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In-Vitro Cytotoxic Activity of Methanol Extract of the *Holarrhena antidysentrica*.

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The present study was designed to investigate cytotoxic activity of methanol extract of the *Holarrhena antidysentrica*. The extract is subjected to brine shrimp lethality bioassay for possible cytotoxicity where a concentration dependent increment in percent mortality of brine shrimp nauplii was produced by the extract indicating the presence of cytotoxic principle in this extract. The cytotoxicity of the extract was found promising. The results of these studies suggest significant cytotoxicity of different extract of *Holarrhena antidysentrica*. The result that obtained from the Crude extract of the of *Holarrhena antidysentrica* have strong cytotoxic activity.

Keyword: *Holarrhena antidysentrica*, Cytotoxicity, Methanol Extract.

1. Introduction

1.1 Plant as a Source of Medicine:

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as “Medicinal Plants”. Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally

important. It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties.

Putting aside the excitement surrounding the human genome, in the near future we may well start to see the emergence of a new class of prescription medicine containing complex mixtures of plant extracts. As

technology advances, the pharmaceutical industry is increasingly focusing on the human genome as a source of the many unanswered questions relating to how disease is prevented, diagnose and treated. It is easy to forget one of the other life forms that has contributed much to our current understanding of medicine and many effective therapies. Plants are an abundant natural source of potential new medicines and as a pharmaceutical company seeks to achieve an optimally balanced R&D portfolio this potential should be kept in mind as a target for new or additional funding. Prior to the advances in synthetic chemistry and the discovery of antimicrobials in the late 19th early 20th centuries, plants provided the major source of medicines. Evidence of their use as long as 50,000 years ago comes from the Middle Eastern grave site of a Neanderthal man containing plant specimens, seven of which are still in use medicinally today by the local population.

1.2 Description of the plant:

The plant is indigenous to India and found all over the country in deciduous forests up to 900 meters. The small tree, 9-12 meters in height and the bark pale, grayish in color. The leaves, 9-18 cm long and 4-8 cm broad, broadly ovate to elliptic. Flowers in terminal corymbs cymes, white, slightly fragrant. The fruit pod, 20 to 40 cm long, in pairs, cylindrical and narrow. The seeds are linear-oblong, 1 cm long. 25-30 seeds per pod, smoky in color.

1.3 Using Information:

The seeds and the skin of the bark is used for medicinal purpose. Kutaja plant is used both internally as well as externally. Externally, to promote healing, the wounds are cleansed with the decoction of its skin. The paste of the skin is also applied on the boils. The oozing wounds are dressed with

its seed powder. The fomentation with the decoction of the skins of kutaja, nimba, sirisa, eranda and arka is given with great benefit in swellings.

1.4 Cytotoxicity:

Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are a chemical substance, an immune cell or some types of venom (e.g. from the puff adder or brown recluse spider).

1.5 Measuring Cytotoxicity:

Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, if they are interested in developing a therapeutic that targets rapidly dividing cancer cells, for instance; or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical. Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as trypan blue or propidium iodide are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components. Alternatively, membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside. One molecule, lactate dehydrogenase (LDH), is commonly measured using LDH assay. Protease biomarkers have been identified that allow researchers to measure relative numbers of live and dead cells within the same cell population. The live-cell protease is only active in cells that have a healthy cell membrane, and loses activity once the cell is

compromised and the protease is exposed to the external environment. The dead-cell protease cannot cross the cell membrane, and can only be measured in culture media after cells have lost their membrane integrity. Cytotoxicity can also be monitored using the 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. In addition to using dyes to indicate the redox potential of cells in order to monitor their viability, researchers have developed assays that use ATP content as a marker of viability. Such ATP-based assays include bioluminescent assays in which ATP is the limiting reagent for the luciferase reaction. Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay and clonogenic assay. A label-free approach to follow the cytotoxic response of adherent animal cells in real-time is based on electric impedance measurements when the cells are grown on gold-film electrodes. This technology is referred to as electric cell-substrate impedance sensing (ECIS). Label-free real-time techniques provide the kinetics of the cytotoxic response rather than just a snapshot like many colorimetric endpoint assays.

2. Methods and Materials

2.1. Cytotoxic screening of the isolated compounds:

2.1.1. Introduction:

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of extracts of *Agave cantula*. Brine shrimp lethality bioassay is easily mastered, costs little and it utilizes small amount of test material. This provides a front line screen that can be

backed up by more specific and expensive bioassays, once the active compound has been isolated. It is evident that brine shrimp lethality bioassay is predictive of cytotoxicity and pesticidal activity. This *in vivo* lethality test has been successfully used as a preliminary study of cytotoxic and antitumour agents.

2.1.2. Test Materials Used for the Study:

Artemia is a genus of aquatic crustaceans known as **brine shrimp**. *Artemia*, the only genus in the family **Artemiidae**, has changed little externally since the Triassic period. The historical record of the existence of *Artemia* dates back to 982 from Urmia Lake, Iran, although the first unambiguous record is the report and drawings made by Schlösser in 1756 of animals from Lymington, England. *Artemia* populations are found worldwide in inland saltwater lakes, but not in oceans. *Artemia* are able to avoid cohabiting with most types of predators, such as fish, by their ability to live in waters of very high salinity (up to 25%).

The ability of the *Artemia* to produce dormant eggs, known as cysts, has led to extensive use of *Artemia* in aquaculture. The cysts may be stored for long periods and hatched on demand to provide a convenient form of live feed for larval fish and crustaceans. Nauplii of the brine shrimp *Artemia* constitute the most widely used food item, and over 2000 tonnes of dry *Artemia* cysts are marketed worldwide annually. In addition, the resilience of *Artemia* makes them ideal animals for running biological toxicity assays and it is now one of the standard organisms for testing the toxicity of chemicals.

2.1.3. Principle:

The modified method of McLaughlin *et al.*, (1998) was employed in this study. Natural sea water from Bar Beach, Lagos

was poured into an improvised hatching chamber made of plastic dish, brine shrimp eggs were added at the closed section of the chamber. The open air section of the chamber was then exposed to fluorescent light for 48 hrs, Sample bottles of the same size used were washed and sterilized before use, Different concentrations (10000, 1000, 100, 10 $\mu\text{g/ml}$) of the extracts of *H.antidysentrica* were prepared using the extracting solvents. The tests were continued out in triplicates, After 48 h's a drop and 4 ml of sea water were added to each of the sample bottles containing the extract Ten brine shrip were carefully counted into each of the sample bottles with the aid of dropping pipette and the volum sea water was made up to 5 ml. A control experiment, ponraining 5 ml of sea water, a drop of DMSO and ten brine shrimp larvae was set up. The experiment was maintained at room temperature for 24 hour under light after which number of surviving larvae were counted and recorded. The data obtained were subjected to Finney's Probitanalysis to determine the LC_{50} of each extract The toxicity expressed by this LC_{50} which is defined as concentration of the oil that kills 50% of the larvae within 24 hour. Percentage mortality was also calculated as number of dead larvae divided by initial number of larvae (10) multiplied by 100.

2.1.4. Test Material:

1. *Artemiasalina* leach(Brine shrimp eggs)
2. Sea salt non ionized(NaCl)
3. Small tank with perforated deviding dam to hatch the shrimp
4. Lamp to attract the nauplii
5. Pipette
6. MicropipetteGlass vials
7. Magnifying glass
8. Test sample from experimental plants

2.2. Procedure:

2.2.1. Preparation of the Simulated sea Water:

38 gram sea salt was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The pH of the sea water was maintained between 8.5 using 1N NaOH solution.

2.2.2. Hatching of Brine Shrimp Eggs:

Artemia Salina Leach (Brine shrimp eggs) collected from the pet shop used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 gm/l) were added to one side of the tank and this side was covered.The shrimp was allowed to one side of tank and this side was covered. The shrimp was allowed for two days to hatch and mature as nauplii. Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These nauplii were taken for this bioassay.

2.2.3. Preparation of Sample Solution:

Clean test tubes were taken. These test tubes were used for different concentration (one test tube for each concentration) of test samples.

10 mg methanol extract were accurately weighted and dissolved in 1000 or 1 ml DMSO in different beaker. Thus a concentration of 10/ml was obtained used as a stock solution.

From this stock solution 10 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$, 300 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ were taken in five test tubes respectively each containing 5 ml sea water and 10 nauplii

2.2.4. Preparation of negative control:

100 μg of distilled water was added to each of three remarked glass vials containing 5

ml of simulated sea water and 10 shrimp nauplii to use control groups.

If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test is considered as in valid as the nauplii died due to some reason other than the cytotoxicity of the samples.

2.2.5. Application of brine shrimp nauplii:

With the help of the Pasteur pipette 10 living nauplii were added to each of the vials containing 5 ml simulated sea water. A magnifying glass was used for convenient count of nauplii. If the counting of the 10 nauplii was not be possible accurately, then a variation in counting from 9-12 might be allowed.

2.2.6. Counting of the Nauplii:

After 24 hours, the vials are observed using a magnifying glass and the number of

survival nauplii in each vial were counted and recorded. From this data, the percentage of mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

Cytotoxicity expressed as LC_{50} was calculated using the formula:

$\text{Log } LC_{50} = \text{Log concentration A} / \text{Log concentration B}$

$\text{Log concentration A} = 50\% - M/N - M$

Where concentration A is the concentration before LC_{50} ,

Concentration B is the concentration after LC_{50}

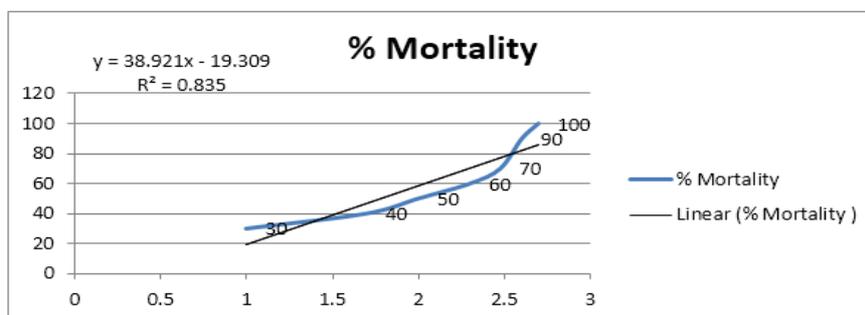
M is the % killed before LC_{50} and

N is the % killed after LC_{50} .

3. Result:

3.1. Result of cytotoxicity study:

Concentration (C) µg/ml	Log C	No. of nauplii taken	No. of nauplii death	% Mortality	LC_{50} µg/ml
10	1	10	3	30	
50	1.6989	10	4	40	
100	2	10	5	50	60.3616
200	2.3010	10	6	60	
300	2.4771	10	7	70	
400	2.6020	10	9	90	
500	2.6989	10	10	100	



Calculation for LD_{50}

We get from the curve $Y = 38.921X - 19.309$ And we know that $Y = 50$

$X = 1.7807 = \text{Log } C$

$C = 60.3616$

4. Conclusion:

The samples showed different mortality rate at different concentration. The mortality rate of brine shrimp nauplii was found to be increased with the concentration for the sample. The median lethal concentration (LC₅₀) was calculated. The LC₅₀ leaf of *H.antidysentrica* is 60.3616(µg/ml).As the extract is able to cause death of brine shrimp nauplii and has a LC₅₀ value of 60.3616(µg/ml) it has good cytotoxic activity. Since the extract is reported to contain a range of compounds, it is difficult to describe these observed activities to any specific group of compounds. Hence, further studies are suggested to be undertaken to pin point the exact compounds and to better understand the mechanism of such actions of *H.antidysentrica* scientifically.

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