



ISSN: 2231-3354
Received on: 05-04-2012
Revised on: 28-04-2012
Accepted on: 03-05-2012
DOI: 10.7324/JAPS.2012.2526

***In Vitro* Evaluation of the Anticancer Effect of Methanolic Extract of *Alstonia scholaris* Leaves on Mammary Carcinoma**

Surya Surendren P., Jayanthi G and Smitha K.R

Surya Surendren P., Jayanthi G
Department of Biochemistry,
Kongunadu Arts and Science College,
Coimbatore, Tamil Nadu, India.

Smitha K.R
Department of Microbiology,
St. Mary's College, Thrissur,
Kerala, India.

ABSTRACT

Breast cancer is the leading cause of cancer associated death among women worldwide. Current cancer treatments include chemotherapy, radiotherapy, and surgery. However, since these conventional methods often have undesirable side effects, new focus towards the use of plant extract to treating cancer with eliminating the side effects. The objective of present study is to assess the anticancer effect of leaves of *Alstonia scholaris*, using the cytosolic marker enzymes like Aspartate transaminase (AST), Acid phosphatase (ACP), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), Gamma - glutamyl transferase (γ -GT), and 5'-nucleotidase (5'-NT) *in vitro* over breast cancer tissue. These are key enzymes in the metabolic pathways and these are the target for the drugs used in chemotherapy. An elevated level of enzyme concentration signals the presence of malignancy. The effect of leaves of *Alstonia scholaris* is also assessed by studying the effect of non-enzymatic antioxidants like vitamin A, E, C *in vitro* over breast cancer tissue. The present study revealed the leaves of methanolic extracts of *Alstonia scholaris* on cancer cells/tumor cells *in vitro* has been justified by its cytotoxic effect and anti proliferative effect.

Keywords: *Alstonia scholaris*, Breast cancer, antiproliferative effect, cytosolic marker enzymes, non-enzymatic antioxidants

INTRODUCTION

Cancer is among the most dreaded of human diseases. It is recognized as a major threat to health since the earliest days of recorded history. It is considered as an adversary of modernization and the pattern of socioeconomic life dominated by western medicine. Cancer still counts as one of the most frequent causes of human fatality, particularly in technically advanced countries. In these countries it accounts for about 15% to 20% of deaths each year. In 1988 over 4,50,000 persons died from cancer in the United States, more than Americans killed in World War II and the Vietnam War combined. (Stephan, L. Wolfe, 1995). The formation of breast cancer is a multi-step process which differs depending on type of disease, a patient's genetic makeup and other factors.

For Correspondence
Smitha K.R.
Department of Microbiology,
St. Mary's College, Thrissur,
Kerala, India.

However, scientists know that many breast cancers are fueled by increased estrogen, which collects and reacts with DNA molecules to form adducts. It is found that resveratrol was able to suppress the formation of these DNA adducts (Rogan, 2008). The principal risk factors for breast cancer include menstrual and reproductive history and family history. The menstrual factors comprise a cluster of associations that point to an important role for functioning ovary in the genesis of breast cancer. Castration, either by surgery or by radiotherapy, substantially reduces a women's breast cancer risk. The reduction is larger the earlier the castration and oophorectomy prior to 35 years to age reduces the risk to one third of that experienced by women undergoing a natural menopause. In addition, women with early menarche and those with late natural menopause appear to be at increased risk (Trichopoulos *et al.*, 1968). The reproductive characteristic most strongly associated with breast cancer risk is the age at which a woman bears her first child. Women who have a first, full term pregnancy before the age of 18 years have only one third the breast cancer risk of those whose first child is delayed, until age 30 years. Women who bear their first child after age 30 years actually have slightly higher risk than do those who remain nulliparous. Heredity plays a role in the development of cancer even in the presence of clearly defined environmental factors.

The use of medicinal plants in modern medicine for the prevention or treatment of cancer is an important aspect. For this reason, it is important to identify antitumor promoting agents present in medicinal plants commonly used by the human population, which can inhibit the progression of tumor (Ganesh Chandra *et al.*, 2003) Cancer chemoprevention is a mean of cancer control by pharmacological intervention of the occurrence of the disease using chemical compounds. Recent events suggest that new emphasis in the development of medical treatment of human disease will be intimately connected to natural products. Recently, a greater emphasis has been given towards the researches on complementary and alternative medicine that deals with cancer management. (Premalatha *et al.*, 2004) Multidisciplinary scientific investigations are making best efforts to combat this disease, but a perfect cure is yet not realized in modern medicine. Any practical solution in combating cancer is of paramount importance. Many herbs have been evaluated in clinical studies and are currently being investigated phytochemically to understand their tumouricidal actions against various cancers. The traditional Indian system of medicine with its evolution through centuries has always fascinated practitioners and researchers on a scientifically proven research background. Herbal medicines have a vital role in the prevention and treatment of cancer. Some herbs protect the body from cancer by enhancing detoxification functions of the body (Bradstreet, 1997). Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. Some herbs reduce toxic side effects of chemotherapy and radiotherapy and are often employed for cancer treatment. The recent upsurge of global interest in herbal medicines can be attributed to the spread of the traditional knowledge of the orient along with the realization and

deeper understanding of the side effects and the warning effectiveness of some the conventional modern medicines such as antibiotics, which once had near-universal effectiveness against serious infections. The traditional Indian system of medicines, Ayurveda, uses about 2,000 plant species, while the Chinese Pharmacopoeia lists over 5,700 traditional medicines, most of which are of plant origin (Nair *et al.*, 2010).

Antioxidants also play an important role in cancer prevention. Cancer cells are "immortal" i.e. they have lost their growth restraining mechanisms and so multiply out of control. This results from alteration of cellular DNA or genetic material, which can be an inherited defect. It was found that free radical damage is the cause of these genetic mutations. Free radicals are molecules that have an unpaired electron. All physical matter is held together by pairs of electron called chemical bonds. When one electron becomes displaced for any reason, a free radical is formed. Free radical "steal" electrons from other molecules in order to restore their own balance. In this process, they create another free radical that can participate in a chain reaction with resultant damage to the involved body tissue. When DNA or genetic material is involved in free radical reactions, mutations or genetic alteration can result. Free radical chain reactions are stopped by the action of antioxidants.

The real foundation for antioxidant nutrition is a diet rich in plant foods. There are many different compounds with in fruits, vegetables, nuts, seeds, legumes, and grains that exert antioxidant activity. This is one of the reasons that plant based diets are associated with good health, including reduced risk to breast cancer.

In the mid-1950's, the pharmaceutical company, Eli Lilly, began screening medicinal plants for antitumor activity, selecting plants used in traditional medicine. The 40th plant they tested in the laboratory, the Madagascar periwinkle (*Catharanthus roseus*), had antitumor activity against leukemia cells. The periwinkle yielded two drugs - vinblastine and vincristine that are still used in cancer chemotherapy. Today, nearly 25% of all prescriptions contain compounds that come directly or indirectly from plants.

Now a days, much importance is given to discover more plants with anticancer effect. The disadvantages of chemotherapy and Immunotherapy in the treatment of cancer have paved the way for herbal treatment for cancer. The advantages of herbal treatment are no side effects, less cost and relapse of the disease is low.

Alstonia scholaris (also known as Devils tree) a tree belonging to the family Apocynaceae, has been used since time immemorial in the folklore and traditional systems of medicine in India, to treat several diseases (Chandra *et al.*, 2003). The plant is grown in the lowland and mountain rainforests of India, the Asia-Pacific, Southern China and Queensland. The plant grows throughout the humid regions of India. especially in West Bengal and west-coast forests of south India. The plant is used in Ayurvedic, Unani and Sidhha /Tamil types of alternative medicinal systems (Dey, 2011). The methanolic extract of this plant was found to exhibit pronounced antiplasmodial activity. The plant is reported to have antimutagenic effect (Arulmozhi *et al.*, 2010). The

present study is done to assess the *in vitro* anticancer effect of leaves of *Alstonia scholaris*, using the cytosolic marker enzymes like AST, ALP, ACP, LDH, γ -GT, and 5'-NT over breast cancer tissue. The effect of leaves of *Alstonia scholaris* is also assessed by studying the effect of non-enzymatic antioxidants like vitamin A, E, C *in vitro* over breast cancer tissue.

MATERIAL AND METHODS

Collection of Carcinoma samples

Cancer sample of breast tissue was collected from a reputed hospital at Coimbatore, Tamil nadu. At the hospital during the surgery, tumor or carcinoma mass was removed from the patient. For biochemical analysis and *in vitro* treatment analysis biopsy samples were directly collected in 0.1M phosphate buffer, pH 7.4. Collected samples were duly preserved and transported to the laboratory safely and in a sterile manner. Mass was weighed and homogenized with 10% homogenizing buffer, 0.1M Tris, pH 7.0. The homogenate was fractionated into three groups for analysis.

Group I - Control (Cancer mass homogenate).

Group II - Homogenate + 3% *A.scholaris* extract.

Group III - Homogenate + 4% *A.scholaris* extract.

Analysis was done after incubating the samples for 3 hours. At the end of the incubation period the samples were assayed for the estimation of cytosolic, lysosomal and membrane markers.

Collection of leaves of *A. scholaris*

Leaves of *A.scholaris* were collected from Western Ghats hills of Kerala.

Extraction of leaves

Dried powder of the plant leaves (25g) were extracted twice with 250 ml of 80% methanol overnight with continuous stirring. The pooled extracts were concentrated and evaporated to dryness under vacuum. The extract was suspended in DMSO and subjected to *in vitro* cytotoxicity.

Biochemical analysis

Estimation of Aspartate transaminase (EC 2.6.1.1)

L-aspartate + 2-oxoglutarate \rightarrow oxaloacetate + L-glutamate. The enzyme is assayed based on the method of King (1965). 0.2 ml of sample and 1.0 ml of the buffer substrate (146 mg of α -Ketoglutarate and 13.3 g of aspartic acid in 1 N NaOH and made up to 1000 ml with phosphate buffer pH 7.4) was incubated for 60 min at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of Dinitrophenylhydrazine reagent (1 mmol/ HCl) and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4N NaOH was added. A set of standard pyruvate were also treated in a similar manner. The color developed was read at 520 nm. The enzyme activity was expressed as units/mg protein in tissues.

Estimation of Alkaline phosphatase (EC 3.1.3.2)

The enzyme is assayed based on the method of King (1965). 4.0 ml of the buffer substrate in a test tube incubated at

37°C for 5 min. Added 0.2 ml of tissue homogenate and incubated further for exact 15 min. Removed and immediately added 1.8 ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2 ml serum to which 1.8 ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of the supernatant added 2.0 ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the colour developed at 700 nm. The enzyme activity was expressed as units/ mg protein in tissues.

Estimation of Acid phosphatase (E.C.3.1.3.2)

The enzyme is assayed based on the method of King (1965). 4.0 ml of the buffer substrate added into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of tissue homogenate and incubated further for exact 60 min. Removed and immediately added 1.8 ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2 ml serum to which 1.8 ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of the supernatant added 2.0 ml of sodium carbonate. Took 4.0 ml of working standard solution and for blank taken 3.2 ml water and 0.8 ml of phenol reagent. Then added 2.0 ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the colour developed at 700 nm. The enzyme activity was expressed as units/ mg protein in tissues.

Estimation of Lactate dehydrogenase (EC 1.1.1.27)

The enzyme is assayed based on the method of King (1965). 1.0 ml buffer substrate and 0.1 ml sample added into each of two tubes. Added 0.2 ml water to the blank. Then to the test added 0.2 ml of NAD. Mixed and incubated at 37°C for 15 min. Exactly after 15 min, 1.0 ml of Dinitrophenyl hydrazine was added to each (test and control). Left for further 15 min. Then added 10 ml of 0.4 N Sodium hydroxide and the color developed was read immediately at 440 nm. A standard curve with Sodium pyruvate solution was taken. The enzyme activity was expressed as units/mg protein in tissues.

Estimation of 5'-nucleotidase (EC 3.1.3.5)

The enzyme is assayed based on the method of (Rathnakumar *et al.*, 2000). The reaction was carried out in two tubes, test (T) and control (C). To the T tube added 1.5 ml of buffer and 100 μ l of manganese solution (20 mmol/L), to the control added 1.3 ml buffer, 100 μ l manganese solution and 200 μ l of nickel chloride solution (0.1 mol/L). Mixed the contents well. Added 200 μ l of serum and 200 μ l of substrate solution to both tubes, mixed and incubated at 37°C for 30 min. Stopped the reaction by adding 2.0 ml of 10% Trichloroacetic acid. Mixed the tube contents thoroughly, centrifuged at 3000 g for 15 min. Pipetted 2.0 ml of clear supernatant from the test and control mixtures. 1.0 ml of working standard and 1.0 ml of water was taken into standard (S) and prepared a reagent blank (B) by mixing 1.0 ml water and 1.0 ml of Trichloroacetic acid. To all the tubes, added 3.0 ml of acetate buffer (2.4 mol/L, pH 4.0), followed by 0.5 ml of ammonium molybdate solution (5g/dl) and 0.5 ml of metol

solution (2g metol in 80 ml water). Mixed well after each addition. After 5 min read the colour developed at 680 nm in a spectrophotometer. The colour was stable for at least 30 min. The enzyme activities were expressed as units/ mg protein in tissues.

Estimation of Gamma -glutamyl transferase

The enzyme is assayed. Add 0.1 ml of sample and 1.0 ml of buffer into the cuvette. Mixed well and incubated for 1 min at 37°C. Add 0.25 ml of the substrate. Mixed and read the increase in absorbance at 405 nm exactly after 1,2,3 minute respectively.

$$\gamma\text{-GT activity (U/L)} = \Delta/\text{min} \times 1421$$

The enzyme activity was expressed as units/L in serum, units/protein in tissues and units/mg creatinine in urine. The enzyme activity was expressed as units/mg protein in tissues.

Estimation of protein

The enzyme is assayed based on the method of Lowry *et al.*, (1951). 0.2 to 1.0 ml working standard solution and 0.1 ml of the sample was taken in each of separate test tubes. The volume in all the tubes was made up to 1.0 ml with distilled water. Added 5.0 ml of alkaline copper reagent (50 ml of 2% sodium carbonate in 0.1 N NaOH and 1.0 ml of 0.5% Copper sulphate in 1% potassium sodium tartarate) to each tube. Mixed well and allowed to stand for 10 min. Then added 0.5 ml of Folin-Ciocalteau reagent (1 part of reagent with 2 parts of water). Mixed well and incubated at room temperature for 30 minutes. A reagent blank was also prepared. After 30 minutes, the blue color developed was read at 660 nm. The results was expressed as mg/g in tissue.

Assay of non-enzymatic antioxidants

Assay of vitamin A

The method is based on the interaction of vitamin A with TFA and the intensity is measured at 620 nm. To 1.0 ml of 10% homogenate 1.0 ml of saponification mixture (2 N /KOH in 90% alcohol) was added and heated under gentle reflux for 20 min at 60°C. 25 ml of water was added to the mixture after cooling to room temperature and the solution was transferred to a separating funnel. It was then extracted thrice with using 25, 15 and 10 ml of petroleum ether (40 - 60°C). The ether extracts were pooled and washed with 50-100 ml of distilled water repeatedly until the wash water was free of alkali. The petroleum ether extract was then dried by adding anhydrous sodium sulphate. The volume of the extract was noted. 3.0 ml of petroleum ether phase was transferred to a cuvette and read at 420 nm against a petroleum ether blank without delay to prevent evaporation of the solvent and destruction of carotenoids by light. Marked this reading as A_1 . The β -carotene working standards were measured at 450 nm. The aliquots were evaporated to dryness at 60°C in a water bath. The residue was taken immediately and 2.0 ml TFA reagent were added to it. The mixture was rapidly transferred to a cuvette and the absorbance was measured at 620 nm exactly after the addition of TFA reagent. Marked this reading as A_2 . The vitamin A working standard was read at 620 nm. The results were expressed as $\mu\text{g/g}$ tissue.

$$\text{Sample} = \frac{A_3 \times \mu\text{g retinol calibrator/cuvette} \times 3 \times \text{total volume}}{A_{620} \text{ retinol calibrator} \times 2 \times \text{gram}}$$

$A_3 = A_2$. A_1 , A_1 = Absorbance of carotene at 450 nm, A_2 = Absorbance at 620 nm due to both carotene and vitamin A, A_3 = Absorbance at 620 nm of vit A, 3 = Volume of petroleum ether from 1.0 ml extract, 2 = Aliquot of the petroleum ether for the assay, 1 = 10% extract taken from initial sample

Assay of vitamin E

Tocopherols can be estimated using Emmerie-engel reaction of ferric to ferrous ions by α -tocopherols, which forms a red colour with Bipyridyl, and read at 520 nm. Into 3 stoppered centrifuge tubes (test, standard and blank) pipetted out 1.5 ml of each tissue extract, 1.5 ml of the standard and 1.5 ml of water respectively. To the test and blank added 1.5 ml ethanol and to the standard added 1.5 ml of water. Added 1.5 ml of xylene to all the tubes, stoppered, mixed well and centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube, taking care not to include any ethanol or protein. Added 1.0 ml of 2,2' dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixtures into spectrophotometer cuvettes and read the absorbance of test and standard against the blank at 460 nm. The in turn beginning with the blank, added 0.33 ml of Ferric chloride solution. Mixed well and after exactly 1.5 minutes read test and standard against the blank at 520 nm. The amount of vitamin E can be calculated using the formula.

Vitamin E ($\mu\text{g/g}$) =

$$\frac{(\Delta A_{520\text{nm}} - \Delta A_{450\text{nm}} \times \text{concentration [S]} \times 0.29 \times \text{Total volume})}{\Delta A_{520\text{nm}} \times \text{Volume for experiment} \times \text{weight of sample}}$$

Assay of vitamin C

The dehydroascorbic acid reacts with Dinitrophenyl hydrazine to form osazone and dissolved in sulphuric acid to give orange red coloured solution which is measured at 540 nm. 1.0 ml of 10% homogenate was precipitated with 5% ice-cold Trichloroacetic acid and centrifuged for 20 min at 3,500 g. 1.0 ml of the supernatant was mixed with 0.2 ml of DTCS reagent (2,4 Dinitrophenyl hydrazine, thiourea and copper sulphate dissolved in 9 N sulphuric acid) and incubated for 3 hours at 37°C. Then 1.5 ml of ice-cold 65% Sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520nm. The results were expressed as $\mu\text{g/mg}$ protein.

RESULT AND DISCUSSION

According to cancer statistics, breast cancer is the leading cause of death from cancer among women. The death rate for this cancer has not changed appreciably during the last 40 years, in spite of greater awareness among the population about breast cancer. About 1 in 14 American women will develop breast cancer during her life and this attack rate may be expected to increase.

Patients with breast cancer have a longer survival time if the disease is detected early, but increase emphasis on early detection has not yet been reflected by a change in overall mortality statistics. All over the world many research works are going on to find a remedy to prevent cancer. Now a days many new techniques and most advanced therapies, like chemotherapy, immunotherapy etc are available to treat cancer. But the main disadvantage of all these treatment measures is that besides treating cancer, they can cause other serious side effect also.

Here lies the importance of using medicinal plants for curing cancer. The main advantage of medicinal plants is that they do not cause much side effects. The medicinal plant used under the study was *Alstonia scholaris*.

The objective of present study is to asses the invitro anticancer effect of leaves of *Alstonia scholaris*, using the cytosolic marker enzymes like AST, ALP, ACP, LDH, γ -GT, and 5'-NT *In vitro* over breast cancer tissue. These are key enzymes in the metabolic pathways and these are the target for the drugs used in chemotherapy. Enzymes are present in much higher concentrations inside than outside the cell. Enzymes are released into the systemic circulation as a result of tumor necrosis or of the change of the membrane permeability of the cancer cells. By the time enzymes are released into the systemic circulation, the metastasis of tumor may have occurred. Enzymes are not unique for a specific organ. Consequently enzymes are most suitable as non-specific tumour markers. An elevated level signals the present of malignancy (Tietz, 1994).

The effect of leaves of *Alstonia scholaris* is also assessed by studying the effect of non-enzymatic antioxidants like vitamin A, E, C invitro over breast cancer tissue.

Cytosolic markers

The cytoplasm contains most of the enzymes that catalyze the entire biosynthetic and degradative metabolism. Progression of neoplastic cells results in quantitative changes in enzymes, as metabolism shifts to enzymes patterns presumably permitting more rapid growth (Potter, 1968). The mammary glands also undergoes wide spread changes in metabolism during malignancy. Cytosolic markers like LDH, 5' NT and AST were chosen and identified for the changes in malignancy and treatment.

Table. 1: Assay of Cytosolic Markers in Cancer and *Alstonia scholaris* Treated Tissues.

Experimental groups	AST (Units/mg protein)	LDH (Units/mg protein)	5'-Nucleotidase (Units /mg protein)
Group I	0.943	0.975	1.148
Group II	0.506a**	0.252a**	0.280a**
Group III	0.323b**	0.435b**	0.419b**

AST: S.E.D. = 0.023, LSD (5%) = 0.050, LSD (1%) = 0.070

LDH: SED = 0.033, LSD (5%) = 0.072, LSD (1%) = 0.101

5'- Nucleotidase: S.E.D. 0.050, LSD (5%) = 0.109, LSD (1%) = 0.153

** = Significant at 1% level, a = comparison between group I and II, b = comparison between group I and III

Group I: Control cancer mass homogenate, Group II: 3% *A. scholaris* treated cancer homogenate.

Group III: 4% *A. scholaris* treated cancer homogenate

Units: AST & LDH: μ moles of pyruvate liberated / min / mg protein.

5'- Nucleotidase μ moles of phosphorus liberated / min / mg protein

Aspartate transaminase (AST)

AST, a cytosolic enzyme was analyzed for any change between neoplastic cells and treated neoplastic cells. It is a liver specific marker enzyme and the elevated levels are indication of liver malignancy. 5 to 10 fold increase is seen in AST level during metastatic cancer. The enzyme level is also increased in hepatobiliary, hepatotoxicity, conditions (Teitz, 1994). Carcinoma of the colon with metastasis to the liver exhibited elevated levels of AST (Cornelia *et al.*, 1975). The level of AST seem to be increased in the control (group I), when compared to non-neoplastic cells. But there is a significant decrease after the treatment with the leaf extract. (Table.1)

Lactate dehydrogenase (LDH)

LDH has been found to be a cytosolic marker. Lactate dehydrogenase, regulates the interconversion of pyruvate to lactate, using NAD as cofactor. LDH the tetrameric protein, forms the center for delicately balanced equilibrium in the metabolism of carbohydrates. LDH also takes part in the biosynthesis of carbohydrates. The high glycolytic rate is important for rapidly proliferating cancers not only as a major energy source but also to provide such cells with precursors for nucleotide and lipid biosynthesis. As a result of this high glycolytic rate, there is an elevated level of LDH during cancerous conditions (Arathi, *et al.*, 2003). Tumor cells have an increased glucose transport and this glucose is metabolized via the anaerobic glycolytic pathway to produce lactic acid. Enzyme is useful in the recognition of neoplastic disease. Malignant tumors are known to have high rates of glycolytic activity leading to high production of lactic acid (Bygrave, 1976). As per table.1, the increased activity of LDH is in group I when compared to group II and group III. The inhibition of cancer mass with varied concentration of the drug significantly reduced the activity of LDH, (group II & III), which would have resulted from the regulation of glycolysis by the extract on reacting with the cells. Elevated serum LDH is also seen in anemia. This elevated LDH levels are due to the destruction of the abnormal red-cell precursors (Elliott and Fleming, 1965). Decrease in the level of LDH activity was observed in N-acetylcysteine (NAC) treated cells infecting with induction in cell proliferation (Sridharan *et al.*, 2001). These reports suggest leaf extract treatment has inhibiting effect over the proliferating mass.

5'-nucleotidase

5'-Nucleotidase is a cytosolic enzyme. It is inhibited in the presence of nickel ions. The substrate for 5'-Nucleotidase is AMP. The activity is elevated in cancer cells as a consequence of increased nucleotide salvage pathway. The activity of the 5'-Nucleotidase in serum is increased the during hepatobiliary disease, and also in malignant infiltration or billiary cirrhosis. The increased activity of this enzyme is a good marker of metastasis . The table.1 depicts the activity of the enzyme to be high in control (group I), which are cancer cells. The reduction in the activity is seen in the treated cells (group II & III). This indicates the reaction of the drug with the neoplastic cells to enhance GSH synthesis.

The activity of the enzyme is decreased in the cancer cells after the treatment with N-acetylcysteine (Sridharan et al 2001). Inference arrived in the analysis of 5'-Nucleotidase in treated cells (group II and III) is that the drug shows an antimetabolic effect by decreasing the 5'-Nucleotidase activity in treated cells.

Lysosomal Marker

Lysosome is an animal cell digestive organelle. It contains 50 different hydrolytic enzymes. These enzymes are produced in the rough endoplasmic reticulum and targeted to lysosome. The lysosomal enzyme activity is greatly, increased indicating that the lysosomal membranes are lysed during involution of normal mammary glands. One among the lysosomal enzyme is the ACP, the chief marker of lysosome.

Acid phosphatase (ACP)

ACP hydrolyses the phosphodiester to monoesters, ACP levels is increased during the malignancy of liver and bone. The activity of acid phosphatase is high in prostate gland than in serum. The elevated levels of ACP are indications of metastasis of prostate cancer. The ACP levels are also increased in cancers of lung, breast, rectal and bone (Teitz, 1994). The table.2 represents the increased activity of ACP in control (group I), and there was a parallel decrease in the activity when the cancer cells were treated with the extract of *A.Scholaris* (group II and III).

Table. 2: Assay of ACP in Cancer and *Alstonia scholaris* Treated Cancer Tissue.

Experimental groups	ACP (Units /mg protein)
Group I	0.021
Group II	0.016a Φ
Group III	0.0026**

S.E.D. = 0.007, LSD (5%) = 0.014, LSD (1%) = 0.020

** Significant at 1% level, Φ = not significant

a = comparison between group I & II, b = comparison between group I & III

Group I: Control cancer mass Homogenate, Group II: 3% *A. scholaris* treated cancer homogenate

Group III: 4% *A. scholaris* treated cancer homogenate.

Units: ACP: μ moles of phenol liberated / min / mg protein.

Membrane markers

G.Block,(1992) has reported remarkable alterations in membrane bound compounds like enzymes, carbohydrate in malignant cells. So the membrane markers like ALP and γ -GT where chosen for the observation of anticancer effect of the leaf extract. As per table 3. Membrane marker level is high in the case of untreated carcinoma tissue. The extract treated tissues showed considerable decrease in the enzyme levels

Table. 3: Assay Of Membrane Markers In Cancer and *Alstonia scholaris* Treated Cancer Tissue.

Experimental groups	ALP (Units /mg protein)	γ -GT (Units /mg protein)
Group I	0.079	4.72
Group II	0.062a**	2.56a**
Group III	0.011b**	1.28b**

ALP: S.E.D. = 0.001, LSD (5%) = 0.003, LSD (1%) = 0.001

γ -GT: S.E.D. = 0.05, LSD (5%) = 0.11, LSD (1%) = 0.15

** Significant at 1% level,

a = comparison between group I & II, b = comparison between group I & III

Group I: Control cancer mass homogenate, Group II :3% *A. scholaris* treated cancer homogenate, Group III: 4% *A. scholaris* 1 treated cancer homogenate.

Units: ALP : μ .moles of phenol liberated / min / mg protein, γ - GT : units / min / mg protein

Alkaline phosphatase (ALP)

ALP, is a membrane bound enzymes cleaves, phosphoric acid from any mono-ester phosphates. ALP levels are increased in liver cancer. Highest levels are seen in patients with osteoblast lesions. Malignant disease is often associated with abnormal plasma enzyme concentration or a change in the normal isoenzyme pattern. Elevated levels are seen in different types of cancers likely, lung, colon and ovarian cancer.

The table.3 represents an increased alkaline phosphatase activity in the control (group I), the neoplastic cells before treatment. The activity of ALP seems to be decreased after the treatment with the drug. This infers the anticancer effect of the extract, that is membrane lysis has occurred and denatured the membrane proteins.

γ -Glutamyl transferase (γ -GT)

GGT is a peptidase enzyme, that catalyses the hydrolytic cleavage of peptide to form amino acids or even smaller peptides. GGT activity is elevated in all types of liver diseases.

High levels of GGT are present in the prostate gland. In general increase GGT activity must first arouse suspicion that the disease is metastatic to the liver. This elevation is due to the depletion of GSH, which may induce the enzyme activity on increased synthesis of its mRNA.

The increased activity is a good marker of metastasis. The control cells shows an increased activity presenting malignant status of breast tissue. The reduction in the activity of the enzyme after the treatment with the extract indicates the enhanced GSH synthesis (group II & III). Sridharan *et al.*, 2001, has shown similar decrease in the activity GGT after the treatment of the cancer cells with N-acetylcysteine (NAC), *in vitro*.

Non - enzymatic antioxidants

Reduction of oxygen in the respiratory chain is often incomplete. Thus, some amount of free radical and other reactive oxygen species are continuously produced even under normal conditions, in all aerobic organisms. In view of disturbances that can be caused by free radicals, organisms have evolved not only antioxidant defense system to protect against them, but also repair system that prevent the accumulation of oxidatively damaging molecules (Sies, 1991). The most important non-enzymatic antioxidants are vitamin A, E & C.

Table. 4: Assay of Antioxidants in Cancer and *Alstonia scholaris* Treated Tissue.

Experimental groups	Vitamin A (μ g / gram tissue)	Vitamin B (μ g / gram tissue)	Vitamin C (μ g / gram tissue)
Group I	11.706	0.211	0.168
Group II	14.202a**	0.491a**	0.341a**
Group III	16.564b**	0.867b**	0.665b**

Vitamin A: SED = 0.151, LSD (5%) = 0.329, LSD(1%)= 0.017

Vitamin B: SED = 0.009, LSD (5%) = 0.329, LSD (1%) = 0.029

Vitamin C: SED = 0.008, SD (5%) = 0.020, LSD(1%)= 0.023

** Significant at 1% level

a = comparison between group I & II, b = comparison between group I & III

Group I: Control cancer mass homogenate, Group II: 3% *A. scholaris* treated cancer homogenate

Group III :4% *A. scholaris* treated cancer homogenate.

Units: μ g / gram tissue

Vitamin A

Vitamin A is a fat soluble vitamin, which is essential for growth, maintenance of visual function, reproduction and differentiation of epithelial tissue. Vitamin A and its deficiency has been associated with a higher incidence of cancer and increased carcinogenesis. A number of epidemiological studies have shown that low dietary intake of vitamin A or carotenoid were correlated with the increased incidence of mortality from lung or breast cancer. Experiments with laboratory animals suggested that vitamin A deficiency may enhance susceptibility to certain forms of chemical carcinogenesis. Vitamin A and its metabolites play a crucial role in regulating the differentiation and proliferation of epithelial cells. Retinoids have been shown suppress the growth and prevent the development of breast cancer in animals (Easwaran, 1999). As per table 4 Vitamin A level in group I (cancer cell) is found to be low when compared to group II and group III (treated samples). Ray *et al.*, 2002 found a significant change in vitamin A level in breast cancer patients after treatment. It suggests that leaf extract could improve the antioxidant status which could inhibit the proliferating cells.

Vitamin E

Vitamin E is thought to be an important chain breaking antioxidant which plays an important role in various stages of carcinogenesis through its contribution and immuno competence, membrane and DNA repair and decreasing oxidative DNA damage (Machlim and Bendich, 1987). *In vitro* studies showed that vitamin E can prevent oxidation of DNA by inhibiting activated neutrophils. Vitamin E can protect the conjugated double bond of β -carotene from oxidation (Kimmick *et al.*, 1997). Ray *et al.*, 2002 have shown lower Vitamin E concentration in breast cancer patients. The table.4 depicts low vitamin E level in group I when compared to treated samples (group II and III), which seems to be representing above mentioned works.

Vitamin C

Vitamin C is an important water soluble antioxidant in biological fluids and it is an essential micronutrient required for normal metabolic functioning of the body. Hydroxylation of aromatic drugs and carcinogens by hepatic cytochrome P450 is enhanced by reducing agents such as vitamin C. Vitamin C neutralizes Reactive Oxygen species and reduce oxidative DNA damage and genetic mutations. It can prevent carcinogenic nitrosamine formation in cancer, another protective function of vitamin C in modification of normal cells into cancer cells. Vitamin C may protect cell against carcinogenesis through several mechanisms in addition to inhibition of DNA oxidation (Frei, 1994).

Ray *et al.*, 2002 showed a significantly low levels of vitamin C in cancer patients. Similar lower levels of vitamin C is seen group I when compared to treated samples (group II and III). (Table.4). Production of O_2 was significantly high in breast cancer patients. Thus higher O_2 content may be one of the possible causative factor to reduce the vitamin A, B and C levels in cancer

patients. At outset markable changes in the level of the enzymes were observed in the results. The improvisation of antioxidant property after treatment was also observed. This clearly indicates that the leaf extract of *Alstonia scholaris* has an immense antimetabolic property and antiproliferating effect over mammary carcinoma.

CONCLUSION

The leaves of *Alstonia scholaris* on cancer cells/tumor cells has been justified by its cytotoxic effect or anti proliferative effect. The cytotoxic potency was proved only in the *in vitro* conditions. The potent anticancer effect at immunochemical level or molecular level or cytogenetic level could be analysed under *in vitro* by cancer cell lines and in *in vivo* condition using experimental rudiments. The potent or principle compound responsible in anticancer effect could be isolated and purified. The purified compound or identical synthetic compound could be studied in the similar manner and followed the potency in curing cancer.

REFERENCES

- Abhijit Dey. *Alstonia scholaris* R.Br. (Apocynaceae): Phytochemistry and pharmacology: A concise review. Journal of Applied Pharmaceutical Science. 2011;01(06): 51-57.
- Arathi G, Sachdanandan P. Therapeutic effect of *Semecarpus anacardium* Linn. nut.milk extract on carbohydrate metabolizing and mitochondrial TCA cycle and respiratory chain enzymes in mammary carcinoma rats". Journal of Pharmacy and Pharmacology. Inc. 2003; 55 : 1283-1290.
- Arulmozhi S, Papiya Mitra Mazumder, Sathiya Narayanan L, Prasad A. Thakurdesai. In vitro antioxidant and free radical scavenging activity of fractions from *Alstonia Scholaris* linn. R.Br. International Journal of PharmTech Research. 2010; 2(1):18-25.
- Block G, *et al.*,. Fruits, Vegetables and Cancer Prevention: A Review of the Epidemiologic Evidence; Nutrition and Cancer. 1992;187:1029.
- Bygrave, F.L., Control Mechanisms in Cancer .Cross W.E. *et al.*, eds, Raven Press, New York (1976) 411-423.
- Carl A. Burtis and Edward R. Tietz Textbook of Clinical Chemistry, Third Edition. Ashwood, eds. Philadelphia (1994) 911-998.
- Cornelia Hoch,Ligeti, Frank J Jaisen., Enzymes in Peripheral and bone marrow serum in patients with cancer. Cancer 1976; 38 : 1336-1343.
- C. K. K. Nair, P. Divyasree and G. Gopakumar. (2010). Ethnomedicinal plants to fight neoplastic diseases. In: Debprasad Chattopadhyay. Ethnomedicine: A Source of Complementary Therapeutics. (pp. 661). Publisher Research Signpost, Trivandrum.
- Eleanor G. Rogan. Cancer Preventive Properties Identified In Resveratrol, Found In Red Wine, Red Grapes. American Association for Cancer Research. (2008).
- Elliott B.A., and Fleming A.F. Source of elevated serum enzyme activities in patients with megaloblastic erythropoiesis secondary to folic acid deficiency. Br. Med. J. 1965; 1: 626-628.
- Easwaran. Simultaneous expression of antioxidants in human breast cancer cell lines. Breast cancer Res. Tr 1999: 40.
- Frei B ,Roberts L , Traber M G., Vitamins E and C in the prevention of cardiovascular disease and cancer in men. Free Radic Biol Med. 2009; 46(11):1558.
- Ganesh Chandra Jagetia , Manjeshwar Shrinath Baliga, Ponemone Venkatesh. Effect of *Sapthaparna* (*Alstonia scholaris* Linn) in modulating the benzo(a)pyrene-induced forestomach carcinogenesis in mice . Toxicology Letters . 2003 ;144:183

Karen Bradstreet. Herbs for Detoxification. Woodland Publishing, Australia (1997) 10-13.

Kimmick G G, Bell R A , Bostock R M., Vitamin E and breast cancer: a review. Nutr. Cancer. 1997; 27:109.

King J. "Methods for determination of enzyme activity". In Practical Clinical Enzymology. Dvon Mortrand Co., Ltd. London. (1965) 106-115.

Lowry O.H., Rosenbrough N.J., Farr A.L and Randall R.J. "Protein measurement with Folin's phenol reagent" J. Biol. Chem. 1951;193 :265-275.

Machlin L J, Bendich A. , "Dye-sensitized photooxidation of tocopherols. Correlation between singlet oxygen reactivity and vitamin E. FASEB J. 1987;1(6):441-44.

Potter, V.R. (1968). Mechanisms of carcinogenesis in relation to studies on minimal deviation hepatomas. In: Exploitable molecular mechanisms and neoplasia, (pp587-610.) Austin. University of Texas Press.

Premalatha Balachandran, Rajgopal Govindarajan. Cancer—an ayurvedic perspective Pharmacological Research. 2005; 51:19–30

Rathnakumar G, Raste A S, Can 5' nucleotidase estimation be a predictor of liver metastases? Indian J Cancer. 2000; 37(1):23-26

Ray and Husain. Oxidants, antioxidants and carcinogenesis. Indian J. Exp. Biology. 2002;1223-1225

Sies, H. Oxidative stress, oxidant and antioxidants, Antioxidant activity of fruit exudates and c-methylated dihydrochalcones from Myrica gale. Plant. A. Med. 1991; 61:515-518.

Sridharan S, Nalini Ramamurthy , Shyamala Devi C.S. *In Vitro* Evaluation of The Anticancer Effect Of N-Acetylcysteine On Oral Carcinoma Cell Line. Indian Journal of Pharmacology 2001; 33: 343-349.

Stephan L Wolfe. Molecular and cellular Biology. 2nd ed. Words worth Publishing Company. (1995) 942-947.

Trichopoulos D., Mac Mohan B., Cole P. Menopause and breast cancer risk. JNCI .1968; 41:315- 329.