RESEARCH ARTICLE

Anticancer Activity of *Acacia nilotica* (L.) Wild. Ex. Delile Subsp. *indica* Against Dalton's Ascitic Lymphoma Induced Solid and Ascitic Tumor Model

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Abstract

The aim of the present investigation was to evaluate the effect of *A.nilotica* extract against Dalton's ascitic lymphoma (DAL) induced solid and ascitic tumors in BALB/c mice. Experimental animals received *A.nilotica* extract (10 mg/kg.bw) intraperitoneally for 10 and 14 consecutive days before induction of solid and ascitic tumors, respectively. Treatment with *A.nilotica* extract significantly decreased the development of tumor and percentage increase in body weight when compared to DAL induced solid tumor control group, also increasing the life span, restoring the total white blood cell count and hemoglobin content and significantly decreasing the levels of serum aspartate transaminase (SGPT), alanine transaminase (SGOT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and nitric oxide (NO) when compared to DAL induced ascitic tumor controls. The treatment also reduced significantly the cellular glutathione (GSH) and nitric oxide levels in treated animals. Histopathological studies also confirmed protective influence. The outcome of the present work indicates that *A.nilotica* extract could be used as natural anticancer agent for human health.

Keywords: *Acacia nilotica* - Dalton's ascitic lymphoma - solid tumor - ascitic tumor - anticancer

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Introduction

Over the past decade, Cancer is the leading cause of death worldwide and it is characterized by uncontrolled growth and spread of abnormal cells. World Health Organization (WHO) reported that there are 7.6 million deaths in 2008 and it is estimated up to 13.1 million deaths in 2030 (Merel et al., 2012). Treatment of cancer varies according to each type, has been facing large number of problems. Several ways in the treatment of cancer have been developed. Currently cancer is treated using surgery, radiation, and chemotherapy which are associated with severe side effects (Garcia et al., 2001; Edy et al., 2012). Even a large number of tumors are scantily responsive to cancer therapeutic drugs and radiotherapy. Identification and development of natural products used for cancer prevention have attracted a lot of attention globally. Herbal extracts with their proven potential and less side effects in therapeutics has replaced the synthetically derived drugs in modern allopathic medication system (Sakthivel and Guruvayoorappan, 2012). Traditionally used large number of medicinal plants and plant products has become the potential source of antitumor agents. Traditional healers of different regions in India particularly Chhattisgarh used Acacia species for treatment of various cancer types of mouth, bone and skin (Kalaivani and Mathew, 2010).

Acacia species is one of the richest resources of bioactive flavonoids, alkaloids, phenolics, saponins, polysaccharides, tannins and terpenoids (Seigler, 2003). The published reports of various biological activities of Acacia species include hypoglycemic, anti-inflammatory, antitumor (Lam and Ng, 2010), antifungal (Lopes et al., 2009), antiplatelet aggregation, spasmogenic and vasoconstrictor, antihypertensive, anti-hepatitis C virus (Lee et al., 2011) antioxidant potential (Singh et al., 2010), wound healing (Tung et al., 2008), antinociceptive activity (Dongmo et al., 2005), chemopreventive and antimutagenic (Meena et al., 2006), anthelmintic activity (Bachaya et al., 2009). Several bioactive agents have been identified from the various species of acacia which includes androstene steroid, gallic acid, ellagic acid, isoquercitin, kaempferol, naringenin, rutin, lupane, niloticane, umbelliferone and catechin (Mutai et al., 2004; Eldeen et al., 2005; Chaubal et al., 2006; Singh et al., 2010).

Among the Acacia species, *Acacia nilotica* (L.) Wild. Ex. Delile subsp. *indica* occupies an imperative place in the indigenous system of medicine against various diseases. Different parts of *A.nilotica* have been used a remedy against variety of diseases in traditional medicine, in particular bark is used to treat diarrhoea, dysentery and leprosy, root is used in the treatment of tuberculosis and leaves are used to treat ulcers. So far several biologically active compounds include umbelliferone, gallic acid,

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niloticane, catechin, kaempferol, rutin, apigenin and two steroids include androstene and β-sitoterol were isolated from different parts of *A.nilotica* (Brahma et al., 2009). Numerous studies in *A.nilotica* showed interesting biological activities (Dongmo et al., 2005; Meena et al., 2006; Tung et al., 2008; Bachaya et al., 2009; Lopes et al., 2009; Lam and Ng, (2010); Singh et al., 2010; Lee et al., 2011) however, no antitumor activity against DAL induced solid and ascitic tumor has been reported. Therefore based on the ethno medical claims, the present study was planned to explore the possible *in vivo* antitumor activity of aerial parts of *A.nilotica* against DAL induced solid and ascitic tumor in BALB/c mice.

Materials and Methods

Collection of Plant Material

The fresh aerial parts of the *A.nilotica* plant were collected from Annur near Coimbatore, India. The plant was authenticated at Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2012-13/tech-459). The voucher specimen of the plant has been retained in the Department of Biotechnology, Karunya University, Coimbatore. The collected plant sample was washed thoroughly with running tap water and completely shade dried under room temperature.

Preparation of extract

The shade dried aerial parts of the plant were subjected to mechanical size reduction. Then the powdered material was extracted with methanol by using soxhlet apparatus. The solvent was removed by evaporation and extract was concentrated by using vaccum rotatory evaporator. The yield of the extract was found to be 11.5%.

Animals

Male BALB/c mice of body weights ranging from 22-25g were obtained from Animals Breeding Station, Mannuthy, Thrissur. The animals were fed with standard pellet diet (Sai Durga feeds, Bangalore, India) and water ad libitum. They were maintained in controlled environment (12:12 h light/dark cycle) and temperature (30±2ºC). All the animal experiments were performed according to the guidelines of the Institutional Animal Ethical Committee, Govt. of India.

Cell line

The DAL cell line was obtained from Amala Cancer Research Institute (Thrissur, India) and was propagated into transplantable tumors in the peritoneal cavity of BALB/c mice. The freshly aspirated cells from the mouse peritoneum were washed with Phosphate Buffer Saline (PBS) under sterile conditions and their concentration was determined using a hemocytometer before transplantation. Animals were inoculated with 1×10^6 cells/mouse.

Drugs and chemicals

Gum acacia was purchased from Hi-Media (Mumbai, India), Drabkin's solution from Nice Chemicals Pvt. Ltd. (Cochin, India). Ethylenediaminetetraacetic acid (EDTA) from Merck. Methotrexate from IPCA Laboratories (Mumbai, India). All other chemicals used were analytical reagent grade.

Toxicity studies

Acute *in vivo* toxicity studies with different concentrations of *A.nilotica* methanolic extract was carried out to determine the LD_{50} value by the Miller and Tainter method (Miller and Tainter 1944). No deaths or adverse effects were detected during the 24 hour observation period in mice treated with up to 2000 mg/kg.bw of *A.nilotica* extract (data not shown). Based on these results and previous literature report (Punar et al., 2006; Ramya et al., 2011), the dose at the concentration of 10 mg/kg.bw was chosen for the experiments.

DAL - induced solid tumor studies

Solid tumors was induced by injecting DAL cell lines (1.5×10^6 cells/mouse) intramuscularly to the right hind limb of BALB/c mice and divided into 3 groups containing 6 mice in each group. Group I served as tumor control and received vehicle PBS alone. Group II was treated with standard drug methotrexate at a dose of 3.5 mg/kg.bw dose. Groups III was treated with extract at 10 mg/kg.bw dose. All the treatments were given IP at 24 hour after DAL tumor inoculation and continued for 14 consecutive days. Initial diameter of the right hind limb was noted using vernier caliper. From the 3rd day onwards, tumor diameter was measured every third day and recorded up to 30 days. The tumor volume was calculated by the following formula: $V = 4/3\ \pi r_1^2 \times r_2$, where v is volume, r_1 and r_2 are the radii of tumors at two different planes. All data were expressed as mean (±SD).

DAL - induced ascitic tumor studies

Animals were divided in to three groups. All the group were inoculated with DAL (1.5×10^6 cells/mouse) and Group I, served as the tumor control. Groups II was treated with standard drug methotrexate at the dose of 3.5 mg/kg.bw, which served as a positive control. Groups III was treated with extract at 10 mg/kg.bw. All the treatments were given IP at 24 hour after DAL tumor inoculation and continued for 14 consecutive days. The blood was collected from each group of animals by using tail vein method at two different points, 10th and 15th day. The hematological parameter of total WBC count and hemoglobin content (Hb) were estimated. The remaining blood was centrifuged and serum was used for the estimation of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and Gamma Glutamyl Transferase (GGT) by using Kit method according to the manufacturer's instruction (AGGAPPE Diagnostics, Kerala, India). Nitric oxide (NO) was measured by using (Green et al., 1982). The ascitic fluid was aspirated from the peritoneal cavity on 10th and 15th day, sonicated for 30 seconds and used for measuring NO and Glutathione reductase (GSH) by using (Szasz et al., 1976). All data were expressed as mean (±SD).

Determination of Mean Survival Time (MST), % Increase of Life Span (ILS) and Average Body weight changes

Animals were divided in to four groups containing 6

animals each. Group I served as Tumor control, Group II served as positive control treated with standard drug methotrexate (3.5 mg/kg.bw), Group III mice was tumor induced and treated with *A.nilotica* extract (10 mg/kg.bw) and Group IV (non-tumor injected group) was treated with extract alone for 14 consecutive days. The animals were monitored daily twice for 50 days. Antitumor effect of *A.nilotica* was determined by monitoring the death pattern of animals due to tumor burden using Mean survival time and calculating the percentage increase in life span (%ILS). The percentage increase in lifespan was calculated using the following formula: ILS (%) = [(Mean survival of treated group - mean survival of control)/Mean survival of control group]×100

The body weight (BW) of the control and treated group animals were measured from 0th day to 30th days of three days interval up to 30 days. All data were expressed as mean (±SD).

Histopathological analysis

A small portion of liver was taken and fixed in to 5% formaldehyde. After several treatments for dehydration in alcohol, sections having 4 µm thickness were cut and stained with haematoxylin and eosin and histopathological analysis was carried out for the treated as well as control group of mice.

Statistical analysis

Values are expressed as mean (±SD). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed By Dunnett'test using Graphpad InStat version 3.0, GraphPad Software, San Diego, California, USA. p-Values (i.e., *p<0.05, **p<0.01) were considered statistically significant compared to DAL tumor control.

Results

Effect of A.nilotica extract on solid tumor development and body weight in DAL induced mice

Treatment with *A.nilotica* extract for a period of 14 consecutive days in the DAL induced solid tumor bearing mice showed a significant (**p< 0.01) reduction in tumor volume (1.12±0.07 mm^3) when compared to the tumor alone group (2.52±0.08 mm^3) on 30th day. The animals treated with the standard drug (methotrexate 3.5 mg/kg BW) were found to be also efficient (0.94±0.09 mm^3) in preventing the development of solid tumor on the same day as shown in Figure 1. Body weight was measured every 3 day interval throughout the period of experiment. By Day 30, there was a significant increase (Day 0) in the body weight (up to 29.1±0.4 g) of the tumor-bearing control animals. In extract treated animals, there was a significant (**p<0.01) reduction in the body weight (25.5±0.41 g) was observed. In contrast, the animals treated with the standard drug (methotrexate) were found to be (24.8±0.42 g) on the same day (Figure 2).

Effect of A.nilotica on MST, % ILS and Average Body weight changes in DAL bearing mice

Administration of extract for 14 consecutive days prolonged the lifespan resulted in significant (**p<0.01) increase in survival time of treated animals (26±0.89) days when compared with untreated tumor controls (16.7±1.08) days. The percentage increase in lifespan of extract treated animals and standard methotrexate was found to be 56% and 79% respectively. Furthermore, the anticancer activity of extract was also evidenced by the significant reduction in percentage increase in body weight (9.45%) when compared with the DAL tumor control (38.9%). The standard methotrexate and treated with extract alone (non tumor animals) also produced the similar results, 13.5% and 12.6% respectively as shown in Table 2.

Effect of A.nilotica on Hematological parameters

Inoculation of DAL cells resulted in a significant increase in the level of total WBC (14.9± 0.70 and 15.7±0.45) on 10th and 15th day respectively when compared with the normal animals (untreated). Administration of extract reversed these changes up to (12.2±0.63 and 12.9±0.37) on 10th and 15th day respectively and this was comparable with the results produced by the standard drug on the same day. Hemoglobin content was significantly reduced in DAL induced animals (10±0.49 and 10.2±0.39) on 10th and 15th day respectively when compared with normal animals (untreated). Both extract and standard drug significantly (**p<0.01) prevented the anaemic condition due to inoculation of DAL cells (14.7±0.35 and 15±0.40) on 15th day respectively. The results evidenced that administration of extract significantly reverses the DAL induced change in total WBC and hemoglobin content shown in Figure 3.

Effect of A.nilotica on serum AST, ALT, ALP, GGT and NO levels in DAL bearing tumor mice

The effect of *A.nilotica* on serum AST, ALT, ALP, GGT

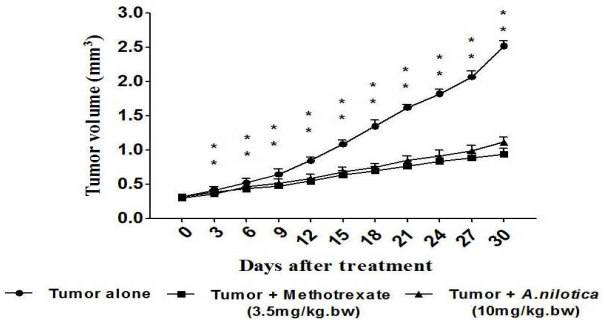

Figure 1. Effect of the Methanolic Extract of *A.nilotica* on Tumor Volume on the Indicated Days After the DAL Cells Intramuscular Injection.

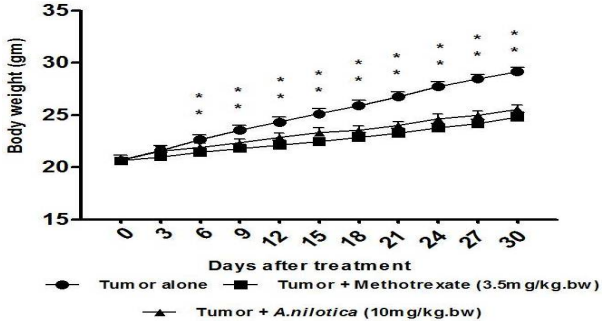

Figure 2. Effect of the Methanolic Extract of *A.nilotica* on Body Weight on DAL Cells Injected-Mice.

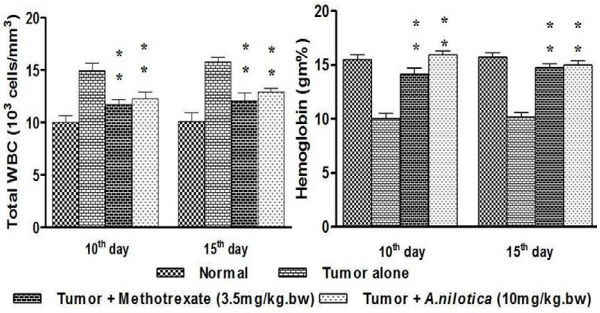

Figure 3. Effect of the Methanolic Extract of *A.nilotica* on Haematological Parameters. Treated animals received fourteen doses of methanolic extract of *A.nilotica* (10 mg/kg.bw).

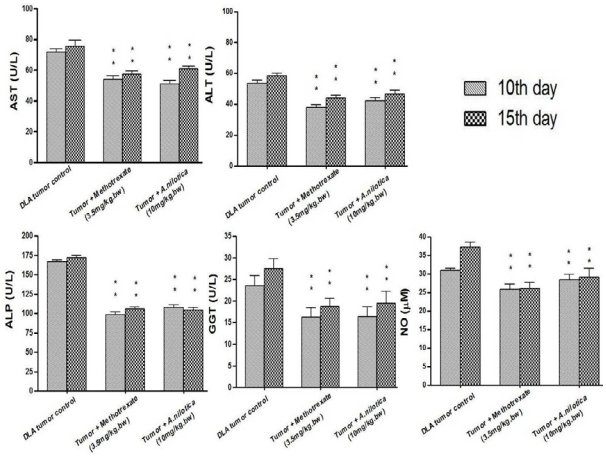

Figure 4. Effect of *A.nilotica* on Serum Biochemical and Antioxidant Parameters in DAL Bearing and Extract Treated Animals.

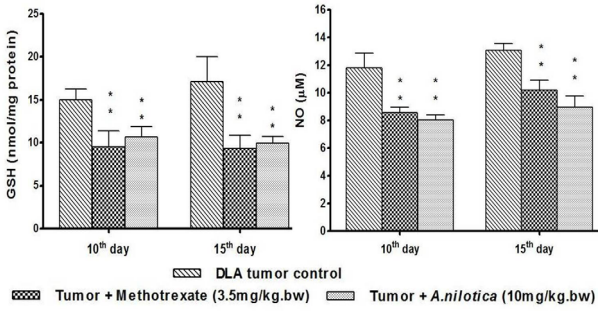

Figure 5. Effect of *A.nilotica* on Cellular GSH and NO Levels of DAL Cells at Tumor Growth in vivo.

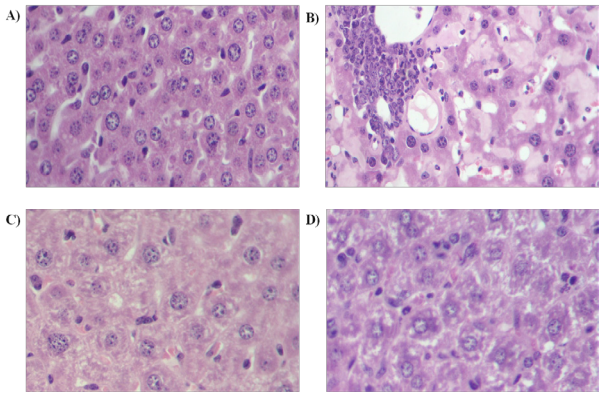

Figure 6. Histopathological Changes in Liver of Experimental Mice. Pictures shown are from representative liver sections collected at the end of the experimental periods (i.e., Day 15 after DAL cells intraperitoneal injection).

Table 1. Effect of *A.nilotica* on Body Weight, Mean Survival Time, Increase in life Span in DLA Bearing Ascites Tumor Models

S.No	Treatment design	MST (in days)	Increase in Lifespan (%)	Percentage increase in Bodyweight (g)
1.	Control	>50	-	10.2
2.	DLA	16.7±1.1	-	38.9
3.	Tumor Control Tumor + Methotrexate (3.5 mg/Kg.bw)	29.8±1.2**	79	13.5
4.	Tumor + *A.nilotica* (10 mg/Kg.bw)	26 ±0.9**	56	9.4
5.	*A.nilotica* Extract alone (10 mg/kg.bw)	>50	-	12.6

*N=6 animals in each group. Values are expressed as Mean±SD, p-values * p<0.05, **p< 0.01 were considered statistically significant from DLA tumor control.

and NO levels in DAL bearing tumor mice are presented in Figure.4. The levels of AST, ALT and ALP in serum on 10th and 15th day were significantly increased in DAL tumor control group as compared to the AST, ALT and ALP of normal group (45.9±1.33, 32.8±1.81, 94.1±2.10 on 15th day) respectively. After administration of extract to the tumor bearing animals, the levels of AST, ALT and ALP were significantly (**p<0.01) reduced as compared to the tumor control groups. The standard drug methotraxate also produced the similar result when compared with the treated group. On the 15th day after the initial tumor challenge, elevated level of GGT and NO (27.4±2.29 U/L and 37.3±1.22 μM respectively) in the serum of tumor bearing control animals as compared to the normal animals (15.9±1.40 U/L and 24.3±0.93 μM respectively) was significantly (**p<0.01) reduced to (19.4±2.70 U/L and 29.1±2.37 μM respectively) after administration of *A.nilotica* extract. This reduction rate was comparable with the standard drug methotraxate was found to be (18.7±1.86 U/L and 26.1±1.69 μM respectively).

Effect of A.nilotica extract on cellular GSH and NO levels in DAL bearing tumor mice

The effect of *A.nilotica* on cellular GSH and NO levels during the tumor progression is represented in Figure 5. The GSH content in DAL bearing tumor cells was found to be maximum (17.1±2.85 nmol/mg protein) on the 15th day. In extract and standard drug treated animals the cellular GSH level was significantly (**p<0.01) reduced up to 9.9±0.84 and 9.3±1.51 nmol/mg protein respectively. The cellular nitric oxide level in the DAL cells was found to be maximum (13.1±0.49 μM) on 15th day of tumor progression. In extract and standard drug treated animals the cellular NO level was found to be 8.9±0.77 and 10.2±0.73 μM respectively.

Effect of A.nilotica on histopathology

The histopathological observation of liver section of normal, DAL tumor control, standard drug methotrexate and extract treated animals collected at the end of the experimental periods (i.e., Day 30 after DAL intraperitoneal injection) were shown in Figure.6. Normal untreated animals showed normal lobular architecture with intact central vein and sinusoids, normal portal

tracts and preserved hepatocytes. DAL induced animals showed necrosis, surrounding fibrosis, perivenular inflammation and vacuole formation. However, mice treated with *A.nilotica* and methotrexate showed reduced vacuole formation and inflammation and almost normal hepatocellular architecture was observed. Histopathological examination showed a protective effect of *A.nilotica* on hepatotoxicity.

Discussion

Cancer is a multi-mechanistic second largest disease in the world require a multidimensional approach for its treatment, control and prevention. Plant based drugs form an important component of total medicines available for treating various human diseases. The use of phytochemicals in cancer prevention has received considerable interest in the past few decades owing to certain discoveries with specific properties include anti-oxidant and anti-inflammation. Recently, a number of anti-cancer agents have become recognized therapies in the clinical setting which include: vinca alkaloids, taxanes, podophyllotoxin, camptothecin and its derivatives (Otsuki et al., 2010). A number of additional plant-derived agents are currently under investigation for example Homoharringtonine, 4-Ipomeanol and ß-lapachone (Adriana et al., 2001).

Among the Acacia species, especially *A.nilotica*, a plant with established medicinal properties was chosen for this study. It has been reported as important medicinal plant used in folk medicine to treat various ailments. Based on this information and previous biological studies, we decided to investigate its anticancer effect against DAL induced solid and ascitic tumor condition. The reliable criteria for judging the quality of any anticancer drug are prolongation of lifespan and its effect on hematopoietic system (Isha et al., 2011). Administration of *A.nilotica* extract at concentration of 10 mg/kg.bw showed increase in mean survival time and percentage increase in life span, decrease in percentage of increase in body weight (due to reduction of tumor burden) when compare to control DAL bearing ascitic tumor group. Myelosuppression and anemia have been frequently observed in ascites carcinoma condition. and similar findings were observed in our present study. In DAL bearing tumor control animals, elevated total WBC count and reduced hemoglobin content was observed. Moreover, *A.nilotica* extract showed a protective effect on hematopoietic system by reversal of total WBC cells and hemoglobin content in DAL bearing animals towards the value of normal group animals when compare to DAL bearing ascitic tumor animals.

To investigate the inhibitory effect on ascitic tumor was local or systemic, the effect of administration of *A.nilotica* extract was tested against solid tumor induced by DAL cell lines. The abnormal mass of tissue that does not contain cyst or liquid is referred as solid tumor and is mostly epithelial in nature (Kushi et al., 2011). We observed significant inhibition of solid tumor volume and reduction of body weight in solid tumor bearing animals when compare to control DAL induced solid tumor animals undoubtly suggests that the inhibitory effect of *A.nilotica* is systemic, not only related to its local cytotoxic effect. This inhibitory effect on tumor volume and protection of hematopoietic system was comparable with the result produced by the standard drug methotrexate. AST and ALT were found in serum and various body tissues but are mostly associated with liver parenchymal cells. The elevated level of AST and ALT will be observed in acute liver damage condition. In addition, the level of ALP will rise with intrahepatic cholestasis and infiltrative diseases of the liver (Gaze, 2007). Similarly in our present study, we observed elevated level of AST, ALT and ALP in DAL induced ascitic tumor animals when compare to DAL induced tumor alone group. Administration of *A.nilotica* extract and standard drug methotrexate exerted a protective effect by reversal of these enzyme levels nearly towards normal value of animals. Gamma glutamyl transferase, an enzyme involved in cellular glutathione homeostasis which is often increased in level in tumor condition. The membrane bound enzyme GGT is expressed highly in embryo livers and decreases rapidly to lowest levels after birth. GGT is highly re-expressed during the development of (HCC) Hepatocellular carcinoma (Pompella et al., 2006; Lei et al., 2012). Treatment with *A.nilotica* significantly lowered the enhanced level of γ- GT in tumor bearing animals when compare to tumor control.

The major non-protein thiol, GSH is required for the tumor cell proliferation and its metabolism (Guruvayoorappan and Kuttan, 2007). Cancer cells have higher GSH levels than the surrounding normal cells, which is characteristic of higher cell proliferation rate and resistance to chemotherapy. Scientific evidence shown that combining GSH depletion using 1,3-Bis(2-chloroethyl)-1-nitrosourea chemotherapy with superoxide dismutase gene therapy could be considerably successful in the treatment of breast cancer. When the intracellular GSH levels are low, the cells are more susceptible to ROS attacks. Increased ROS might activate different intracellular oncogenic pathways which lead to activation of tumorigenesis process (Weydert et al., 2008). However, the excessive levels of ROS stress can also be toxic to the cancer cells. Therefore, changing ROS levels by GSH modulation is a way to selectively kill cancer cells without causing toxicity to normal cells (Makiya, 2008; Trachootham et al., 2009). Administration of *A.nilotica* extract significantly reduced the level of intracellular GSH in extract treated DAL tumor cells when compare to the non-extract treated DAL bearing animals. Moreover, the treatment with extract also reduce the level of Nitric oxide production in serum and tumor cells when compare with tumor control animals. Since, nitric oxide is an important regulator of tumor growth and involved in various pathophysiological process includes inflammation and carcinogenesis (Hong, 2002).

Phenolics and flavonoids display a wide range of biological and pharmacological properties and normally scavenge the free radicals and play an essential role in prevention and therapy of cancer. It is well documented that *A.nilotica* is one of the rich source of these flavonoids and phenolics. For example, the polyphenolic compound

Kaempferol displayed radical scavenging activity in different in vitro assays (Rajbir et al., 2008). Niloticane isolated from the bark of the *A.nilotica* showed anti-inflammatory property by inhibition of Cyclooxygenase enzymes which is involved in inflammatory process (Eldeen et al., 2010). ß-sitosterol also showed anti-oxidant and anti-inflammatory activity, which is used in the treatment of inflammatory disorders, breast cancer and colon cancer (Padmasri and Sarada, 2011). Similarly, gallic acid and catechin showed protective effect against N-nitrosodiethylamine-induced hepatocarcinogenesis (Brahma et al., 2009). Umbelliferone is also reported as potential scavenger of free radicals which is present in bark and leaves of *A.nilotica* (Rajbir et al., 2010). Recently, a report indicates that apigenin can act as potential chemopreventing agent due to induction of leukemia cell cycle arrest. Apigenin inhibited phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) pathway in HL60 and induced caspase-dependent apoptosis (Rajbir et al., 2010). Androstene also exhibits dose dependent anti-inflammatory property against TPA (12-O-tetradecanoylphorbol-13-acetate) induced mouse ear edema (Chaubal et al., 2006). The anti-angiogenic effect of rutin and its regulatory effect on the production if VEGF, IL-1β and TNF-α in tumor associated macrophages was also demonstrated (Guruvayoorappan and Kuttan, 2007).

The outcome of present investigation undoubtedly indicate that, the treatment with *A.nilotica* was effective on inhibiting the tumor progression in *in vivo* models, most likely because of high content and synergistic activity of specific constituents present in the extract such as umbelliferone, gallic acid, niloticane, catechin, kaempferol, rutin, apigenin, androstene and β-sitoterol derivatives may exert these preventing effects. However, the exact molecular mechanism by which *A.nilotica* mediates its antitumor activity is still not clear. Further we planned to identify more precisely the lead component responsible for anti tumor activity and to unveil the molecular mechanism behind its therapeutic action.

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