Antigenotoxic and Antiangiogenic Activity of Alternanthera Sessilis, Alstonia Scholaris and Anogeissus Acuminata Leaf Extracts

Dr. Rashmi Mohapatra
Associate Professor, Department of Botany, KISS Deemed to be University, Bhubaneshwar – 751024, India

Abstract **- Bioactive properties of Alternanthera sessilis, Alstonia scholaris and Anogeissus acuminata are extensively studied for their biological activities and being used in the treatment and management of human diseases in the traditional medical system. However, cytotoxic and genotoxic activities are yet to be explored. The present study was conducted to evaluate the cytotoxic and genotoxic effect in peripheral blood lymphocytes in vitro. As reports on the anticancer activities of these plants are scanty and expression of angiogenic factors reflect the aggressiveness of tumor cells, this study was undertaken to assess the pro/anti-angiogenic activity of these plant extracts in chick embryo chorioallantoic membrane. The presence of aberrant metaphase chromosome and mitotic index were evaluated in peripheral blood lymphocyte in vitro for genotoxicity and cytotoxicity assay. Genotoxicity and cytotoxicity assessments were carried out at the dose levels of 75, 150 and 300 µg/ml while antiangiogenic activities were studied at 25, 50 and 100 µg/ml concentration. Experiments were performed in triplicates for each concentration. Genotoxicity and Cytotoxicity test resulted in the absence of toxicity at doses between 75 µg/ml and 300µg/ml. Leaf extracts of all the three plants showed antiangiogenic activity at the dose level tested and it was dose specific. The percentage of inhibition of neovascularization in A.sessilis extract were 26%,82% and 91% and in A.scholaris 91%,97%,98%, at the dose level of 25, 50 and 100 µg/ml where as in A.accuminata the percentage reduction are 65%,76%,88% from lower to higher concentration. The selected doses of the plant extract were not genotoxic and cytotoxic but they have a high potential antiangiogenic activity.**

Index Terms **- Antiangiogenesis, Chromosomal aberration, Human lymphocytes, Mitotic Index.**

INTRODUCTION

Medicinal properties of plants have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods such as Ayurveda, Siddha and Homeopathy and the secondary metabolite constituents serve as an important source of drug leads. Currently, there has been an increased demand for natural herbal products globally because of their lesser side effects as compared to the contemporary system of medicine. Toxic substances synthesized by plants, which act as a defense against infections, insects and herbivores also affect the organisms that feed on them. However, approximately 60-80% of the world population still relies on traditional medicines for the treatment of common illnesses. Therefore, it is necessary to ensure relatively safe use of medicinal plants.

Angiogenesis is a physiological process, which leads to the generation of new blood vessels from pre-existing capillaries and circulating endothelial precursors [4]. It has a significant role in various physiological and pathological processes such as embryonic development, the transition of tumors from a benign step to a malignant one, wound healing, chronic inflammation and metastasis [5]. Regulation of angiogenesis is dependent on numerous factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF), tumour necrosis factor-α (TNF-α), and interleukin-8 (IL-8)[3]. The objective of this study was to investigate the antigenotoxic and angiosupressive effect of three medicinal plants *i.e., Alternanthera sessilis*L., *Alstonia scholaris* (L.) R.Br. and *Anogeissus acuminata (Roxb. Ex DC.) Wall. exGuill. &Perr.* on human peripheral blood lymphocytes (*in vitro*) and chick embryo respectively.

Alternanthera sessilis (L.) R. BR known as sessile joy weed belongs to family Amaranthaceae. It is an annual or perennial prostate herb with spreading branches with short petioled simple leaves and small white flowers, found throughout the hotter part of India. It is a well-known medicinal plant with several pharmacological properties such as anti-inflammatory, antiarthritic, antimicrobial, antidiabetic, antioxidant and anti-diarrhea [14; 7; 17; 18]. *A. sessilis* is considered as Kaya Kalpa drug according to Siddha literature (*i.e.*, the drug which prevents and cures chronic diseases and rejuvenates the body) and used as compatible diet. It is recognized to have galactogouge properties and useful in night blindness due to its high carotene content.

Alstonia scholaris (commonly known as Sapthaparna) belongs to family Apocynaceae. It is a tropical evergreen tree and grown in the low land and mountain rainforests of India, the Asia –Pacific and is native to Indian sub-continent. It is also known as Devils tree and has been used since time immemorial in the folk lore traditional systems of medicine in India. Preclinical studies on cultured neoplastic cells [9] and tumor-bearing mice [10] has demonstrated anticancer activity of *A.Scholaris*. In addition to this, the plant has a promising place in Ayurvedic system of medicine due to various medicinal values like antidiabetic, antibacterial, hepatoprotective, anti-inflammatory and analgesic effects. The presence of several bioactive compounds such as alkaloids, flavonoids, saponins, steroids, and phenolic compounds witness the ample amount of medicinal potential of this plant. The plant parts have been used in the treatment of chronic diarrhea, fever and dysentery. The bark extract is a useful remedy for treating asthma, lung cancer, hypertension, and pneumonia while the leaf extracts are used against fever. In folk medicine, the herb is given to lactating mothers to increase the lactation and to overcome post-delivery weakness and indigestion. Phytochemical studies reveal rich source of alkaloids (about 180 alkaloids) isolated, so far and only a few have been assessed for biological activities. Ethonomedical practice suggest it to be of use in treating cancer and preclinical studies performed with cultured neoplastic cells [10] and tumor bearing animals [9] having validated these observations. In addition to cytotoxic effects, *A.scholaris* has also been observed to possesses radio protective [6; 8; 2].

Anogeissus accuminata (*A. acuminata*) is a moderate size tree with small leaves and are used in traditional and tribal medicine of South India to treat painful inflammatory conditions. It has anti-inflammatory [11], analgesic [16], hypoglycemic [12] and free radical scavenging activity [19]. It has been reported that the leaves and barks contain flavonoids and oleo resins and many oleo resins are used in anti-inflammatory and analgesic agents in modern medicine. Volatile oils, resins, flavonoids and terpenoids isolated from plant extracts are known to produce anti-inflammatory and analgesic effects [15].

MATERIALS AND METHODS

Preparation of plant extracts:

Leaves of the plant *A. sessilis, A. scholaris* and *A.acuminata*, were collected in the winter season from the surrounding area of Bhubaneswar, Odisha. The plants were authenticated and voucher specimens were preserved at KISS, Deemed to be University with collection number KISS/BOT/2021/1-3. Fresh healthy leaves were separated from stems, thoroughly washed 2-3 times with water and dried in shade at room temperature. The dried plants (200gms) were milled to a fine powder with the help of a blender and stored at room temperature in closed containers in the dark until used. The powdered plant leaves were extracted with methanol: distilled water (80:20 (v/v) six times at room temperature by maceration method. The extracts were concentrated to dryness in the rotavapour till free from the solvents. Later the weight of the extracts were noted and kept in a desiccator.

CYTOTOXIC AND GENOTOXIC ASSAY

Blood sample collection:

Venous blood from six healthy volunteers (age 21-23) was drawn with informed consent in vacutainers. Non-smoking individuals with no known illness or recent exposures to genotoxic agents (e.g. chemicals, ionizing radiations) were the criteria used for sample collection.

Lymphocyte culture

RPMI-1640 supplemented with Fetal Bovine Serum (10% v/v), 1mM glutamine and 2mM $NaHCO_3$ were used for the culture of PHA stimulated whole blood cells. The lymphocyte cultures were exposed to plant

extracts of different concentrations (75, 150 and 300µg/mL) and incubated for 24h at 37ºC. At 67thhr of incubation period the dividing cells were arrested at a stable metaphase stage by adding 0.025µg/mL colchicine to each culture vials. The supernatant was aspirated, after gently tapping the pellet containing cells. A 5ml of pre-warmed (37°C) hypotonic solution (0.075M KCl) was added to the tubes and the contents were mixed gently using a Pasteur pipette and incubated for 5 minutes at 37°C.

Experiments were performed in triplicates for each concentration and incubated at 37°C in a chamber containing 5% CO_2. The cultures were incubated further for 5 hours at 37°C. Lymphocyte cultures were then harvested at 72 hrs. The cells were collected by centrifugation at 1000 rpm for 10mins and washed twice with RPMI 1640.

Preparation of metaphase chromosome:

Slides were prepared by gently placing a drop of the cell suspension on a cleaned and pre -chilled glass slide fixed with freshly prepared Carnoy's fixative (3:1:, Methanol: Glacial acetic acid). The slides were dried immediately by using the hot plate at 40°C. Staining was achieved with 10% (v/v) Giemsa in phosphate buffer pH 6.8 for 5 to 8 mins and examined under the microscope for mitotic index and metaphase spreads.

Scoring and microphotography:

A minimum 100 good metaphase spreads were analyzed from each culture and for each concentration under the oil immersion objective lens (100X) of the light microscope (Olympus, CX 31, USA). The slides were screened for structural aberrations in control and all the treated groups. Upon evaluating the slides, different types of chromosomal aberrations were detected in cultures exposed to plant extracts under investigation. The selected metaphase spreads were photographed using Cytovision software.

Evaluation of mitotic index to assess the cytotoxicity:

The mitotic index was used to determine if the plant extracts from various solvents at varying concentration has material produced any cytotoxicity. About 3 to 4 slides were prepared from each blood culture. Approximately 5000 cells were counted per culture to determine the mitotic index. The mitotic index was calculated as follows:

$$\text{Mitotic Index} = \frac{\text{Number of lymphocytes in metaphase}}{\text{Total number of lymphocytes counted}} \times 100$$

METHODOLOGY FOR ANTIANGIOGENESIS

Filter Paper Disc Preparation. Paper discs (approximately 5 mm in diameter) were made from Whatman filter paper and were sterilized by autoclaving for 15 minutes. One-day-old fertilized broiler eggs were collected from the hatchery, and the eggs were cleaned with70 percentage ethanol. The eggs were then incubated for ten days at 37^{0}C and 70% humidity. On the 10thday the eggs were exposed to different concentration of leaf extracts. A small window of 1x1 cm was made in the shell at the air space part of the egg. About 2ml of albumin was sucked out from the egg to bring their membrane down. Through the window of each egg, sterile disc of paper containing different concentrations (25µg/ml, 50µg/ml, 100µg/ml) of crude extracts were implanted inside the egg and at the junction of blood vessels of the CAM subsequently. The opening was sealed with parafilm, and the eggs were incubated again at 37°C for 72 hours. On the 12th day of incubation, the CAMs were harvested by removing the hard shell leaving intact the soft membrane covering the embryo. Four quadrants of the CAM in the area were drawn. The blood vessel branch point at each area of the quadrant was counted manually in a clockwise direction.

STATISTICAL ANALYSIS

One-way analysis of variance was employed to compare the mean difference in the percentage of Mitotic Index & chromosomal aberrations and neovascularization between control and treated groups. The difference with $p<0.05$ between experimental group was considered statistically significant.

RESULTS AND DISCUSSION

Mitotic Index was reported to be a good indicator to access the cytotoxic level, whereas chromosomal aberration was used to test mutagenicity. Fig 1 shows the data on the effect of various concentration of

methanolic plant extracts on the mitotic index in peripheral blood lymphocytes. Upon treatment with plant extracts at varying concentrations and evaluation of the harvested metaphase plates, reveals that there is no significant cytotoxic effect on the lymphocytes.

Chromosome abnormalities are considered as one of the most important cytogenetic parameter for the manifestation of genotoxicity. The result of the present study reveals no induction of chromosomal aberration at the doses tested in the selected medicinal plant extracts (Table 1) as compared to control. Generally, plants produce hazardous secondary metabolites, which may be used as their defensive mechanism. Following that several reports have indicated the toxicity of some plants used in traditional medicines if consumed above certain concentrations though their potentials to cure diseases were also mentioned. [13] Therefore, despite the study about the efficacy and mechanisms of plants to cure diseases, it is also vital to study the potential cytotoxic and genotoxic effect they might have. Antioxidant effect and modulatory action of *A.scolaris* against cytogenetic induced damage has been observed earlier [1], which is in accordance with the current findings.

During the present investigation, plant extracts used are non-mutagenic at the dose levels ranging from 75, 150, whereas *A.Sessilis* and *A.Scholaris* at the dose level of 300 µg/ml observed to be mutagenic as measured by the *in-vitro* chromosomal aberration assay using human blood lymphocyte. The *in-vitro* chromosomal aberration assay using human blood lymphocyte is an easy and accurate test procedure as compared to other genotoxicity-based studies.

The chicken chorioallantoic membrane (CAM) assay has been widely used for developmental and post-developmental studies of angiogenesis. Over recent years, more attention has been focused on the antiangiogenic and antitumor effect of non-toxic compounds from natural products. Angiogenesis mainly depends on proper activation, proliferation, adhesion, migration and maturation of endothelial cells. Angiogenesis inhibition plays a vital role to diminish invasive tumour growth and metastasis. Usually, angiogenesis is tightly controlled by a balance of angiogenesis factors and inhibitors and occurs only in embryonic development, wound healing and the female reproductive cycle. Angiogenic diseases result from new blood vessels growing either excessively (e.g., cancer, diabetic retinopathy and psoriasis) or insufficiently (e.g.,chronic wounds and ischemic heart disease). To date, the stimulation of angiogenesis using angiogenesis peptides has produced encouraging clinical outcome in treating coronary artery disease.Inhibition of angiogenesis by antiangiogenic compounds result in the lack of new blood vessel formation and sometimes in the disappearance of pre-existing vessel networks. In this direction, plant is being actively explored as a source of the new chemical substance that can inhibit angiogenesis.

In the current study inhibition of CAM neovascularization in chick embryo *in ovo* was observed by the treatment of CAM with 25, 50 and 100 µg/ml of *A.scholaris*, *A.accuminata* and *A.sessilis* leaf extracts. Fig 2A and 2B represents normal vascularization in the untreated and solvent treated CAM which consisted of primary, secondary and tertiary microvessels. In comparison to the control, there is a significant (P=0.01) reduction of vascularization in the CAM treated with the plant extracts at all the doses. However in *A.sessilis* treated CAM the reduction of vascularization were less as compared to other two leaf extracts taken for the study. The percentage of inhibition of neovascularization in *A.sessilis* extract (Fig 2C; 2D; 2E) were 26%,82% and 91% and in *A.scholaris* 91%,97%,98%,(Fig 2F,2G,2H) at the dose level of 25, 50 and 100 µg/ml where as in *A.accuminata* the percentage reduction are 65%,76%,88% (Fig 2I,2J,2K) from lower to higher concentration. In all the plant extract treated group there was significant (P<0.05) reduction in vascularization as compared with the control group. The branch points that were observed and counted were lesser at the greater dose level in *A. scholaris* treated embryo it is more effective than other exposed groups. The results indicate that the plant extracts could suppress angiogenesis *in ovo*. These plants may have the potential to be a useful deactivator of numerous serious diseases characterized by regulated angiogenesis.

CONCLUSION AND RECOMMENDATION

The result of the study shows the use of *A.sessilis*, *A.scholaris* and *A.accuminata* extracts has a potential antiangiogenic property. These plants are not cytotoxic and not genotoxic as revealed by the results

obtained from the analysis of Mitotic Index and Chromosomal aberration analysis.

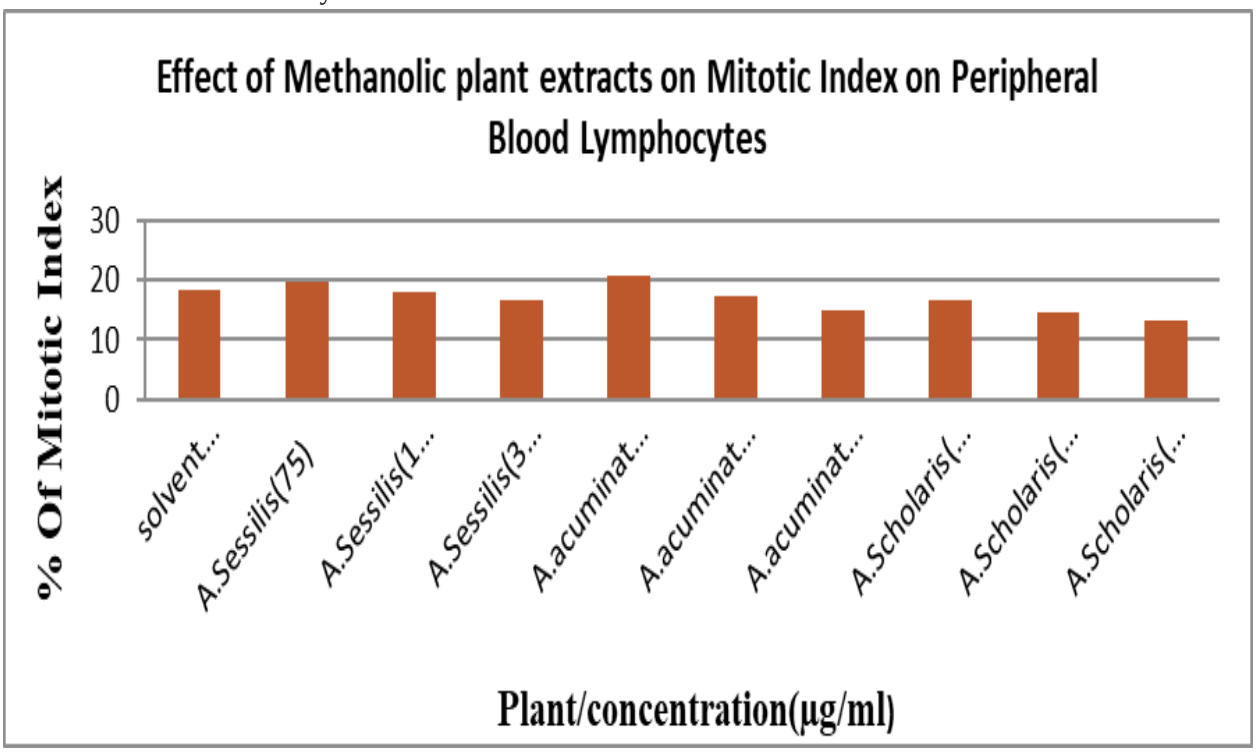

Fig1.Effect of Methanolic plant extracts on Mitotic Index (Mean±SD)

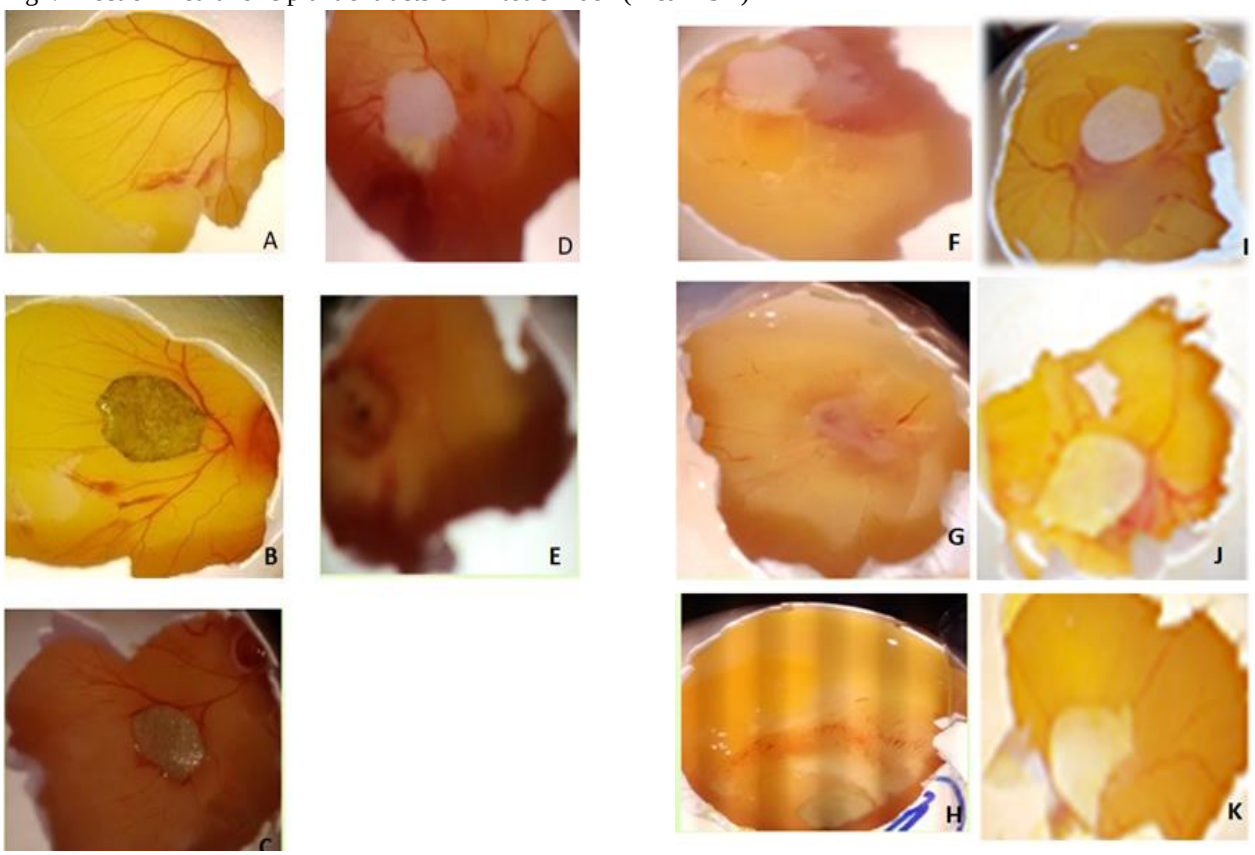

Fig2. A-control; B-solvent control; C-A.sessilis (25µg/ml);D- A.sessilis (50µg/ml);E-A.sessilis(100µg/ml) F-A.scholaris (25µg/ml);G- A.scholaris (50µg/ml); H-A.scholaris (100µg/ml);I-A.accuminata(25µg/ml); J-A.accuminata(50µg/ml); K-A.accuminata(100µg/ml)

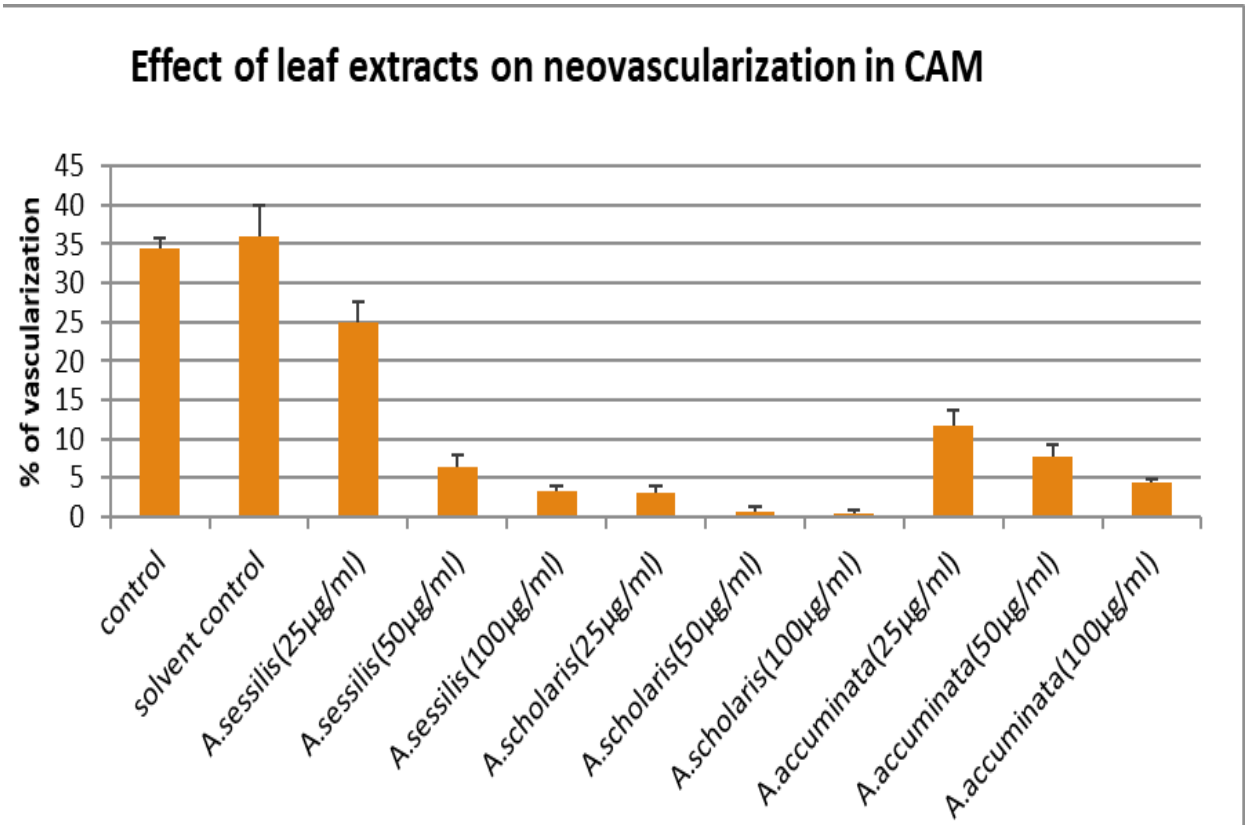

Fig 3. Effect of leaf extracts on neovascularization in CAM

Table 1: Effect of plant extracts on peripheral blood lymphocytes

Treatment/Dose(µg/ml)	Total no. of aberrant metaphases (N)	Single aberrant cells (N)	Multiple aberrant cells(N)	Mean % of CA Mean ± SD
Control	9	9	0	0.30 ± 0.13
Solvent control (Methanol)	11	10	1	0.35 ± 0.13
A.Sessilis (75)	9	9	0	0.27 ± 0.15
A.Sessilis (150)	10	10	0	0.23 ± 0.10
A.Sessilis (300)	11	11	0	5.80 ± 0.03
A.acuminata (75)	14	14	0	0.24 ± 0.10
A.acuminata (150)	17	15	2	0.32 ± 0.10
A.acuminata (300)	9	9	0	0.20 ± 0.58
A.Scholaris (75)	8	7	1	0.50 ± 0.36
A.Scholaris (150)	6	6	0	0.43 ± 0.32
A.Scholaris (300)	7	7	0	4.10 ± 0.47

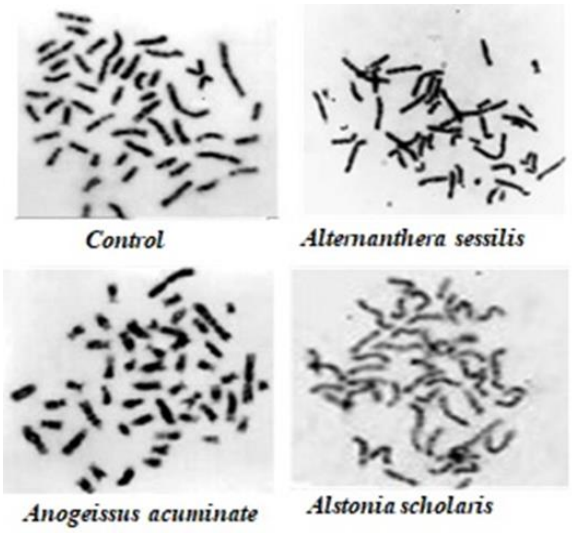

Fig 3. It shows Metaphase chromosomal aberrations. Healthy individual with no aberrations (Control), Metaphase spread with arrow mark indicates breaks, gaps and fragments (Alternanthera sessilis-300µg/ml), Metaphase spread with no aberrant chromosome (Anogeissus acuminate-300µg/ml) and Metaphase spread with arrow mark indicates break (Alstonia scholaris-300µg/ml).

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