT

Types of yeasts:

We have 3 types of Yeasts:

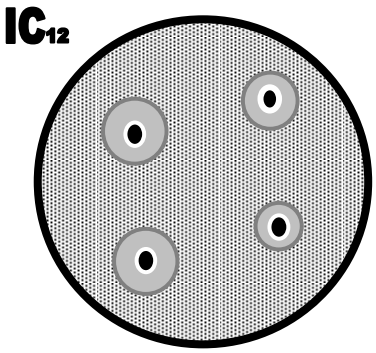
1. Wild type compounds affected this yeast are antifungal compounds.
2. This type is deficient in Topoisomerase I enzyme but has Topoisomerase II.
3. This type is deficient in Topoisomerase II enzyme but has Topoisomerase I..

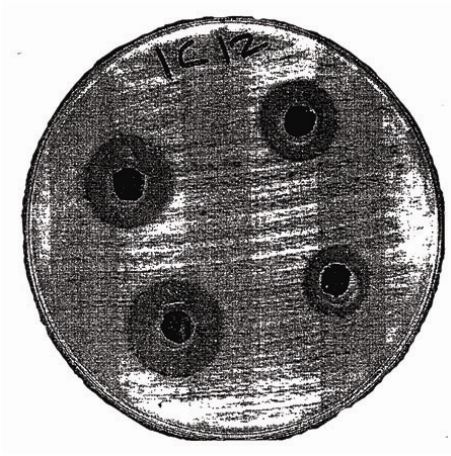
Expression of results:

The activity is measured using the IC_{12} value. IC_{12} value is Concentration of material resulted in 12 mm inhibition zone.

Procedures:

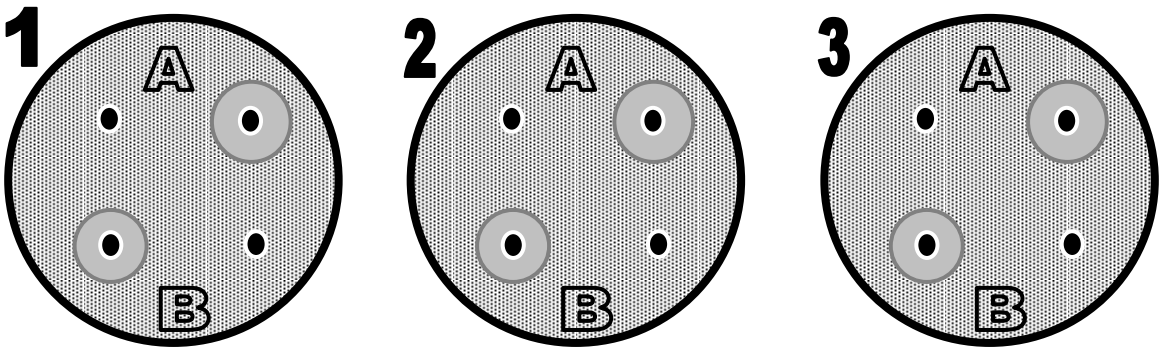
1- Wells of 100 µl capacity are done in the agar plates.
2- Different concentrations of the test materials are introduced in the wells.
3- After incubation for 48 hours the inhibition zones are measured by ruler.
4- The values are used to calculate the IC_{12} value by the aid of computer program.



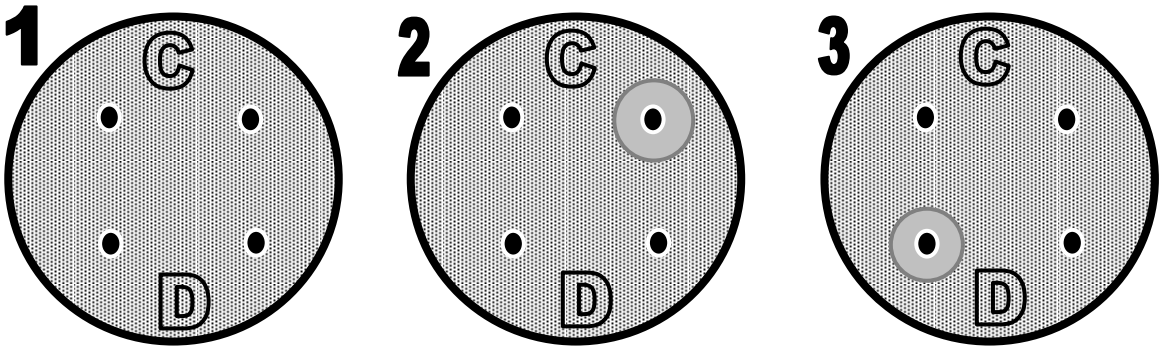


Data Interpretation:

The possible results are shown in the figures below.



- Products A and B in the above figure are active against the three strains. That means they are both antifungal agents and anticancer.

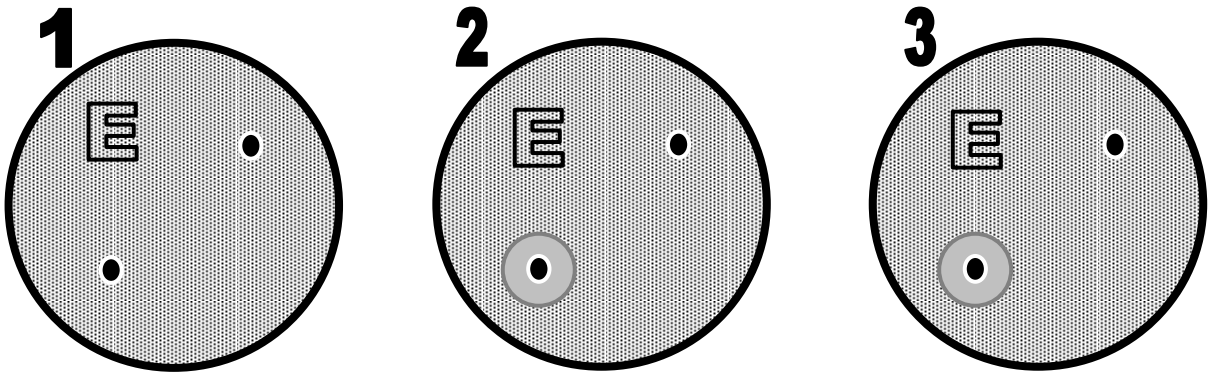


- Product C active against strain 2 only. It is deficient in Topoisomerase I.

Any products active against strain 2 only are described as Topoisomerase II inhibitors.

- Product D active against strain 3 only. It is deficient in Topoisomerase II.

Any products active against strain 3 only are described as Topoisomerase I inhibitors Inhibitors.



- Product E is active against strains 2 and 3 but inactive against 1 so it is DNA damaging agent with no specific mechanism.

Antioxidant Screening of Natural Products Using TLC

Oxidation of a wide range of chemical compounds and the production of radicals at the cellular level are believed to be involved in the cause of many cancers and may also be important factors causing cardiovascular disease. This is why there is so much interest in antioxidants.

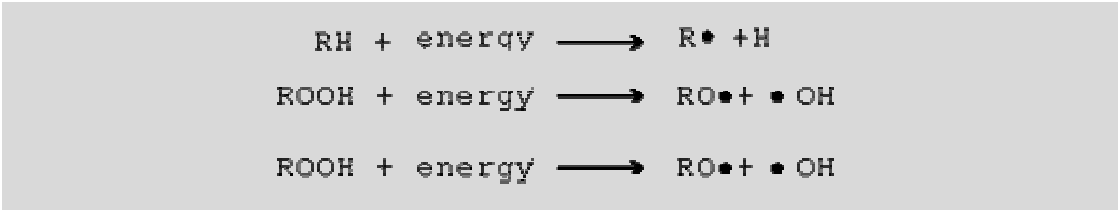
Plant derived natural products such as flavonoids, terpenoids, and steroids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antitumor activity. Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative. Antioxidants are compounds that help to inhibit many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxy nitrate there by preventing or delaying damage to the cells and tissues. There is some synthetic antioxidant compounds, such as butylated hydroxyl toluene (BHT) and butylated hydroxyanisole (BHA), commonly used in processed foods. However, it has been suggested that these compounds have side effects

Medicinal plants possessing natural antioxidant polyphenolics such as anthroquinones, flavonoids, aromatic acids and tannins have been shown to have reactive oxygen species (ROS) scavenging and lipid peroxidation preventing effects. In addition, it has been reported that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human diseases.

How Antioxidants Work

The key role played by antioxidants in the body is their ability to react with radicals. When this happens the destructive properties of the radical is eliminated. A free radical is a chemical compound that contains one or more unpaired electrons. Radicals can be produced by exposure to energy such as radiation or may be the product of

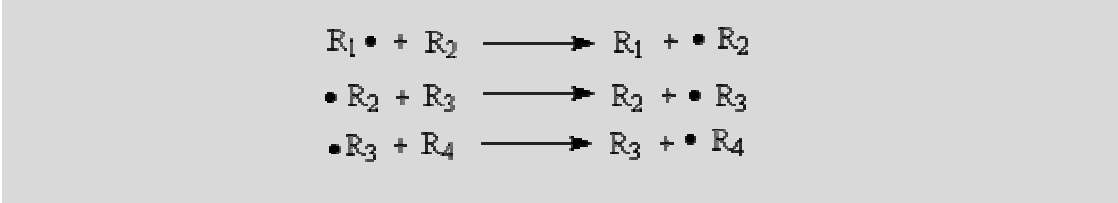
incomplete reactions in the cells that produce electrons that have escaped. The step that produces a radical is called the initiation step. Radicals are usually represented in chemical formula by a single dot. Below are three examples of initiation steps that illustrate the production of radicals.



In nature electrons are usually paired. In radicals they are not, and so radicals generally are more reactive than non-radicals. Because they are reactive, radicals search out ways of pairing up their odd electron. In their haste to pair up their electron, radicals often attack nearby chemical compounds. These chemical compounds may be involved in important enzyme reactions, may be components of cell walls or may be part of a DNA molecule. If their chemical structure is changed, their function in the body may be lost and the result can be disease or infection. This process is usually long term, but more and more evidence is pointing to the benefits of reducing oxidative damage in body tissue.

A radical can donate its odd electron to another molecule, it can rob an electron from another nearby molecule or it can combine with another radical. When two radicals combine this is called a termination step.

If the initial radical donates or steals an electron, a second radical is produced which can then in turn react. This can continue in a series of propagation steps, until termination occurs.



Cell membranes are particularly sensitive to radical reactions, since cell membranes are composed of fatty acids which can easily form peroxyl radicals. Fortunately alpha-tocopherol is also located in cell

membranes. Electron transfer from the peroxyl radical to alpha-tocopherol restores the fatty acid, thus retaining the cell membrane structure and function. The tocopherol radical that is formed is more stable because of its chemical structure. Tocopherol is less reactive than other radicals and so it doesn't immediately try to rob an electron from nearby molecules . It has time to move to the surface of the cell membrane where it can pick up an electron. It becomes alpha-tocopherol again and can return to react with other membrane radicals.

It is the ability of alpha-tocopherol to pick up and transport electrons that makes it an anitoxidant. All antioxidant agents work in this way to protect the cells in the body from the damage of radicals.

EXPEREMENT

Conditions:

- Plant extract rich in antioxidant are:
  - Orange or lemon peel (dry and coarsely powder).
  - Green Tea leaves (coarsely powder).
  - Buchu leaves.
  - Liquorices.
  - Gall.

- Standard antioxidant (References):
  Gallic acid, Ellagic acid, Arbutin, Hydroquinone & Rutin (at concentration = 5-10 mg/5 ml MeOH).

- Solvent System:

  - For orange peel, Lemon peel, green tea leaves and Buchu leaves is (for flavonoids) :

Ethylacetate : Formic acid : H_2O (8 : 1 : 1)

Or $CHCl_3$: Ethylacetate (6 : 4)

- For Tanins is :

  n-butanol : glacial acetic acid : H_2O (4 : 1 : 5)

▣ Detection :

1. ◇ DPPH solution (20 mg/100 ml in MeOH), must be freshly prepared. **(DPPH= 1,1 Diphenyl-2-picrylhydrazyl)**

   ◇ Spray reagent for tannins is: (5% alcoholic $FeCl_3$)

2. Heat in Oven at 60^oC or hot plate at 60^oC.

Procedure:

1. Spotting of the plate.
2. Develop the plate.
3. Dry plates at open air for 15-20 seconds.
4. Dip the plate in DPPH which in Petri dish.
5. Dry plate in dark for 90 seconds at room temperature then for 30 seconds at 60^oC.
6. Free radical scavenging zone were identified immediately as yellow area against violet purple background plate.