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Cytotoxic (*In-Vitro*) Effect of Methanol and Petroleum Ether Extracts of the *Aerva lanata*.

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The present study was designed to investigate cytotoxic activity of methanol and petroleum ether extracts of the *Aerva lanata*. Both extracts were 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 extracts indicating the presence of cytotoxic principles in these extractives. The cytotoxicity of the extracts were found promising. The results of these studies suggest significant cytotoxicity of different extracts of *Aerva lanata*. The result that obtained from the Crude extract of the of *Aerva lanata* have strong cytotoxic activity.

Keyword: *Aerva lanata*, Cytotoxicity Activity

1. Introduction:

Medicinal plants are various plants used in herbalism and thought by some to have medicinal properties. The definition of Medicinal Plant has been formulated by WHO (World Health Organization) as follows- "A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs." 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.

1.1.1 Toxicological studies

Toxicology is the branch of pharmacology, which deals with the comprehensive study of poisonous and adverse effects of bioactive substances on living organisms along with their detection,

prevention and treatment of poisonings. All drugs are toxic at higher doses and people vary greatly in their sensitivities to the drugs. So the safe and appropriate dose for someone may be an over dose for another individual. Moreover, even the therapeutic doses, many drugs have unavoidable toxic effects (**Klaassen, C.D. 1991 and Plaa, G.L. 1988**).

In order to develop and assess the safety and efficacy level of a new drug, toxicity studies are essential. No drug is used clinically without its clinical trial as well as toxicity studies. Toxicological data helps to make decision whether a new drug is adopted for clinical use or not,

Toxicological studies are used:

- a) To determine or evaluate the efficacy, safety or toxicity of a new drug.
- b) To determine the factors those modify drug safety and efficacy.
- c) To get information about drug metabolism.
- d) To reveal the toxic effects on different organs.
- e) To predict therapeutic dose
- f) To establish dose interval time
- g) To determine lethality (LD₅₀) bioassay.

Toxicity studies are carried out on experimental animals like mice, guinea pigs, dogs and monkeys under various conditions of drug administration.

1.1.2 Types of toxicity studies

I. Acute toxicity studies

Acute toxicities are those which occur rapidly as result of exposure to a relatively large quantity of a drug, administered as a single dose. The effects are quite dramatic occurring shortly after the drug administration drug is characterized by untoward reactions having serious symptoms and short course of existence, which may follow the administration of a single dose (or an over dose) of the drug (**Satoskar, R. S. et al. 1995**).

During screening of a new drug, acute toxicity test are done to estimate the nature and extent of acute toxicity.

II. Sub- acute toxicity studies

Sub- acute toxicity studies are done by giving repeated dose of a drug in sub- lethal concentration to animal species for a period of 14 to 21 days. The aim is to predict toxic effect that may occur during chronic administration of the drug. A Variety of parameters are monitored during this period and at the end of the study, tissues and organs are examined from histopathological changes.

III. Chronic toxicity studies

Chronic toxicity studies refer to the toxic effects of a drug over a extended period of time during which animal may receive repeated doses of the drugs in an apparently safe level. Study of a chronic exposure is often used to determine the carcinogenicity and mutagenic potential of the drug

1.1.3 Toxicity:

Sum of adverse effects or the degree of danger posed by a substance to living organisms. It is expressed generally as a dose response relationship involving the quantity of substance to which the organism is exposed and the route of exposure skin (absorption), mouth (ingestion), or respiratory tract (inhalation). Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ (organotoxicity), such as the liver (hepatotoxicity). By extension, the word may be metaphorically used to describe toxic effects on larger and more complex groups, such as the family unit or society at large.

Toxicity is classified usually as

- 1) Acute toxicity : harmful effects produced through a single or short-term exposure.
- 2) Chronic toxicity : harmful effects produced through repeated or continuous exposure over an extended period.
- 3) Sub-chronic: harmful effects produced through repeated or continuous exposure over twelve months or more but less than the normal lifespan of the organism.

1.1.4 Types of toxicants:

There are generally three types of toxic entities; chemical, biological, and physical:

1. **Chemical toxicants:** include inorganic substances such as lead, mercury, asbestos, hydrofluoric acid, and chlorine gas, organic compounds such as methyl alcohol, most medications, and poisons from living things.
2. **Biological toxicants:** include bacteria and viruses that can induce disease in living organism. Biological toxicity can be difficult to measure because the "threshold dose" may be a single organism. Theoretically one virus, bacterium or worm can reproduce to cause a serious infection. However, in a host with an intact immune system the inherent toxicity of the organism is balanced by the host's ability to fight back; the effective toxicity is then a combination of both parts of the relationship. A similar situation is also present with other types of toxic agents.
3. **Physical toxicants:** are substances that, due to their physical nature, interfere with biological processes. Examples include coal dust and asbestos fibers, both of which can ultimately be fatal if inhaled.

1.1.5 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).

1.1.6 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 commonly measured molecule is lactate dehydrogenase (LDH). 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 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, reassuring. 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.

2.1 Description of study plant *Aerva lanata*:



Fig 01: *Aerva lanata*

Amaranthaceae family of the genus *Aerva* that sometimes flowers in the first year. *Aerva lanata* belonging to Amaranthaceae family is a common weed which grows wild everywhere in plains of India. The root has camphor like aroma. The dried flowers which look like soft spikes, are sold under the commercial name as Buikallan or Boor. Decoction of the flowers is said to cure stones in any part of the stomach and that of the root is diuretic and cure for kidney stones.

2.1.2 Distribution

A. lanata prefers damper sites than *A. javanica* and can be found in open forests on mountain slopes, on waste and disturbed ground, deserted cultivation and coastal scrub and at altitudes from sea level to 900 meters (3,000 ft).

Native

Northeast Tropical Africa: Ethiopia, Somalia
 East Tropical Africa: Kenya, Tanzania, Uganda
 West-Central Tropical Africa: Cameroon, Rwanda, Zaire
 West Tropical Africa: Côte d'Ivoire, Ghana, Liberia, Nigeria, Sierra Leone, Togo
 South Tropical Africa: Malawi, Mozambique, Zimbabwe
 Southern Africa: South Africa - Natal, Transvaal
 Western Indian Ocean: Madagascar
 Arabian Peninsula: Saudi Arabia
 Indian Subcontinent: India, Sri Lanka, Bangladesh

2.1.3 Using information:

<i>Aerva lanata</i>	
<i>Aerva lanata</i> var. <i>rotundifolia</i>	
2.1.1 Scientific classification	
Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Core eudicots
Order:	Caryophyllales
Family:	Amaranthaceae
Subfamily:	Amaranthoideae
Genus:	<i>Aerva</i>
Species:	<i>A. lanata</i>

The plant is said to be diuretic and demulcent. Its diuretic action is said to be very effective in the treatment of urethral discharges and gonorrhoea and is of value in cases of lithiasis and as an anthelmintic. A trace of alkaloid has been detected.

Food: The whole plant especially the leaves are edible. The leaves are put into soup or eaten as spinach or as a vegetable. The plant provides grazing for stock, game in and chickens.

Leaves: A leaf-decoction is prepared as a gargle for treating sore-throat and used in various complex treatments against guinea-worm. to wash Babies that have become unconscious during an attack of malaria or of some other disease are washed with a leaf decoction at the same time smoke from the burning plant is inhaled. The leaf-sap is also used for eye-complaints. An infusion is given to cure diarrhoea and in an unspecified manner at childbirth, and on sores.

Root: The root is used in snake-bite treatment.

Leaves are used in toothache and fever. Roots are also used in fever. The latex of the plant is used to stop hemorrhage. Bark is anti-dysenteric; used in menstrual and renal complaints.

2.1.4 The aim of study:

The aim of study was to find out the bioactive chemical constituents and to evaluate the antioxidant, cytotoxicity and antimicrobial activity of the petroleum ether and methanol extracts of *Aerva lanata*.

To observe the biological effects, such as

Toxicological Studies: i.e.

Cytotoxic effect on brine shrimp

In order to develop and assess the safety and efficacy level of a new drug, toxicity studies are essential.

3.1 Method and Materials:

3.1.1 Cytotoxicity Studies

3.1.1.1 Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS etc) of the compounds. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial and pharmacological activities of natural products. Natural products extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here in vitro lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening an fractionation in the discovery of new bioactive natural products, Generally the median effective dose (ED₅₀) values for cytotoxicities are one tenth (1/10) of median lethal dose (LD₅₀) values in the brine shrimp test (McLaughlin, J. L. 1990 Personae, G. 1980, Meyer, B. N. et al., 1992 and McLaughlin, J. L. et al., 1988).

3.1.1.2 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by the addition of calculated amount of DMSO (dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of sea water. The samples of different concentrations are added to pre-marked vials with a micropipette. The vials are left for 24 hours and sthen nauplii are counted again to find out the cytotoxicity of the test

agents. These data are processed in a simple program for profit analysis to estimate LC₅₀ values with 95% confidence intervals for statistically significant comparisons of potencies.

3.1.1.3 Test materials

- 1) *Artemia salina* Leach (brine shrimp eggs)
- 2) Sea salt non ionized NaCl)
- 3) Small tank with perforated dividing dam to hatch the shrimp
- 4) Lamp to attract the nauplii
- 5) Pipette (1 ml and 5 ml)
- 6) Micropipette (5-50µl &10 to 100 µl)
- 7) Glass vials (5ml)
- 8) Magnifying glass
- 9) Test sample for experimental plants

3.1.1.4 Procedure

3.1.1.4.1 Preparation of the simulated sea water

37 grams sea salt (nonionized NaCl) was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The p^H of the sea water was maintained between 8.5 using 1 NNaOH solution.

3.1.1.4.2. Hatching of brine shrimp eggs

Artemia salina Leach (brine shrimp eggs) collected from the pet shop was 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 shrimps were allowed to one side of tank and this side was covered. The shrimp were allowed for two days to hatch and mature as nauplii (larvae). 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.

3.1.1.4.3 Preparation of the sample solution

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

5 mg of petroleum ether methanol,extract s(*Aerva lanata*) were accurately weighed and dissolved in 1000µl or 1 ml DMSO (dimethyl sulfoxide)in

different beaker. Thus a concentration of 5 mg/ml was obtained which used as a stock solution . From this stock solution 500 µg/ml, 300µg/ml, 100µg/ml, 50µg/ml, 25µg/ml and 10µg/ml were taken in six test tubes respectively each containing 5ml sea water and 20 nauplii.

3.1.1.4.4 Preparation of control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used.

- I. Positive control
- II. Negative control

3.1.1.4.5 Preparation of the positive control group:

Positive control in cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study, vincristine sulphate was used as the positive control. 3 mg of vincristine sulphate was dissolved in 1.8 ml of distilled water to get a concentration of 5 mg/ml. This was used as stock solution of vincristine sulphate. With the help of a micropipette 10, 50, 100, 150 and 200 µl of the stock solution were transferred in 5 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of vincristine sulphate in the vials became 10, 50, 100, 150 and 200µg/ml respectively. The experiment was repeated three times.

3.1.1.4.6 Preparation of the negative control group:

100 µl of distilled water was added to each of the three remarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the samples.

3.1.1.4.7 Application of brine shrimp nauplii

With the help of the Pasteur pipette 20 living nauplii were added to each of the vials containing 5 ml of simulated sea water. A magnifying glass was used for convenient count of nauplii. If the counting of the 20 nauplii was not possible accurately, then a variation in counting from 19-21 table might be allowed.

3.1.1.4.8 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 (LC₅₀) 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 LC₅₀ was calculated using the formula:

$$\text{Log LC}_{50} = \text{Log Concentration A} + \frac{\text{Log Concentration B} - \text{Log Concentration A}}{N - M} \times M$$

Log Concentration A = 50% - M / N - M

Where concentration A is the concentration before LC₅₀, Concentration B is the concentration after LC₅₀,

M is the % killed before LC₅₀, and N is the % killed after LC₅₀.

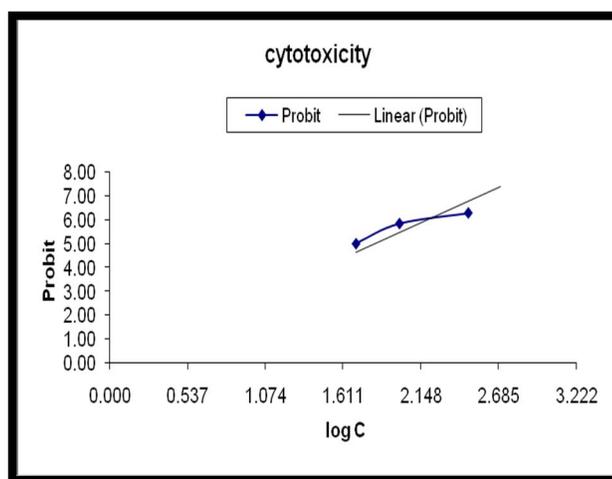
4.1Result:

The results of brine shrimp lethality bioassay are shown in the table 01.02 & Test samples showed different mortality rate at different concentration. The mortality rate of brine shrimp nauplii was found to be increased with the increase with the concentration of the sample. The median lethal concentration (LC₅₀) was calculated. The LC₅₀ values of petroleum ether and methanol of *Aerva lanata* are 40.85 µg/m and 49.997 µg/ml .respectively

So, it is evident that the petroleum ether and methanol extract of *Aerva lanata* were cytotoxic as well as biologically active.

Table 01: Effect of *Aerva lanata* P(etroleum ether extracton shrimp nauplii

Conc. $\mu\text{g/ml}$	Log C	No. of nauplii taken	No. of nauplii dead	% mortalit y	Probit	LC50 $\mu\text{g/ml}$	Value of x
0	0	20	0	0	-		
10	1	20	6	30	4.75		
50	1.699	20	10	50	5.00	40.85	1.611196
100	2.000	20	16	80	5.84		
300	2.477	20	18	90	6.28		
500	2.699	20	20	100			

**Fig 02:** Effects of various concentrations of Petroleum ether extract of *Aerva lanata* on the viability of brine shrimp nauplii after 24 hrs of incubation.**Table 02:** Effect of *Aerva lanata* (Methanol extract) on shrimp nauplii.

Conc. $\mu\text{g/ml}$	Log C	No. of nauplii taken	No. of nauplii dead	% mortalit y	Probit	LC50 $\mu\text{g/ml}$	Value of x
50	1.699	20	10	50	5.00	49.997	1.698949
100	2.000	20	18	90	6.28		
300	2.477	20	20	100			
500	2.699	20	20	100			

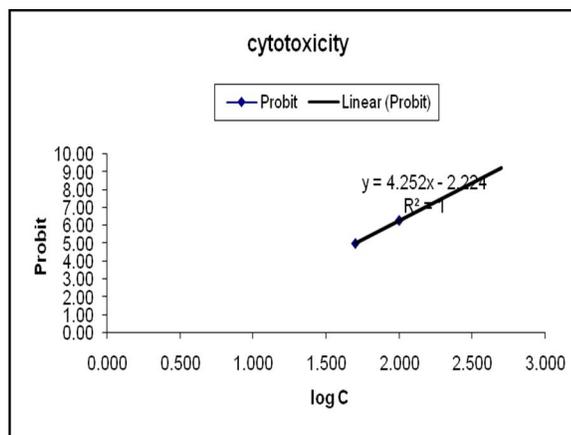


Fig 03: Effects of various concentrations of methanolic extract of *Aerva lanata* on the viability of brine shrimp nauplii after 24 hrs of incubation.

5. Discussions

The present study was aimed at investigating the cytotoxicity of the methanolic and petroleum extracts of *Aerva lanata*. The cytotoxicity of extracts has been evaluated by using a sensitive in vitro Brine shrimp lethality bioassay. From result it can be well predicted that the crude extracts have considerable cytotoxicity.

The present results suggest that all the tested plant extracts have moderate to strong cytotoxic activity. Since a variety of constituents is present in the extracts studied, it becomes difficult to describe the cytotoxic properties selectively to any one group of constituents without further studies, which are beyond the scope of this paper.

6. Conclusion

In conclusion, the present study, using in vitro experiments established that petroleum ether and methanol of *Aerva lanata* inhibits the bacterial growth. In case of anticancer drug preparation related as a good this plant extracts may be a candidate as it has notable cytotoxic effect. The petroleum ether and methanol extracts of *Aerva lanata* possess cytotoxic activity. It was observed that brine shrimp died at the concentrations of 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 300 µg/ml and 500 µg/ml. LC50 was found at the dose of 50 µg/ml in case of petroleum ether extract, at the dose of 100 µg/ml in case of methanol extract. This is only a preliminary study but the plant can be further

in order to find screened against various diseases in order to explore its unexplored efficacy and can be a potential source of biologically important drug candidates.

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