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RESEARCH ARTICLE

MOSQUITO LARVICIDAL AND CYTOTOXIC STUDIES ON *ANNONA RETICULATA* L.

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Abstract

Mosquitoes transmit serious human diseases, causing millions of deaths every year. Natural products of plant origin with insecticidal properties have been used in recent years for control of a variety of pest insects and vectors. This study was based on assessment of mosquito larvicidal activity to determine the efficacy of acetone, chloroform and methanol extracts of leaves, bark, fruits and seeds of *Annona reticulata* L. These extracts were tested against fourth instar larvae of *Aedes aegypti*, causing Dengue fever. Seven concentrations (500, 250, 125, 62.5, 31.25, 15.63 and 7 mg/l) of various extracts of plant parts were tested against the larvae. The larval mortality was recorded for 24hr. Cytotoxic activity of *A. reticulata* was also studied. The chromosomal abnormalities observed were of both clastogenic and non-clastogenic types. The study reveals that the leaves, bark, fruits and seeds extracts of *A. reticulata* possess both cytotoxic and larvicidal activities. The use of natural products that reduces mosquito populations at the larval stage can be used to vector control. In addition to addressing the needs for treatment of water near human habitation, natural products can be utilized as mosquito insecticides, which may promote localized control of persistent vector borne diseases. The study suggests the promising potential of *A. reticulata* L. as an alternative source of a sustainable, biodegradable and environment friendly solution for the control of dengue vector, *A. aegypti*.

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Introduction:-

Plants have been one of the important sources of medicines since the beginning of human civilization. According to the WHO survey 80% populations living in the developing countries rely exclusively traditional medicines for their primary health care needs. Exploration of chemical constituents of plants and their pharmacological screening may provide us the basis for developing novel agents.

Mosquitoes are the most important single group of insects in terms of public health importance. They are well-known vectors for transmission of vector-borne diseases affecting human beings particularly malaria and lymphatic filariasis (Hubalek and Halouzka, 1999). Dengue is the most rapidly spreading mosquito borne viral disease in the world. In the last 50 years, incidence has increased 30 fold with increasing geographic expansion to new countries and in the present decade, from urban to rural settings. *Aedes aegypti* is an important vector of dengue fever in India (Dutta et al., 1998). Dengue virus and the resulting severe form of dengue hemorrhagic fever, has experienced an

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expanding geographic range in recent decades as the *A. aegypti* vector has returned to regions where it was once eliminated (Gubler, 1998).

The target of mosquito reduction efforts on the larval stage has the advantage of controlling the vector prior to spreading the disease and interrupting the life cycle before it can cause devastation. In the absence of an effective preventive measures or vaccine, the best approach should be the interruption of disease transmission by either killing, preventing mosquitoes from biting people or killing the larva at the breeding sites of vectors. The discovery and use of conventional pesticides like DDT and malathion against adult mosquitoes in the last five decades established a shift but the development of resistant mosquito strains leads to ecological imbalance. This has necessitated to the continued effort for search and development of ecofriendly, biodegradable and low cost larvicides and adulticides for killing larva and adult mosquitoes respectively from natural sources (ICMR Bulletin, 2003). The use of plant derived compounds for mosquito control has been reported since 1933. Medicinal plants with larvicidal properties have paramount importance for the local control of mosquito.

Cytology is believed as a dependable tool for solving taxonomic problems and for elucidating systematic relationships, phylogeny and evolution of related plant groups. The information like chromosome number, structure, morphology and behaviour during mitotic and meiotic division has been of considerable value in understanding interrelationships and delimitations of taxa.

Cytotoxic study has got great significance now a days because of its application in cancer therapy. Chemical compounds of plant origin were among the first tested for mutagenic activity. The effect of plant extracts in the induction of chromosomal abnormalities has been studied by many workers. Cytotoxic and mitodepressive effects of several plant extracts and phytochemicals have been found in literature. Chromosomal aberrations have been considered as reliable indicators of mutagenic activity (Mohandas and Grant, 1972).

Annona reticulata L. (Annonaceae) is a medicinal plant commonly known as custard apple. It is a tropical fruit tree originating from Asia and America. The fruit is known as the 'Aristocrat of Fruits' for its nutritional value. This plant species is traditionally used in treating of different disorders such as constipation, fever, ulcers, cancer and tumour. It is also reported to exhibit antimicrobial, antioxidant, antibacterial and hepatoprotective. The main aim of the present study is to investigate the various extracts of *A. reticulata* for their larvicidal activity against *A. aegypti*.

Seven Annonaceae members have been studied so far for their mosquito strong activities. Only 4 species from 150 species of genus *Annona* studied for larvicidal activities (Das et al., 2007; Promsiri et al., 2006; Rodrigues et al., 2006; Saxena et al., 1993). Less number of studies so far reported on cytologic studies of *A. reticulata*. The present study is an attempt to find out the larvicidal and cytotoxic activity of the various parts of *A. reticulata*.

Materials And Methods:-

The leaves, bark, fruits and seeds of *A. reticulata* were collected locally in and around Kattachira of Alappuzha district in Kerala, dried in shade at room temperature and powdered with the help of mechanical device. The dried powder 15g was extracted with 250ml of 100 % solvents viz. acetone, chloroform and methanol for 6 h in a Soxhlet apparatus. The extract so obtained was concentrated and evaporated to dryness in a water bath for complete removal of solvents and stored at 4°C in a refrigerator. Dried residues obtained were 10g they were used for larvicidal experiment and *A. cepa* assay.

Larvicidal Activity:-

The eggs of *A. aegypti* were collected locally and the colonies were cultured and maintained in the laboratory at 27±1°C; 65-70% relative humidity and 10 h photoperiod. Fourth instar larvae of selected mosquito species were kept in a trough and different concentrations of various plant part extracts were added.

Seven concentrations of acetone, chloroform and methanol extracts (500, 250, 125, 62.5, 31.25, 15.63 and 7 mg/l) were prepared using distilled water. Thirty larva of *A. aegypti* were introduced in different test concentration of plant extracts along with a set of control containing distilled water without any test solution. After adding the larvae, the glass dishes were kept in laboratory at room temperature. By counting the number of dead larvae for 24 hrs of exposure, the mortality rate and the medium lethal concentration were obtained. Dead larvae were removed as soon as possible in order to prevent decomposition, which may cause rapid death of the remaining larvae.

Cytotoxic Assay:-

The test plant material selected for the cytotoxic assay is *A. cepa* L. $2n = 16$. Healthy and uniform sized bulbs of *A. cepa* were purchased from the local market, selected and washed in distilled water. These selected bulbs were planted in pure sandy soil without manure to prevent other chemically induced chromosomal alterations. After 2 days the roots sprouted out.

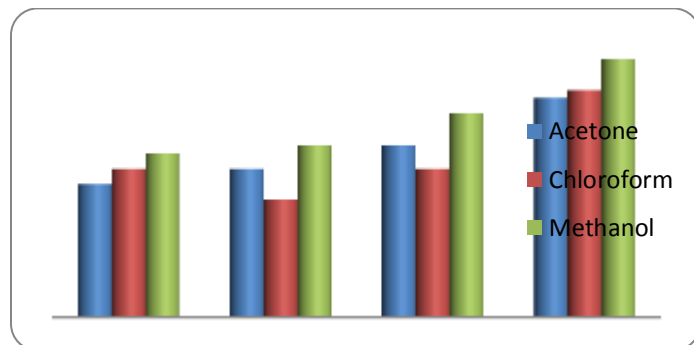
Cytotoxic activity of various concentrations of acetone, chloroform and methanol extracts of leaf, bark, fruit and seed of *A. reticulata* L. were investigated during the study. Different concentrations of the extracts viz., 500, 250, 125, 62.5, 31.25, 15.63 and 7 mg/l (w/v) were prepared in distilled water and after preliminary analysis it was confirmed that higher concentrations were found to be extremely toxic and hence were eliminated. So, the lower concentrations such as 500, 250, 125, 62.5, 31.25, 15.63 and 7 mg/l were selected. Both positive and negative controls were used. Distilled water was taken as negative control and an organophosphorus pesticide, methyl parathion (0.01%) as the positive control.

Germinated bulbs of *A. cepa* with healthy roots (1 - 2 cm) were collected at the time of peak mitotic activity, washed thoroughly with distilled water. The onion bulbs were kept at the rim of the bottle in which different extracts were taken in such a manner, that only the roots remain completely immersed in the solution. A few root tips were cut from each sample after the treatment for time duration 2 h, washed thoroughly with distilled water and immediately fixed in modified Carnoy's fluid (1 acetic acid : 2 alcohol) for one hour.

After fixing, all the root tips were transferred to 70% ethyl alcohol and kept under refrigeration. Mitotic squash experiments were conducted with the help of improved techniques (Sharma and Sharma, 1960). The root tips were washed in distilled water and hydrolyzed with 1N HCl for 5 - 10 minutes to separate the cells during squashing. The root tips were then washed thoroughly in distilled water and stained with 2% acetocarmine for 3 hours. After staining the roots were destained with 45% acetic acid, squashed and mounted on clean glass micro slides. All the slides were scanned under Olympus EPL3 integrated camera attached to Olympus CX20i research microscope.

Results:-

The larvicidal activity of leaves, bark, fruits and seeds of *A. reticulata* using acetone, chloroform and methanol extracts of various parts were evaluated.



Among the various leaf, bark, fruit and seed extracts maximum average total mortality rate was found with methanol extract 60%, 62.86%, 74.29% and 94.29% respectively. 500, 250, 125 and 62.5 mg/l of chloroform seed extract and 500 and 250 mg/l of acetone seed extract showed 100% larval mortality rate. Among the plant parts studied, seed extracts showed maximum mortality rate.

Treatment with crude extract of *A. reticulata* on *A. cepa* root meristem showed many mitotic abnormalities when compared with control. The abnormalities observed were metaphase and anaphase clumping, diagonal metaphase, diagonal anaphase, diagonal telophase, chromosome bridge formation, multiple bridge formation at anaphase, chromosome pulverization, fragmentation of chromosomes at metaphase and anaphase, misorientation of chromosomes, disturbed chromosomes, ball metaphase, scattering of chromosomes at anaphase, chromatin erosion, bizarre prophase, early movement of chromosomes, star metaphase and anaphase, c-metaphase, disturbed anaphase,

unipolar movement and interphase lesion. Mitotic indices were found to be less than that of the control. The frequency of abnormalities were found to be increasing and mitotic indices were found to be varying with the increasing concentration of the plant extract. Diagonal metaphase, sticky metaphase, ball metaphase, star metaphase, disturbed metaphase, clumped metaphase, bridge anaphase, single and double lesion at interphase were the most frequently observed abnormalities.

Table 1: Cytotoxicity of higher (500 mg/l) and lower (7 mg/l) concentrations of leaf, bark, fruit and seed of *A. reticulata* L. on *A. cepa* assay (2hr)

Control	Distilled Water						Methyl parathion					
Mitotic Index %	71.10±0.85						7.06±0.21					
% of aberrations	-						57.35±0.71					
	Leaf						Bark					
	Acetone		Chloroform		Methanol		Acetone		Chloroform		Methanol	
	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l
Mitotic Index %	7.9±0.42	7.2±0.17	8.8±0.25	4.2±0.56	33.3±0.37	34±0.09	12.5±0.18	34.3±0.38	9.9±0.84	17.1±0.36	20±0.47	12.8±0.27
% of aberrations	8.2±0.23	7.2±0.64	10.4±0.32	5.1±0.24	10±0.59	29.4±0.45	9.6±0.22	5.2±0.23	9.2±0.51	17.1±0.16	5±0.39	3±0.34
	Fruit						Seed					
	Acetone		Chloroform		Methanol		Acetone		Chloroform		Methanol	
	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l	500mg/l	7mg/l
Mitotic Index %	27.7±0.23	15.8±0.16	8.2±0.25	17.5±0.72	27.7±0.46	21.7±0.52	11.7±0.31	3.2±0.46	45.4±0.65	4.8±0.17	4.3±0.21	8.6±0.19
% of aberrations	24.6±0.45	3.3±0.06	4.4±0.33	9.1±0.28	24.6±0.83	10±0.14	5.8±0.28	3.2±0.53	42.4±0.25	11.2±0.34	2.4±0.29	25.9±0.37

Diagonal Metaphase



Star Metaphase



Sticky Metaphase



Ball Metaphase



Disturbed Metaphase



Clumped Metaphase



Bridge anaphase



Single Nuclear Lesion



Double Nuclear lesion



Discussion:-

Mosquito borne diseases are one of the most public health problems in the developing countries. It can be controlled by preventing mosquito bite using repellent, causing larval mortality and killing mosquitos (Das et al., 2007). The effects of various extracts were studied in a dose dependent manner. Among all the concentrations of various plant part extracts studied, methanolic seed extract of *A. reticulata* L. was found to have higher rate of larvicidal rate against *A. aegypti*. Crude extracts of leaves or bark of these plants have been tested earlier by several investigators and larvicidal and antifeedant activities have been shown against *A. aegypti* (Magadula et al., 2009).

Plants may be an alternative sources of mosquito control agents. The role of larvicidal activities of hexane, chloroform, ethyl acetate, acetone and methanol dried leaf and bark extracts of *Annona squamosa* L., *Chrysanthemum indicum* L. and *Tridax procumbens* L. against the malaria vector, *Anopheles subpictus* and Japanese encephalitis vector, *Culex tritaeniorhynchus* was reported by Kamaraj et al. (2011). The high potency of *Annona squamosa* and *Syzygium cumini* as a larvicidal against mosquito species of *A. aegypti* is demonstrated after the preliminary screening with crude leaf extracts (Kaushik et al., 2009). The significant activity demonstrated by extracts of *Annona squamosa* and *A. senegalensis* suggest that the two plants may have strong killing effects against insects particularly mosquitoes, hence giving a promise source of larvicidal agents. From the study, it is evident that leaf, bark, fruit and seed extracts (acetone, chloroform and methanolic) of *A. squamosa* can be developed as ecofriendly larvicides.

Allium test has been widely used in screening plant extracts to reveal its cytotoxic potential. This plant bioassay is now considered as one of the most efficient approaches routinely used to determine the toxic effects of chemical compounds in the environment (Leme and Marin-Morales, 2009). Many mitotic abnormalities were observed in the treatment with different concentrations of the crude extracts of *A. reticulata* L. and on *A. cepa* root meristem, when compared with the control. The frequency of abnormalities showed steady increase with increase in the concentration of the extract as well as with increase in the time of exposure of *A. cepa* root meristem to the extract of *A. reticulata*. The mitotic index showed variations with the duration of exposure and concentration and no strict correlation was observed. The mitotic poisons may cause metabolic imbalances which may interfere with the synthesis, state and structure of nucleic acids including physiological effects and structural changes in chromosomes during cell division which may lead to mitotic delay and mitotic inhibition (Soni et al., 1982). The changes brought about by the viscosity of the cytoplasm are primarily responsible for the chromosome abnormalities (Sharma, 1980).

Nuclear lesions are induced by certain plant derived chemicals in *A. cepa* root tip cells (Mercykutty and Stephen, 1980). The normal organization of chromatin in the nucleus and chromosome segregation is genetically controlled (Franklin and Cande, 1999). Metaphase clumping occurs due to the effect of a C mitotic agent. Increased concentrations of sodium salt of mimosine also was found to be hastening the onset of clumping (Pritchard and Van, 1997). Stickiness may result from the entanglement of chromatin fibres, which fail to condense properly in preparation for mitosis (Mc Gill et al., 1974). Breaking up of chromosomes followed by proximal chromatid reunion results in dicentric anaphase bridge that can also be attributed to the general stickiness of chromosomes (Gomurgen, 2000). The formation of ball metaphase may be due to the localized activity of spindle apparatus at the centre, so that the chromosomes were arranged in such a way that their centromeres remain at the equator and arms radiating in different directions and orienting in the form of a ball (Morgan, 2006). The improper functioning of spindle apparatus causes the diagonal orientation of chromosomes (Das et al., 1968). Star shaped arrangement may be due to the clumping of daughter chromosomes into star like structures near the polar region of the cell (Amer, 1965). Chromosome scattering could be attributed to the interference of the rhizome extracts with tubulin during polymerization of the microtubular subunits forming the spindle apparatus (Mathur and Chua, 2000).

All the concentrations of *Annona* extracts studied for larvicidal activity showed clastogenic and non clastogenic aberrations. Also, the mortality rate of mosquito larva and percentage of chromosomal aberrations increases with higher concentrations. The larval mortality is due to the action of plant secondary metabolites on the chromosome leading to clastogenic/non clastogenic aberrations.

Conclusion:-

The leaf, bark, fruit and seed extracts of *A. reticulata* possess cytotoxic and larvicidal activities. Their potential application in managing mosquito larvae would therefore be a promising undertaking. However, further work is required to isolate the active constituents in good amounts in order to test them for their cytotoxic and larvicidal potential. The use of natural products that reduces mosquito populations at the larval stage can be used to vector control. The selection of natural products that limit the environmental impacts of pesticides due to shorter latency

may be in preventing the evolution of resistance. In addition to addressing the needs for treatment of water near human habitation, natural products utilized as mosquito insecticides may provide a useful tool to promote localized control of persistent vector-borne diseases.

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