CHEMOTHERAPEUTIC EFFICIENCY OF SAPONIN EXTRACTED FROM ARTEMISIA PALLENS WALLS WITH REFERENCE TO DALTON’S LYMPHOMA ASCITES TUMOR MODEL.

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ABSTRACT

Plants produce a diverse range of bioactive molecules, making a rich source of different types of medicines. Natural products play an important role in drug development programs in the pharmaceutical industries. Extracts of Artemisia pallens was screening analyzed for their therapeutic capacity against cancer cells. Phytochemical screening of the leaf extract of Artemisia pallens showed the presence of alkaloid, phenols, glycosides, saponin, steroids and triterpene. Purified saponin was administrated in the dalton’s lymphoma ascites tumor induced mice model. The present work is carried out in order to evaluate the efficacy of the saponin as formulated medicine for the treatment of cancer. Saponin significantly increased the survival time in the tumor mouse model by about 50% in comparison with tumor controls. Saponin had a role to decrease the volume of ascitic fluid in tumor-bearing mice by 80%, thereby returning body weight to normal. Cytotoxicity might be reduced due to the protection of white blood cell and platelet counts in ascitic fluid from the tumor-bearing mice were brought to near-normal range. Molecular changes revealed the possibility of apoptosis induction by the saponin treatment in tumor-bearing mice led to a significant reduction in the number of malignant cell clumps for the treated group when compared with the control group, reflecting the potential of saponin to have cytotoxic effects in tumor cells, without affecting normal cells. Antitumor properties of sapanin suggested may be effective alternative drug component in the treatment of cancer.

KEYWORDS: Artemisia pallens, Antitumor, Ascite Dalton’s lymphoma, Sapanin.
INTRODUCTION
Phytomedicine and chemosynthetic pharmaceutical research find themselves in a race to develop new medicines with no side-effects for therapeutic and preventive application. The standard drug therapy for cancer in classical medicine uses cytostatic chemotherapeutics, which, in ideal conditions, arrest or destroy tumour cells. Most of the cytostatic drugs possess severe side-effects and reduce the quality of life.[1] Apart from some exceptions, these drugs are not able to heal cancer patients or to extend their life span by more than 5 or 10 years. The new strategy follows a quite different concept. This multi target therapeutic concept requires a cocktail consisting of several individual or multivalent drugs, which, in a concerted and synergistic way, might be able to arrest the tumour growth.

*Artemisia pallens* Walls. ex DC, commonly known as Davana, is an aromatic herb found abundantly in humid habitats in the plains all over India. *Artemisia pallens* is found in Nilgiri hills and has been used by the tribal people for various ailments.[2] Artemisia species are invariably found as small fragrant shrubs or herbs and most of them yield essential oils. *Artemisia umbelliformis* is traditionally used to treat loss of appetite and digestive spasms, also some other *Artemisia* species were used as stomachic, stimulant, flavoring, antioxidant, antibacterial, anti-inflammatory, antispasmodic, carminative etc. *Artemisia pallens* walls leaves and flowers yield an essential oil known as oil of Davana. Several species yield essential oil and some are used as fodder, some of them are a source of the valuable antihelmintic drug santonin. *Artemisia species* are popular among gardeners as cultivated ornamentals.[3] *Artemisia pallens* has been widely used in Indian folk medicine for the treatment of diabetes mellitus and cancer.[4] The search for novel drugs is still a priority target for cancer therapy due to the fact that chemotherapeutic drug resistance is becoming more and more frequent.

Chemotherapy is the treatment of cancer with drugs (anticancer) that can destroy cancer cells. It interferes with cell division in various possible ways with the duplication of DNA or the separation of newly formed chromosomes most forms of Chemotherapy target all rapidly dividing cells and are not specific for cancer cells.[5] Drug targeting is an approach to improve the therapeutic index of drugs by manipulating the disposition of the drug in the body. Ascitic fluid plays a crucial role in DLA and is a collection of pleomorphic cells with hyper chromatic nuclei that are clumps of malignant cells. Researchers are focused to develop novel therapeutic and diagnostic modalities for human use.[6] The present study demonstrates the
efficacy of natural active compound from *Artemisia pallens* plant and the purified phyto material formulated as an antitumor agent using Dalton’s lymphoma ascites (DLA) cell lines in vitro and in vivo. The objective of the present study was to determine the effects of Sapanin on Dalton’s lymphoma ascites (DLA) tumorigenesis in mice model.

**MATERIALS AND METHODS**

**Plant material**

*Atremisia pallens* were collected from in and around Ootacamund, belonging to the district Nilgiris of Tamil Nadu state. The plant was identified and authenticated by Medicinal Plants Survey and Collection Unit, Ootacamund, Tamil Nadu, India.

**Extraction procedure**

The dried plant material was powdered (10gms) and passed through sieve no. 20 and extracted separately using methanol by soxhlation. The extracts were concentrated to dryness under reduced pressure and controlled temperature. All extracts were preserved in refrigerated condition till further use.

**Phytochemical analysis of plant extracts for active components**

Phytochemical screening of the extract was carried out according to the methods described by[7] and Trease and Evens[8] for the detection of active component like saponins, tannins, alkaloids, tannins, glycosidese etc.

**Saponins:** (i) Frothing test: 2cm$^3$ of the extract was vigorously shaken in the test tube for 2 minutes. Frothing was observed. (ii) Emulsion test: 5 drops of olive oil was added to 3cm$^3$ of the extract in the test tube and vigorously shaken. Presence of stable emulsion formed indicates the presence of saponins.

**Experimental animal maintenance and tumour transplantation**

In vivo studies were conducted on female Swiss albino mice aged 5–6 weeks, weighing cages (five mice per cage) at an ambient temperature of 25 ± 2°C with a 12-hour light and 12-hour dark cycle. The mice were fed with commercially obtained rodent chow and water *ad libitum*. The animals were allowed to acclimatize to the laboratory environment and were then randomly subjected to the experiment. All the experiments were carried out as per the guidelines of the institutional animal ethics committee and had prior approval from the same committee. Ascites Dalton’s lymphoma tumor was maintained in vivo by intraperitoneal (ip)
transplantation of 1X10^6 tumor cells per animal (0.25 volumes, in phosphate-buffered saline, PBS). PBS was prepared by adding 0.15 M NaCl to 0.01 M sodium phosphate buffer, pH 7.4. The DLA cell lines were maintained in mice models by aseptic serial transplantation in Swiss albino mice from tumor-bearing mice after the 10th day of ascites induction.\textsuperscript{[9]} Dal ton’s Lymphoma ascites was obtained from Amala Cancer Institute, Trisshur, Kerala Dt.

**Experimental schedule given below**

The mice were divided into four groups, with six animals in each group: Group 1, blank nontumor mice (nontumor, untreated); Group 2, tumor control mice (tumor induced, untreated); Group 3, tumor-induced mice treated with Saponin at a concentration of 100 µl in aqueous solution via intraperitoneal (IP) injection for 15 days at two days intervals; On the 17th day of mice in all the groups mice were subjected for experimental studies, fasting blood samples were collected from 3 animals from each batch and the rest of the animal were kept to check the survival time of DLA bearing mice, subsequently animals were sacrificed by anesthesia and organs tissue were isolated (liver, blood, ascites tumor) frozen in liquid nitrogen and stored at -80°C until biochemical analysis could be completed. If any death, of the animals in different groups were recorded daily and the survival pattern of the animals were determined for different group. The parameters such as survival time, packed cell volume, body weight, haematological parameters like RBC count, WBC count, were studied during the period of experiment (data not given).

**Measure of Viability**

This procedure can be performed along with the cell counting procedure but cell density may require adjustment in order to obtain approximately 10^6 cells/ml. Mix 1 drop of trypan blue with one drop of the cell suspension and allow 1 - 2 minutes for absorption. Prepare haemocytometer and load chambers as described in "Cell Quantitation". Count both the total number of cells and the number of stained (dark) cells Calculation: percent viability= \( \frac{\text{Total cell counted} - \text{Stained cells}}{\text{Total cells counted}} \times 100 \). The ascites tumor cells are obtained by aspirate with Phosphate buffer saline in the peritoneal Cavity of DLA bearing mice. The cells were then mixed with 0.4% Trypan blue in the ratio of 1:1 and the cells were counted using the Haemocytometer. (Live cells do not take stain whereas the dead cells get stained).

**Extraction of total RNA**

Ascites tumor cells were washed with PBS then homogenized with RNA ZOL (2ml per 100mg tissue) with few strokes in a glass Telflon homogenizer. The use of guanidium to lyze
cells was originally developed to allow purification of RNA from cells rich in endogenous ribonuclease. Guanidium denatures protein and thus inactivates any ribonucleases were present. RNA Extraction was carried out 0.2ml Chloroform was added to 2ml of tissue homogenate and the samples were tightly closed and shaken vigorously for 15 seconds and let them stay on ice for 15 minutes. The suspension was centrifuged at 12,000rpm (-4°C) for 15 minutes. RNA precipitation step was continued transfer of the aqueous phase to a fresh tube, added an equal volume of iso-propanol and stored the samples for 45 minutes at -20°C. Samples were centrifuged for 15 minutes at 12,000rpm (-4°C). RNA precipitate (often invisible before centrifugation) formed a white pellet at the bottom of the tube. The supernatant was removed and washed the RNA pellets twice with ice cold 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 12,000rpm (-4°C) and the pellets were dried under vacuum for 10-15 minutes. The RNA pellet was dissolved in 1mM EDTA, pH 7.0. Diethyl pyrocarbonate (DEPC) treated and RNase free solutions should be used for RNA solubilization. The final preparation was free from DNA and protein and pure RNA samples checked by measuring its OD at 260nm. RNA pellet was suspended in DEPC treated water and read at UV spectrophotometer, purity and quantity of RNA was calculated by A260/A280 ratio.

RESULTS AND DISCUSSION
Artemisia species invariably found as small fragrant shrubs or herbs and most yield essential oils. Phytochemical analysis of plant extracts for active components showed the presences of Saponin. Medicinal plants provide a wide array of hope through its phyto compounds which are behind to act in synergistic manner, providing excellent healing touch with no desirable side effects, provided its quality is assured. The effect of saponin were analyzed and it was observed posses highest pharmaceutical potential as antimicrobial and anticancer activity. Artemisia pallens extracts may be used to treat cancer.

Figure 1: Morphological View of A. PALLENS
Microscopical studied on leaf Anatomy showed the leaf is dorsiventral with isolateral mesophyll tissue (Figure 2). Thick mass of sclerenchyma cells occurs both on the upper and lower sides of the vascular bundle containing essential fluid had potential value for drug preparation. Crude plant extracts has been shown to be valuable sources of novel anti-cancer drugs. Drug development has implemented a large-scale project of acquisition and testing of compounds isolated from Indian medicinal plants.

Figure 3: Effect of Saponin on ascitic tumor volume and body weight.

Legend: X axis represented treatment groups in which C- indicates control (nontumor mice); T-indicates tumor mice (tumor induced, untreated); TS-indicates tumor-induced mice treated with Saponin at a concentration of 100 µl IP injections for 15 days at two days intervals. Each value represents the mean n=6.

To investigate the efficiency of plant based component saponin at a concentration of 100 µl IP injections for 15 days at two days intervals has administrated to tumour induced mice as mention in the methods. The injected doses were normalized to be 500 mg/kg and the tumour were calculated measured and staining data clearly revealed apoptotic cells Figure 4. The
treatment effect is confirmed by tumor staining that reveals significant apoptotic cells and very few proliferation active cells in the treated tumor. Thus, staining results clearly confirmed the treatment efficacy of saponin by inhibiting proliferation and inducing apoptosis of tumor cells. Saponin evidenced by its ability of slowing down tumor growth at administrated purified compound of saponin even at low dose affords markedly improved treatment efficacy over clinical.

Figure 4: Tumor staining for understanding of treatment effects.

Legend: Tumor from mice after different treatments indicated. 1- control mice; 2- tumors induced untreated mice; 3- tumor induced carboplatin-treated mice; 4- tumor induced combination of carboplatin and saponin treated mice 5- tumor induced saponin treated mice at final stage were undergoing apoptosis. Samples used for experiments were taken from tumor-bearing mice 12 d after initiation of treatment.

Bioactive material delivered to cancer cells is directly responsible for tumor suppression at a low dose of treatment. Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam-forming properties in aqueous solution. The presence of saponins has been reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anti-cancer properties.

Angiogenesis is the formation of new blood vessels to the tumor site is mainly induced and regulated by VEGF.\textsuperscript{[10]} Blocking angiogenesis is now considered to be a promising approach for anticancer therapy. Tyrosine kinase plays an important role in cancer angiogenesis and crucial for supplying vasculature to provide nutrient and remove waste material. Saponin compound was found to both inhibit the in vitro growth of ascites tumor transformed cells and reduce tumor size. In general antiangiogenic drugs stops new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass, thus shrinks the tumor by limiting blood supply.
One approach to address the problem may be to test these inhibitors in patients where serial ascitic/pleural fluid sampling in patients with malignant ascites or pleural effusions.\textsuperscript{[11]} Alternatively, circulating tumour cells can be harvested from the peripheral blood and subjected to target inhibition analysis which evaluated the effect of a drug on its target tissue.

The intact total RNA isolated and the yields were assessed to check the yield and quality of RNA content of experimental samples. However, measuring RNA level was critically important to have a good quality of RNA in any of expression study. RNA assessment was carried out to ensure the reliability of the RNA samples also total RNA sample has shown distinct 18S and 28S subunit spikes, with 2:1 ratio (28:18S). Gene expression can be assessed by measuring the quantity of the final product of protein. It has been clear that cells transcribe more RNA than they accumulate, implying the existence of active RNA degradation systems. In general, RNA is degraded at the end of its useful life, which is long for a ribosomal RNA but very short for excised introns or spacer fragments and is closely regulated for most mRNA species. RNA molecules with defects in processing, folding, or assembly with proteins are identified and rapidly degraded by the surveillance machinery. RNA degradation is a prevalent activity, many of the enzymes and cofactors involved in RNA processing and degradation is multifunctional.\textsuperscript{[12]} Eukaryotic mRNAs carry protective 5′-cap structures that must be removed prior to 5′ exonuclease degradation. The regulated turnover of mRNAs is a key factor in the control of gene expression and an apparently universal feature of mRNA metabolism. Similarly, most characterized examples from the seemingly large numbers of unstable non-protein-coding RNAs (ncRNAs) undergo rapid and continuous degradation.\textsuperscript{[13]} This “constitutive” degradation makes these classes of RNA distinct from the many stable RNA species.

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Figure 5: Total RNA isolated from experimental mice.

Legends: Total RNA isolated from mice serum samples after different treatments indicated as follows: Lane 1 (control mice), lane 2 - tumor induced untreated mice; 3- tumor induced carboplatin-treated mice; 4- tumor induced combination of carboplatin and saponin treated mice; 5- tumor induced saponin treated mice.

The presence of bands in the Fig 5 shows the presence of RNA in the samples. The mice were treated with different combinations of drug and the changes in the RNA content were determined by agarose gel electrophoresis. The size of the band elucidates roughly the amount of RNA as seen in the figure as degradation is obtained in the RNA content by tumor induced untreated mice treatment with lane 2 and lane 4 showed the content of RNA is seen to be increasing. The increased in lane 5 in fig 5 recovered DNA contents in tumor induced saponin treated mice was noticed with time.

Total RNAs were prepared from DLA cells of peritoneal fluid revealed that saponin treatment in tumor-bearing mice led to a significant reduction in the number of malignant cell clumps for the treated group when compared with the control group (Figure 5), reflecting the potential of saponin to have cytotoxic effects in tumor cells, without affecting normal cells.

*Artemisia pallens* extracts contained saponin which showed dose-dependent cytotoxicity against DLA cells cytokine, leading to induction of apoptosis which was further confirmed through resulting nuclear fragmentation. Interferons possess direct tumor cytotoxicity and a capacity for immune modulations. They may act indirectly to recruit and activate leukocytes, augment expression of cell surface molecules, and induce the production of other intermediate cytokines. Studies have demonstrated that human interferon alpha and beta inhibit tumor growth in rodent glioma models. The viability of tumor cells in ascitic fluid can lead to further aggravation of disease and hence the morphology and number of cells in ascitic fluid of the controls and tumor-treated mice were observed by histologic analysis.
CONCLUSION
Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times. Successful inhibition of tumoral angiogenesis is an important step towards cancer therapy discovery, knowing very well that it plays a major role in tumor growth and metastasis. Tumor cells which are not able to stimulate angiogenesis in the presences of saponin leads to stop growing, the treatment efficacy of saponin-based drug delivery vehicles can be further improved because the current product is not yet fully optimized for tumor targeted drug delivery, also expected to further enhance treatment efficacy. As a result, new treatment strategies are emerging that target steps in the molecular pathogenesis of these tumors. One of the important challenges for the future is the development and implementation of sound clinical research methods that will enable investigators to identify active treatment regimens. This background knowledge of tumoral angiogenesis has created the right platform for scientists to come out with therapeutic methods that target the inhibition of tumoral angiogenesis. An effective method that inhibits tumoral angiogenesis will cut supply of oxygen and nutrients to tumor cells and therefore prevent their growth and metastasis. Present work have been able to identified inhibitors to tumoral angiogenesis, Moreover, the nonspecific inhibition on VEGF induced downstream pathways that brought about some side effects such as inhibition on growth or cell cycle arrest indirectly correlate with VEGF expression particularly on hypertension and endothelial bleeding. However we believe the development of novel compound based drug against VEGF will offer a promising solution to cancer therapies.

REFERENCES


