RESEARCH ARTICLE

Acacia ferruginea Inhibits Tumor Progression by Regulating Inflammatory Mediators-(TNF-a, iNOS, COX-2, IL-1ß, IL-6, IFN-γ, IL-2, GM-CSF) and Pro-Angiogenic Growth Factor-VEGF

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Abstract

The aim of the present investigation was to evaluate the effect of *A ferruginea* extract on Dalton's lymphoma ascites (DLA) induced tumours in BALB/c mice. Experimental animals received *A ferruginea* extract (10 mg/kg.b.wt) intraperitoneally for 14 consecutive days after DLA tumor challenge. Treatment with extract significantly increased the life span, total white blood cell (WBC) count and haemoglobin (Hb) content and decreased the level of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (γ-GT) and nitric oxide (NO) in DLA bearing ascites tumor models. In addition, administration of extract significantly decreased the tumour volume and body weight in a DLA bearing solid tumor model. The levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and granulocyte monocyte-colony stimulating factor (GM-CSF), as well as pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) were elevated in solid tumour controls, but significantly reduced by *A ferruginea* administration. On the other hand, the extract stimulated the production of interleukin-2 (IL-2) and interferon-gamma (IFN-γ) in animals with DLA induced solid tumours. Increase in $CD4^+$ T-cell population suggested strong immunostimulant activity for this extract. GC/MS and LC/MS analysis showed quinone, quinoline, imidazolidine, pyrrolidine, cyclopentenone, thiazole, pyrazole, catechin and coumarin derivatives as major compounds present in the *A ferruginea* methanolic extract. Thus, the outcome of the present study suggests that *A ferruginea* extract has immunomodulatory and tumor inhibitory activities and has the potential to be developed as a natural anticancer agent.

Keywords: *Acacia ferruginea* - antitumor activity - Dalton's lymphoma ascites - pro-inflammatory cytokines - LC/MS

Asian Pacific J Cancer Prev, **14 (6)**, 3909-3919

Introduction

Cancer is the leading cause of death worldwide and the World Health Organization (WHO) reported there were 7.6 million deaths (≈13% of all deaths) in 2008 and they estimated this will reach 13.1 million deaths by 2030. Chronic inflammation could lead to cellular proliferation – a process that in and of itself increased the risk for aberrant cell formation and, ultimately, development of cancer (Mantovani et al., 2008; Grivennikov et al., 2010). During tumorigenesis, tumor-infiltrating inflammatory cells will produce variety of cytokines. It has been reported that pro-inflammatory cytokines including TNF-α, Interleukins (IL-1β, IL-6) and GM-CSF contribute to carcinogenesis by persuading the survival, growth, proliferation, differentiation and metastasis of tumor cells (Lazar et al., 2000; Lawrence, 2007). Conventional cancer therapies include surgery and radiation if the tumor is diagnosed at initial stage and chemotherapy is the treatment of choice for advanced tumors. Although these treatments are effective, it associated with severe adverse events include drug resistance and dose-limiting toxicities such as immunosuppression. Thus, there is need to develop new therapeutic options with low toxicity and minimal side effects. In fact, single antitumor drug may be ineffective because of its unique molecular target with in the tumor cell. Therefore, presence of multiple compounds in well characterized plant extract with synergistic activities may tackle this difficulty.

Development of drugs from natural sources that prevent or inhibit tumor growth by down-regulating select inflammatory factors has become of keen interest in the field of drug discovery and anti-cancer therapies. Throughout history, plants have been the most consistently successful source of traditional medicines and continue to provide new remedies and to promote human health and well being. Several traditionally-used medicinal plants and plant products have become potential sources

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of anti-cancer agents. Acacia is the second largest genus in the family *Leguminosae*, comprising >1200 species. Traditional healers in different regions in India have routinely used Acacia species for treating various cancer of the mouth, bone and skin (Kalaivani and Mathew, 2011). Extensive research on *Acacia* has been carried out over the past few decades because of their reputed pharmacological effects and low toxicity. Various biological activities for these Acacia species have been reported, covering a wide gamut of beneficial effects (Dongmo et al., 2005; Meena et al., 2006; Singh et al., 2006; Tung et al., 2008; Bachaya, 2009; Lopes et al., 2009). Acacia is a rich natural source of bioactive flavonoids, alkaloids, phenolics, saponins, polysaccharides, quinones, tannins, and terpenoids (Seigler, 2003). To date, several biologically active natural products have been identified from the various species of *Acacia*; these include androstene steroid, gallic acid, ellagic acid, isoquercitin, kaempferol, naringenin, rutin, lupane, niloticane, umbelliferone and catechin (Chaubal et al., 2003; Mutai et al., 2009; Eldeen et al., 2010; Rajbir et al., 2010). Earlier studies from our laboratory shown potential anticancer activity of *A nilotica* against DLA induced tumor models (Sakthivel et al., 2012).

Among the various Acacia species, *Acacia ferruginea* (in family Mimosaceae) is one medicinal plant used for various purposes. Traditionally, bark decoction from *A ferruginea*, in conjunction with ginger is frequently used as an astringent for the teeth (Suresh and Rao, 1999), also as antidiarrhoeal, haemostatic; used for treating excessive mucous discharges, haemorrhages, stomatitis, irritable bowel syndrome, antileprotic drug (Rajanna et al., 2011) and also used to treat skin disease mainly scabies (Das, 1983). To our knowledge, neither phytochemicals nor biological activities of *A ferruginea* extract have yet to be documented. Thus, in this study, we sought to characterize the major phytoconstituents of this particular plant as well as to ascertain the anti-tumor activity of *A ferruginea* extract in a murine experimental model.

Materials and Methods

Collection of plant material

The fresh aerial parts of the plant were collected from Annur, near Coimbatore, India. The plant was identified and authenticated at Botanical Survey of India, Coimbatore (No: BSI/SRC/5/23/2011-12/Tech-687). A voucher specimen was retained in the Department of Biotechnology, Karunya University, Coimbatore. The remainder of the harvested plant samples were washed thoroughly with water and shade-dried at room temperature.

Preparation of extract

The shade-dried aerial parts of the plant were subjected to mechanical size reduction. The powdered material was then extracted with methanol in a Soxhlet apparatus. Traces of the solvent were ultimately removed by evaporation and the final extract concentrated using a vaccum rotatory evaporator. The percentage yield of the extract was 12%. The crude extract thus obtained, as a thick semisolid mass, was stored in the refrigerator for use in the various experimental protocols.

Animals

Male BALB/c mice (4-6 weeks-old, 22-25 g) were obtained from the Small Animals Breeding Station, Kerala Agricultural University (Mannuthy, India). All mice were maintained in a controlled sterile environment main-tained at a constant temperature (24±2ºC), 50% relative humidity, and a 12-hr light/dark cycle. All mice had *ad libitum* access to standard diet pellets (Sai Durga Feeds, Bangalore, India) and filtered water. All experiments performed here were based on the rules and regulations assigned by, and had the approval of, the Animal Ethical Committee of the Government of India.

DLA cell line

DLA cells were obtained from the Amala Cancer Research Institute (Thrissur, India) and propagated in the peritoneal cavity of naive BALB/c mice. For instillation into mice in the various treatment groups, cells freshly-aspirated from these mice were washed with phosphate-buffered saline (PBS, pH 7.4) to remove cell debris and dead cells under sterile conditions. The viability of the cells were checked by Trypan Blue assay and the viable cells (10^6 cells) were incoculated via intraperitoneal (IP) injection.

Preliminary screening for phytochemicals

Qualitative phytochemical screening of the methanolic extract was carried out according to the methods of (Harbone, 1973). The extract was screened for alkaloids, flavonoids, phenolics, saponins, glycosides, quinones, steroids and tannins.

*GC/MS analysis of **A ferruginea** extract*

GC/MS analysis of the extract was performed using a Thermo GC-Trace Ultra VER: 5.0 (Bremen, Germany). For MS detection, the MS DSQ II electron ionization mode with ionization energy of 70 eV was used, with a mass range at m/z 50-650. A TR-5MS capillary column (30m×0.25mm, film thickness=0.25µm) was used for the analysis. The column temperature was programmed from 80-250°C at a rate of 8°C/min, with the lower and upper temperature being held for 3 and 10 min, respectively. The GC injector and MS transfer line temperatures were set at 280 and 290°C, respectively. GC was performed in the splitless mode. Helium (at flow rate=1.0 ml/min) was used as the carrier gas. A 1 µl injection volume was used. Major and essential compounds were identified by retention times and mass fragmentation patterns using data of standards from the National Institute of Standards and Technology (NIST) & Wiley 9.0 library (Vinod and Guruvayoorappan, 2012).

*LC/MS analysis of **A ferruginea** extract*

LC/MS analysis of the extract was performed using a 3200 Q-Trap system equipped with a degasser, binary pump, auto sampler, and column heater (Applied Biosystems, Foster City, CA). The system was coupled with Sciex turbo ion spray triple mass spectrometer (Darmstadt, Germany). Data acquisition and mass

spectrometric evaluation were carried out in a personal computer with data analysis software (Analyst -1.4.1. and Light sight software1 (IDC, Framing-ham, MA). Chromatographic separation was carried out on Eclipse XDB - C_8 column (5-μm, 150×4.6 mm; Agilent Technologies, Santa Clara, CA). Gradient elution was applied with a mobile phase comprised of 95% Solvent A (0.1% acetic acid in water) and 5% Solvent B (0.1% acetic acid in acetonitrile) for 1 min, followed by an 11-min step gradient from 5% B to 100% B; thereafter, it was kept for 4 min with 100% B. Finally, elution was achieved with a linear gradient from 100% B to 5% B for 2 min. The flow-rate used throughout was 200 µl/min; sample injection volume was 10 µl. The following parameters were used throughout the MS experiment: for electrospray ionization with positive ion polarity, capillary voltage was set to 20 V, transfer capillary temperature to 300°C, nebulizer pressure to 40 psi, and the drying gas flow to 15 L/min. Data were acquired in the positive ion MS mode in the range of 50-1000 m/z (Vinod and Guruvayoorappan, 2012).

Acute toxicity test

Acute *in vivo* toxicity studies with different concentrations of *A ferruginea* methanolic extract was carried out according to OECD 423 (Organization for Economic Co-operation and Develpoment) Ecobichon, 1997 (Loganayaki and Manian, 2011). The general behaviors such as motor activity, tremors, convulsions, aggressiveness, changes in mucous membrane, sedation, mydriasis, lacrimation, diarrhea, rising fur and coma were observed for the first hour and after 24 h of test drug administration. No deaths or adverse effects were detected during the 24-hr observation period in mice treated with up to 200 mg/kg body weight (b.wt) of *A ferruginea* extract (data not shown). Based on these results, a dose of 10 mg/kg.b.wt was chosen for use in all of the experiments hereafter.

DLA-induced ascites tumor studies

Ascites tumors were induced by IP injection of DLA cells (1.5×10^6 cells/mouse). In these studies, mice were in one of four groups (n=6/group): Group I mice served as Normal (Untreated); Group II mice served as solid tumor control and received PBS vehicle only (i.e., no drug, no extract); Group III was treated with methotrexate (3.5 mg/kg.b.wt); Group IV mice were treated with extract (10 mg/kg.b.wt). All treatments were given IP (as 100 μl injections) starting 24 hr after DLA injection and were done daily for 14 consecutive days thereafter.

*Determination of the effect of **A ferruginea** extract on mean survival time (MST), %increase of life span (ILS) and average body weight changes*

For these studies, dedicated sets of mice were treated with tumor cells and the various drugs/extract regimens as outlined above; the animals were then monitored daily for 50 days. Anti-tumor effects of the extract were determined by monitoring mortality due to tumor (MST) and any percentage increase in lifespan (%ILS) relative to the survival of mice that received tumor cells but no other treatment. The latter was calculated as: ILS (%)=100×(mean survival of treated group-mean survival of control)/mean survival of control group. Body weights (BW) of all animals were measured from day 1 to 15 and the average increase in body weight on day 15 was determined.

*Determination of the effect of **A ferruginea** extract on key liver marker and oxidative stress marker enzymes*

Blood was collected from each animal (via tail-vein) on day 10 and 15. Total WBC count and hemoglobin content (Hb) were estimated and the remaining blood was centrifuged and serum prepared for estimation of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (GGT) activities by using a kit from Span Diagnostics (Surat, India). Nitric oxide (NO) in the serum was measured by the method of (Green et al., 1982). Ascites fluid was also aspirated from the peritoneal cavity on day 10 and 15. The aspirated cells were washed to remove dead cells, and then the remaining cells were suspended in RPMI 1640 medium and placed in sterile glass dishes to allow resident macrophages to adhere. After 2 hr at 37°C, the culture medium containing the 'purified' DLA cells was gently removed, and then centrifuged to pellet the cells. The cells were then re-suspended at 10^7 cells/ml in RPMI 1640; 100 μl aliquots of the sample were then removed and sonicated for 30 sec to rupture the cells present. This material was then assessed for NO and reduced glutathione (GSH) content using the protocol of (Szasz et al., 1976).

Histopathological analysis

At day 15 of the study, mice with DLA-induced ascites were euthanized via cervical dislocation. At necropsy, a small portion of liver was recovered from each mouse and fixed in 5% formaldehyde solution. After several steps to induce dehydration in alcohol, sections of 4-µm thickness were prepared and stained with haematoxylin and eosin (H&E). Thereafter, histopathological analysis was carried out using a EVOS-xl CORE light microscope (AMG, Bothell, WA). All samples were analyzed in a blinded manner. A certified histopathologist performed all analyses/interpreted the observed outcomes.

DLA-induced solid tumor studies

Solid tumors were induced by intramuscular injection of the DLA cells (1.5×10^6 cells per mouse) into the right hind limb of the mice. In these studies, mice were in one of four groups (n=6/group): Group I mice served as Normal (Untreated); Group II mice served as solid tumor control and received PBS vehicle only (i.e., no drug, no extract); Group III was treated with methotrexate (3.5 mg/kg.b.wt); Group IV mice were treated with extract (10 mg/kg.b.wt). All treatments were given IP (as 100 μl injections) starting 24 hr after DLA injection and were done daily for 14 consecutive days thereafter.

*Determination of the effect of **A ferruginea** extract on solid tumor volume and body weight*

Limb initial diameter was measured using a vernier

caliper. From day 3 post-DLA injection onwards, tumor diameter was measured every 3 day, up to day 30. Tumor volume was calculated as: $V=4/3\pi r_1^2 \times r_2$, where r_1 and r_2 are tumor radii measured in two planes. Body weights (b.wt) of all mice were measured at 3 day intervals from day 0 to 30.

*Determination of the effect of **A ferruginea** extract on cytokines (TNF-α, IL-1β, IL-2, IL-6, IFN-γ, GM-CSF), inflammatory mediator (iNOS) and pro-angiogenic growth factor (VEGF) using Enzyme-Linked Immunosorbent Assay (ELISA) kit in mice with DLA-induced soild tumor*

To study the effect of *A ferruginea* extract on cytokines level, blood samples obtained from the above experiment at two time intervals, (i.e., day 15 and 30) was centrifuged and serum separated for estimation of TNF-alpha, iNOS (USCN Life science, USA), IL-1β, IL-2, IL-6, IFN-γ, GM-CSF and VEGF (Koma Biotech, Korea) using standard sandwich ELISA kit specific for murine cytokines according to the manufacture's instruction.

Immunophenotyping of lymphocyte subsets by flow cytometry

The whole blood obtained from the above experiment on day 30 was diluted at a ratio 1:10 with (1×) lysis buffer (BD Pharm Lyse) to lyse the RBCs, mixed well, and incubated for 10 min at room temperature in the dark. Tubes were centrifuged for 5 min at 600×g, supernatant aspirated, cells washed again with 2 mL of sheath fluid (BD FACS Flow, BD Biosciences, USA), and spin down at 200×g for 10 min to aspirate the supernatant. Viable cells obtained were adjusted to a cell concentration of 10^7/mL in falcon tubes. CD4, and CD8 positive cells were determined by direct immunofluorescence using the following antibodies: anti-CD4 (PE) and anti-CD8 (PerCp) purchased from Becton-Dickinson, USA. After incubation for 30 minutes, the cells were washed three times with sheath fluid and analyzed immediately on a FACS Calibur (Becton Dickinson, USA) for evaluating lymphocyte subsets. For each sample, 10,000 gated cells were analyzed using CellQuest 3.0 Software (Becton Dickinson, San Jose, USA). Data presented are representative of those obtained in independent experiments done in triplicates.

Statistical analysis

All data values are expressed as mean (±SD). Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed By Dunnett's test, using Graphpad InStat version 3.0, (GraphPad Software, San Diego, CA). Results from non-tumor control mice were considered statistically significant compared to those from DLA tumor control hosts at p-values **<0.05 (or in some cases, *<0.01).

Results

Preliminary phytochemical analysis

Preliminary phytochemical screening of the extract was carried out to determine the presence of consitutents that could be responsible for any observed biological activities in these studies. The analysis indicated that there was an abundance of flavonoids, phenols, terpenoids, alkaloids, anthraquinones and tannins in the extract, glycosides and saponins were present in trace amounts.

*GC/MS analysis of **A ferruginea***

The GC/MS chromatogram of the extract is shown in Figure 1. GC/MS analysis resulted in identification of 18 different compounds as shown in Table 1. Derivatives of quinone (37.3%), quinoline (22.9%), imidazolidine (6.4%), pyrrolidine (4.5%), and cyclopentenone (3.5%) were identified as major components. Hexadecanoic acid, propanoic acid, pyridine, pyrazole and pyrimidine derivatives were also identified in the methanolic extract.

*LC/MS analysis of **A ferruginea***

Table 1. GC/MS Analysis of the Methanolic Extract of *A ferruginea**

Retention time	Name of the Compound	Molecular Formula	Molecular Wt	% of relative Area
6.99	1,4,5,6-tetrahydropyrimidine	$C_4H_8N_2$	84	2.14
11.31	5-Fluoropentanenitrile	C_5H_8FN	101	2.29
12.05	1-Methyl-4-(1-imidazoyl)-1,2,3,6-tetrahydropyridine	$C_9H_{13}N_3$	163	1.58
12.56	Butyl ester, hydroxyl acetic acid	$C_6H_{12}O_3$	132	1.22
14.7	2-Hydroxy-3-tert-butyl-2-methylpropionitrile	$C_8H_{15}NO$	141	1.56
15.21	4-hydroxy-3-t-butyl-2methyl-2-cyclopentenone	$C_{10}H_{16}O_2$	168	3.5
15.68	Propanoic acid, butyl ester (CAS)	$C_7H_{14}O_2$	130	0.84
16.59	1-(2,3-O-Anhydro-5-O-trityl-a-D-lyxofuranosyl)-2-pyrrolidino-4-pyrimidine	$C_{32}H_{31}N_3O_4$	521	1.03
18.92	7-Amino-3-carbethoxy-1,8-napthyridine-2(1H)-2-one	$C_{11}H_{11}N_3O_3$	233	0.91
20.07	1,8-Bis(1'-aza-4',7.10',13'- tetraoxacyclopentadecan-1'yl)-9,10- anthraquinone	$C_3H_{46}N_2O_{10}$	642	37.28
21.89	Hexadecanoic acid, methyl ester (CAS)	$C_{17}H_{34}O_2$	270	2.45
22.76	3,4-dihydronathalene-1-oxo-2(H),3'-(2',5'-diphenyl-4'-methyl-thio-5'-pyrrolidine	$C_{20}H_{18}N_2O_4$	350	4.5
23.65	3-Chloro-2,2,5,8-tetrahydroxy-3-methyl-2,3-dihydro-1,4-napthoquinone	$C_{11}H_9ClO_6$	272	0.68
24.97	2-(Methylthio)-5-(furylmethylidene)-N(3)-(2'-chlorobenzyl-4-imidazolidine	$C_{16}H_{13}ClN_2O_2S$	400	6.44
25.43	N,N-Dimethyl-2-Pyridine Methanamine	$C_8H_{12}N_2$	136	1.42
29.78	2,3,4,6-tetra (3,5-dimethyl pyrazol-1'-yl)-4-pyrazole-1'-yl) pyridine	$C_{28}H_{31}N_{11}$	521	0.95
30.86	5-(3,4-Dimethylphenyl)-4-phenylisoxazole	$C_{17}H_{15}NO_{34}$	281	0.78
39.19	Bis[2,2-(4-methylquinoline)]	$C_{20}H_{16}N_2$	284	22.94

*R_i=6.99, 11.31, 12.05, 12.56, 14.70, 15.21, 15.68, 16.59, 18.92, 20.07, 21.89, 22.76, 23.65, 24.97, 25.43, 29.78, 30.86 and 39.19, min representing 2.14, 2.29, 1.58, 1.22, 1.56, 3.50, 0.84, 1.03, 0.91, 37.28, 2.45, 4.50, 0.68, 6.44, 1.42, 0.95, 0.78 and 22.94% of the relative area respectively. Major component identification was aided by comparison against reference standards in NIST and Wiley 9.0 library

The LC/MS chromatogram of the extract is shown in Figure 2 and Table 2 presents the retention times, mode (+/-), λ max, and molecular weights of the respective components iden-tified. In the LC/MS analysis, a positive molecular ion at $[MS+H]^+$ at an *m/z* of 146.5 correspon-ded to carboxamidine derivatives, 1H-pyrazole-1-carboxamidine monohydrochloride and 4-iodo-1-benzothiophene-2-carboximidamide hydrochloride at an *m/z* of 338.6 was observed. Imidazole and thiazole derivatives such as 2-phenyl-4,4,5,5-tetramethylimidazoline-oxyl-1-oxyl-3-oxide, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl] phenyl] ben-zene sulphonamide, 2-(2'-methyl-*n*-propyl)-4,5-dimethyl-D3-thiazoline and thiazole,2,5-dihy-dro-4,5-dimethyl-2-(2-methylpropyl) were observed in a positive molecular ion at *m/z* values of 233.3, 481.3, and 171.3 respectively. At *m/z*=633.3 and 381.1, 4-octadecanyl-amino-benzoyl-α-phenoxy-N-(2-chlorophenyl)-acetamide and coumarin derivatives, 7-hydroxy-4-methyl-,bis(2-chloroethyl) phosphate in a negative molecular ion at $[MS-H]^+$ and catechin derivatives at *m/z*=289 was also seen.

*Effect of **A ferruginea** on mean survival time (MST), %increase of lifespan (ILS) and %increase in body weight in mice with DLA-induced ascites*

Administration of extract for 14 consecutive days significantly prolonged lifespans/survi-val time (MST) of treated mice (27.2±1.5) days compared to that of their untreated tumor-injected counterparts (16.7±1.0) days; this represented a 63% increase in lifespan. The percentage increase in lifespan of methotrexate-treated mice was 79%. The percentage increases in body weights in the ascites-bearing mice were also analyzed. The results indicate that there was a significant reduction in the net changes between the extract vs. non-extract-treated DLA-injected hosts control (17.1 vs. 38.8%) over the study period, whereas mice treated with methotrexate shown 13.6% (Table 3).

*Effect of **A ferruginea** on hematological parameters in mice with DLA-induced ascites tumor*

Inoculation with DLA cells resulted in a significant increase in the of total WBC levels (14.9±0.7 and 15.5±0.4×10^3 cells/ml) on day 10 and 15, respectively, as compared to values seen in naive mice. Administration of extract mitigated these changes; values only reached 12.2±0.6 and 12.8±0.4×10^3 cells/ml on day 10 and 15, respectively. These outcomes were comparable with results produced by methotrexate (11.6±0.5 and 12.5±0.8×10^3 cells/ml) on day 10 and 15, respectively. Hemoglobin content was also significantly reduced in DLA-injected mice (10.0±0.5 and 10.2±0.3 gm% on day 10 and 15, respectively (Figure 3), when compared with values in naive animals. Both extract and standard drug significantly prevented the anaemic condition (Hb values of, respectively, 14.6±0.4 and 14.6±0.4 gm% on day 15).

*Effect of **A ferruginea** on serum AST, ALT, ALP, GGT and NO levels in mice with DLA-induced ascites tumor*

The effect of *A ferruginea* on serum AST, ALT, ALP, GGT, and NO levels in DLA-injected mice are presented

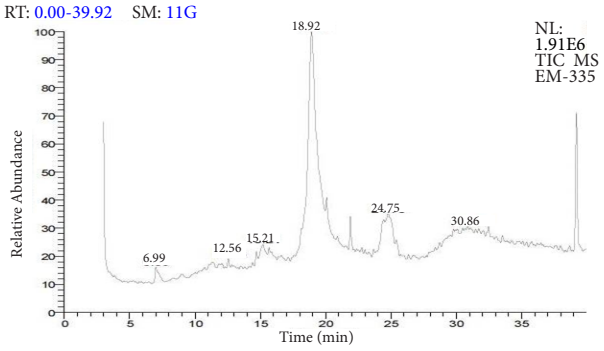

Figure 1. GC/MS Chromatogram of Methanolic Extract of *A ferruginea*

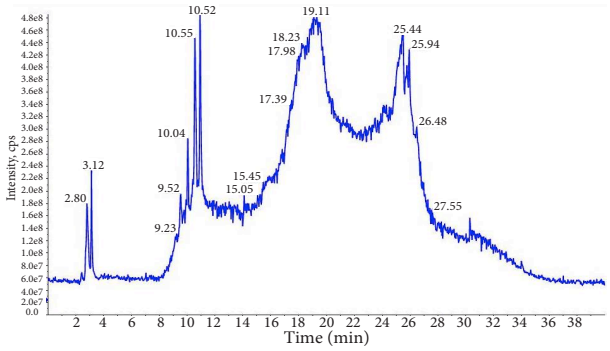

Figure 2. LC/MS Chromatogram of Methanolic Extract of *A ferruginea* at 254 nm

Table 2. Identification of Compounds in *A ferruginea* Methanolic Extract Using Their Retention Time and MS Data Derived in LC/MS Analysis*

R_t** (min)	λmax (min)	Molecular Weight	Mode (+/-)	Compounds (Tentative ID)	Molecular Formula
2.8	254	146.5	+	1H-pyrazole-1carboxamidine monohydrochloride	$C_4H_6N_4.Hcl$
3.12	254	130.4	+	Unknown	-
9.52	254	233.3	+	2-phenyl-4,4,5,5-tetramethylimidazolineoxyl-1-oxyl-3-oxide	$C_{13}H_{17}N_2O_2$
10.04	254	481.3	+	N-(2,2,2-Trifluoroethyl)-N-[4-[2,2,2-Trifluoro-1-hydroxy-1-	$C_{17}H_{12}F_9NO_3S$
10.55	254	171.3	+	2-(2'-Methyl-n-propyl)-4,5-dimethyl-D3-thiazoline; Thiazole,2,5-dihydro-4,5-dimethyl-2-(2-methylpropyl)	$C_9H_{17}NS$
10.92	254	338.6	+	4-iodo-1-benzothiophene-2-carboximidamide hydrochloride	$C_9H_7IN_2S.Hcl$
18.23	254	497.3	+	4-Epianhydrochlorotetracycline hydrochloride	$C_{22}H_{21}ClN_2O_7.Hcl$
19.11	254	633.3	+	4-octadecanylamino-benzoyl-alpha-phenoxy-N-(2-chlorophenyl)-acetamide	$C_{39}H_{51}ClN_2O_4$
25.44	254	381.1	-	Coumarin, 7-hydroxy-4-methyl-,bis(2-chloroethyl)phosphate	$C_{14}H_{15}Cl_2O_6P$
25.94	254	290.27	-	Catechin, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	$C_{15}H_{14}O_6$

*Identification of compounds was aided by correlation with previous literature reports. **R_t refers to LC/MS chromatogram shown in Figure 2

Table 3. Effect of *A ferruginea* on Body Weight, Mean Survival Time, Increase in life Span in DLA Bearing Ascites Tumor Models

Treatment design	MST (in days)	Increase in Lifespan (%)	% increase in Bodyweight (g)
Normal	>50	-	10.29
DLA tumor Control	16.66±1.03	-	38.77
Tumor+Methotrexate (3.5 mg/kg.bw)	29.83±1.16[b]	79.05	13.58
Tumor+*A ferruginea* (10 mg/kg.bw)	27.16±1.47[b]	63.02	17.1

*Values are expressed as Mean±SD (n=6/group). Values significantly different from tumor (non-extract-treated) control ([a]p<0.05,[b]p<0.01)

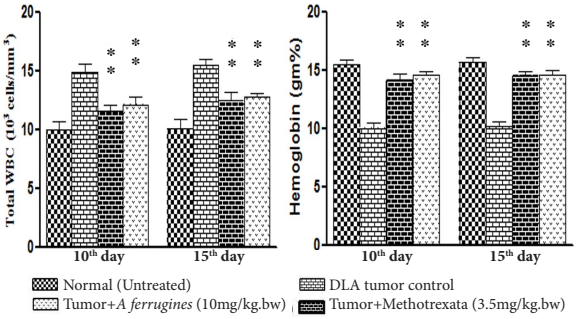

Figure 3. Effect of Methanolic Extract of *A ferruginea* on Haematological Parameters. Treated animals received 14 doses of extract (at 10 mg/kg.b.wt). Blood samples were collected from tail vein on day 10 and 15 after start of extract administration. Total WBC counts and hemoglobin content were assessed with each sample collected. Values shown are means (±SD). Values significantly different from tumor (non-extract-treated) control (*p<0.05,**p<0.01)

in Figure 4. Serum levels of AST, ALT, and ALP on day 10 and 15 post-injected were significantly increased in DLA-tumor control mice (i.e., 75.2±2.9, 57.9±1.9, 171.9±2.9, respectively, on day 15) as compared to values in naive hosts (Group I), (45.9±1.3, 32.9±1.8, 94.1±2.1, respectively, on day 15). In extract-treated tumor-injected hosts, the levels of AST, ALT and ALP were significantly reduced (i.e., 59.8±2.0, 45.0±2.1, 110.5±3.3, respectively, on day 15) as compared to those in the tumor-injected controls. Methotrexate also produced a similar result (i.e., 57.1±1.9, 43.8±1.9, 104.8±2.3, respectively, on day 15).

On day 15 after tumor injection, elevated levels of GGT and NO (27.4±2.1 U/L and 36.2±1.2μM, respectively) were found in the serum of DLA-injected controls as compared to in naive hosts 15.9±1.4 U/L and 24.4±0.9 μM, respectively). These levels were significantly reduced to 17.4±2.2 U/L and 29.6±2.4 μM, respectively due to administration of the *A ferruginea* extract. This reduction was comparable to that achieved with methotrexate (i.e., 19.5±2.8 U/L and 26.5±1.4 μM, respectively).

*Effect of **A ferruginea** extract on GSH and NO levels in ascites fluid cells*

The GSH content in aspirated DLA tumor cells on day 15 after injection was found to be 17.2±2.8 nmol/mg protein. In extract- and methotrexate-treated animals, the cellular GSH level was significantly reduced to 8.7±0.8 and 9.3±1.5 nmol/mg protein, respectively (Figure 5). The nitric oxide level in the aspirated cells on day 15 was 13.1±0.5 μM. In extract and drug-treated mice, the NO

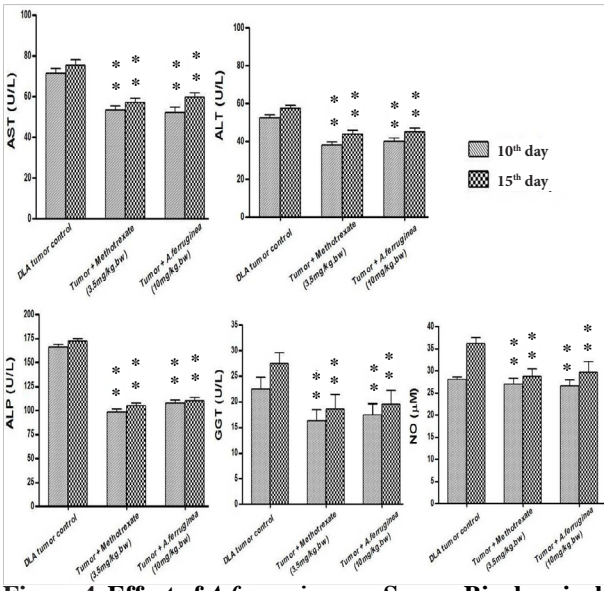

Figure 4. Effect of *A ferruginea* on Serum Biochemical and Antioxidant Parameters in DLA-Bearing and Extract-Treated Mice. Blood samples were collected from tail vein on day 10 and 15. AST, ALT, ALP, GGT and NO levels were assessed. Values shown are means (±SD). Values significantly different from tumor (non-extract-treated) control (*p<0.05,**p<0.01)

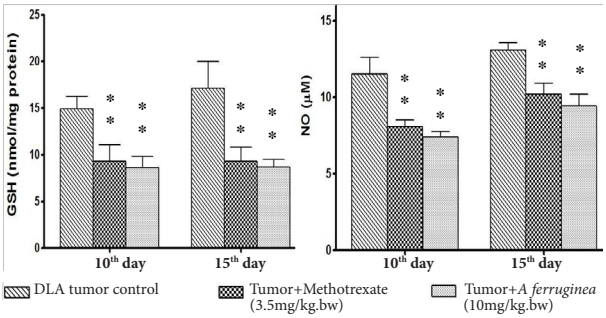

Figure 5. Effect of *A ferruginea* on Cellular GSH and NO Levels in DLA Bearing Tumor Mice. Ascites fluid was collected on day 10 and 15 after intraperitoneal injection of DLA cells. The isolated solution was sonicated and lysates then assayed for GSH and NO. Values shown are means (±SD). Values significantly different from tumor (non-extract-treated) control (*p<0.05, **p<0.01)

levels were seen to be lowered to 9.4±0.8 and 10.2±0.7 μM respectively.

*Effect of **A ferruginea** on histopathology in ascites-bearing hosts*

Representative liver sections from normal (PBS), DLA tumor control, methotrexate-, and extract-treated mice that were collected at the end of the experimental periods (i.e., day 15 post-DLA injection) are presented in Figure.6. PBS mice yielded tissues with normal lobular arch-itecture and with an intact central vein and sinusoids, normal portal tracts, and intact hepatocytes. DLA-injected mice (control) samples evidenced necrosis, fibrosis, nuclear debris, and a peri-venular inflammation containing several polymorphonucleated cells. Vacuole formation and local inflammation was significant in the tissues from DLA-injected hosts. In comparison, mice treated with *A ferruginea* extract or methotrexate had

livers with a reduced vacuole presence and inflammation and an almost normal hepatocellular architecture.

*Effect of **A ferruginea** extract on solid tumor development and host body weight*

Treatment of DLA-injected mice with *A ferruginea* extract on 14 consecutive days led to a significant reduction in tumor volume (0.81±0.07 mm^3) compared to that seen in tumor-injected mice that did not receive any extract at all (2.52±0.06 mm^3) by day 30 of study; Figure 7A). Mice treated with the methotrexate also displayed effective reduction (0.88±0.07 mm^3) in tumor development. Body weight was measured at 3 day intervals throughout the period of experiment. By day 30, there was a significant increase (vs. day 0) in weights (up to 29.17±0.33g) of the tumor-bearing controls (Figure 7B). In contrast, in extract-treated tumor-injected mice, significantly lower comparative weights (25.55±0.19 g) were noted. Mice treated with methotrexate were also of a similar 'lower' weight by this time (24.92±0.29g). It is likely that the greater weights in the non-extract-treated mice were attributable to increases in the tumor mass itself.

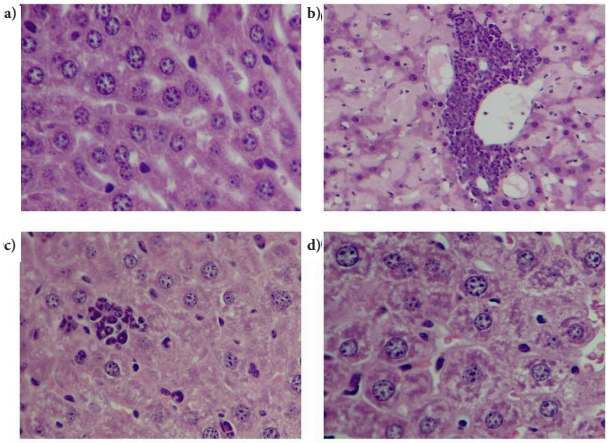

Figure 6. Histopathological Changes in Liver of Experimental Mice. Pictures presented are representative liver sections collected at end of experimental periods (i.e., day 15 after DLA injection). (A) Normal (no tumor, no extract); (B) tumor only; (C) tumor+*A ferruginea* extract; (D) tumor+methotrexate

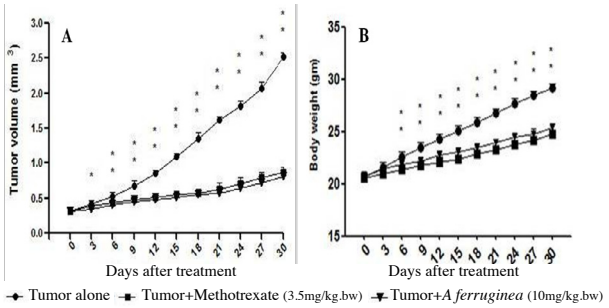

Figure 7. Effect of *A ferruginea* Extract on Tumor Volume (A) and Body Weight (B) on Indicated Days After DLA Intramuscular Injection. Treated animals received 14 doses of extract (10 mg/kg.b.wt). Tumor radii in extract- and non-extract-treated controls were measured at 3-d intervals using a vernier calliper. Body weights of treated and non-extract-treated controls were also measured at 3-d intervals. Values shown are means (±SD; in mm^3) from 6 mice/treatment group. Values significantly different from tumor (non-extract-treated) control (*p<0.05, **p<0.01)

*Determination of the effect of **A ferruginea** extract on cytokines profile and inflammatory markers in mice with DLA-induced soild tumor*

The effect of *A ferruginea* extract on TNF-α, iNOS, IL-1β and IL-6 production on day 15 and 30 in mice with DLA-induced soild tumor is depicted in Figure.8. Serum TNF-α, iNOS, IL-1β, IL-6, GM-CSF and VEGF

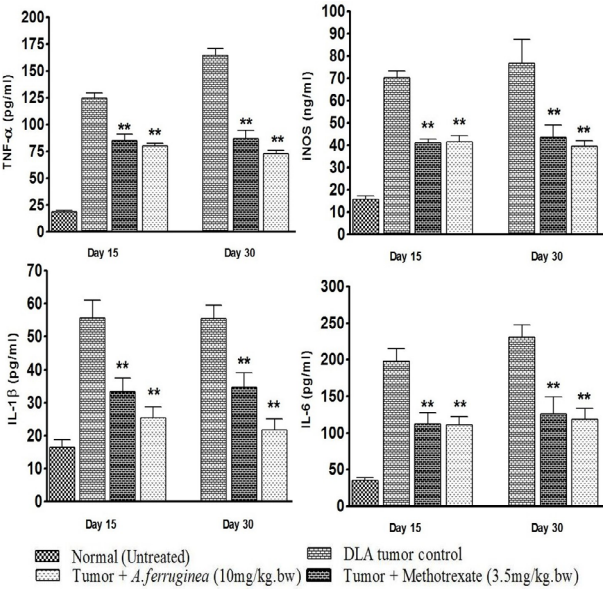

Figure 8. Effect of *A ferruginea* Extract on TNF-α, iNOS, IL-1β and IL-6 Production in Mice with DLA-Induced Soild Tumor. Blood samples were collected from tail vein on day 15 and 30, the level of cytokines were assessed using standard ELISA method. Values shown are means (±SD). Values significantly different from tumor (non-extract-treated) control (*p<0.05,**p<0.01)

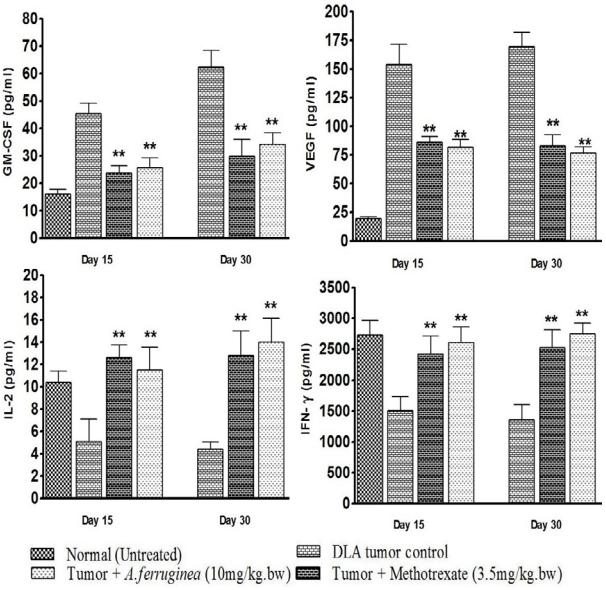

Figure 9. Effect of *A ferruginea* Extract on GM-CSF, IL-2, IFN-γ, and VEGF Level in Mice with DLA-Induced Soild Tumor. Blood samples were collected from tail vein on day 15 and 30, the level of cytokines were assessed using standard ELISA method. Values shown are means (±SD). Values significantly different from tumor (non-extract-treated) control (*p<0.05,**p<0.01)

level was found to be elevated where as IFN-γ and IL-2 level were reduced in DLA-injected hosts on day 15 and 30 respectively. Administration of *A ferruginea* extract in DLA-injected hosts significantly reduced the Serum TNF-α, iNOS, IL-1β, IL-6, GM-CSF and VEGF level on day 15 and 30. Similarly, reduced level of IFN-γ and IL-2 were significantly enhanced in the DLA-injected mice treated with *A ferruginea* extract on day 15 and 30. The effect of *A ferruginea* extract on GM-CSF, IL-2, IFN-γ, and VEGF level on day 15 and 30 in mice with DLA-induced soild tumor is depicted in Figure.9. Mice treated with the methotrexate also displayed effective result in cytokine profiling.

*Determination of the effect of **A ferruginea** extract on lymphocyte subsets in mice with DLA-induced soild tumor*

Immunophenotyping for lymphocyte subsets with CD markers showed significant increase in the proportion of T lymphocyte population in *A ferruginea* treatment (Figure 10). The increase was prominent in the $CD4^+$ cells and showed an increased CD4/CD8 ratio (1.67), shown in Table 4. It was found that extract treatment significantly enhanced CD4 and CD8 count (56.5±0.78 and 33.7±0.97) respectively in comparison with DLA-injected hosts (32.7±0.71 and 26.3±0.14) respectively.

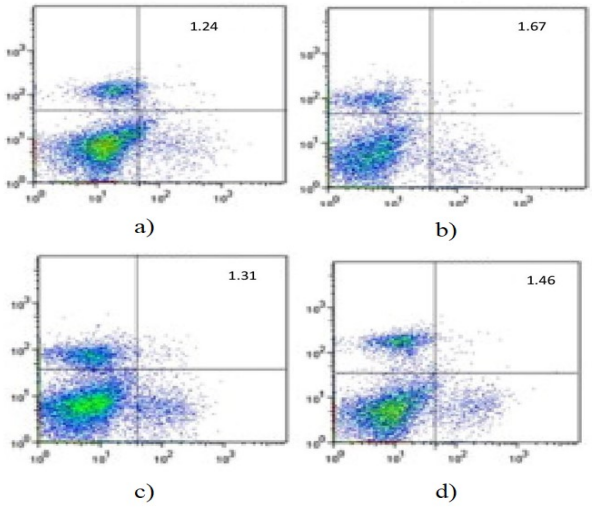

Figure 10. Effect of *A ferruginea* Extract on Lymphocyte Subsets in Mice with DLA-Induced Soild Tumor. The whole blood obtained from the above experiment on day 30, CD4 and CD8 positive cells were measured using Flow cytometry. Data presented are representative of those obtained in independent experiments done in triplicates. (A) tumor control; (B) tumor+*A ferruginea* extract; (C) tumor+methotrexate; (D) Normal (no tumor, no extract)

Table 4. Determination of the Effect of *A ferruginea* Extract on Lymphocyte Subsets in Mice with DLA-Induced Solid Tumor

Treatment design	CD4	CD8	CD4/CD8 ratio
Normal	41.77±0.62	28.49±0.61	1.46
DLA tumor control	32.69±0.71	26.29±0.14	1.24
Tumor+Methotrexate (3.5 mg/kg.bw)	45.40±0.66[b]	34.28±0.68[b]	1.31
Tumor+*A ferruginea* (10 mg/kg.bw)	56.52±0.78[b]	33.69±0.97[b]	1.67

*Data presented are representative of those obtained in independent experiments done in triplicates on day 15 of the experiment. Values significantly different from tumor (non-extract-treated) control ([a]p<0.05,[b]p<0.01)

Discussion

Cancer is a complex disease, and has become a major public health problem around the world. As a treatment for cancer, chemotherapy is successful but still faces a variety of challenges due to poor selectivity and/or toxicities that affect all rapidly proliferating and dividing cells, including lymphatic, red blood, epithelia, and bone marrow cells (Mahato et al., 2011). Several natural product drugs of plant origin have been proposed for use against cancer; galantamine, nitisinone, and tiotropium have been examined in late-phase clinical trials (Balunas and Kinghorn, 2005). Our interest in recent years has been in examining the potential anti-cancer effects of natural products based on their abilities to act against inflammatory mediators. There are several reports that plants belonging to genus *Acacia* have been widely used in the management of pain, inflammation, and treatment of cancer in folk medicine (Bukhari et al., 2010). In the current study, we for the first time provide evidence that *A ferruginea* extract has potent anti-tumor activity *in vivo*.

Our preliminary phytochemical analysis of the *A ferruginea* methanolic extract revealed a presence of numerous flavonoids, phenolics, steroids, terpenoids, alkaloids, saponins, and tannins. Polyphenols, which include mainly flavonoids and phenolic acids, have been reported to impart a protective role against a wide range of cancers, including those of the lung, breast, colon, stomach, and mouth (Araujo et al., 2011). These bioactive natural products act either by blocking initiation or suppressing/arresting promotion and progression of cancers (Ziech et al., 2012). Polyphenols and alkaloids exhibit considerable activity against a wide range of cancers. Of these, mahanine (a plant carbazole alkaloid) has been shown to inhibit cancer cells by inducing apoptosis of both androgen-sensitive (LNCaP) and androgen-independent (PC-3) cancer cells by reducing phosphorylation of PIP3 dependent kinase-1 (PDK-1), deactivating Akt, and down-regulatiing expression of pro-apoptotic Bcl-Xl (Yue and Wang, 2011). The wide range of biological and pharmacological activities of flavonoids in tumor cells is also well known (Hodek et al., 2002). Overall, emerging evidence has shown that the diverse classes of plant metabolites like flavonoids, phenolics, alkaloids, etc. can interfere with the promotion and progression phases of carcinogenesis, thereby inhibiting the premalig-nant/malignant transformation of initiated cells (Zhao et al., 2010).

In DLA tumor-bearing mice, a regular and rapid increase in tumor volume (i.e., accumulation of ascites fluid) and reduced host lifespan is usually noted. Administration of *A ferruginea* extract prolonged host life span; a concomitant reduction in body weight (reflecting a reduced tumor burden) in extract-treated DLA-injected mice animals also suggests the induction of a significant anti-tumor activity by mechanisms as-yet to be defined. Many reports have provided evidences that a presence of tumors in experimental animals affects functions of vital organs, particularly the liver and kidney, even when the tumor does not have direct contact with these organs. Often, the hepatocellular necrosis observed in

cancer-bearing hosts results in significant elevations in serum AST and ALT (released from liver). Increased serum levels of ALP (hyper alkaline phosphatasemia) has also been observed with intrahepatic cholestasis, bile duct obstruction, or infiltrative diseases of the liver (Gaze, 2007). In the current study, elevated level of serum AST, ALT, and ALP were noted in DLA-injected control mice. The significant reversal of these changes towards normal values once again indicated the anti-cancer effect of the *A ferruginea* extract as well as a hepatoprotective action against potential damage induced by the DLA tumor cells. Lastly, in hosts with cancer (i.e., ascites), myelosuppression and reductions in hemoglobin levels (anemia) due to iron deficiency are also frequently observed. The prevention of a drop in hemoglobin content and a reversal of the tumor-induced changes in total WBC counts towards a normal range after administration of the extract again indicate that the extract imparts a significant anti-cancer activity. These results also support the notion that the *A ferruginea* extract might also be providing a protective role for the hematopoietic system as well as some measure of immunostimulatory activity.

Cancer cells have higher total glutathioine (GSH) levels than normal cells; this is a characteristic of a higher cell proliferation rate (and often a resistance to chemotherapy). Studies have shown that combining GSH depletion using 1,3-bis(2-chloroethyl)-1-nitrosourea along with superoxide dismutase gene therapy could be successful in the treatment of breast cancer (Weydert et al., 2008). The theory behind this is that when intracellular GSH levels are low, the cancer cells are more susceptible to the effects of reactive oxygen species (ROS). This seems somewhat counter-intuitive in that while ROS might activate different intracellular oncogenic pathways that lead to activation of tumorigenic processes, the excessive levels of ROS can also be toxic to the cells that have already been transformed. In this study, administration of *A ferruginea* extract resulted in significant reductions in the intracellular GSH levels in DLA cells recovered from the treated mice. It is known that plant extracts containing antioxidants have been shown to cytotoxicity among cancer cells by inducing apoptosis (Trachootham et al., 2009). Whether their reduction in GSH content might have caused the DLA cells in our mice to be more susecptible to ROS generated by peritoneal/local macrophages (and thus more susceptible to apoptotic events) remains to be resolved. Similarly, effects of the extract on local macrophage formation of ROS need to be examined. While it is known that methotrexate has an inhibitory effect on ROS formation by some cell types (i.e.,synoviocytes) (Sung et al., 2000) other studies showed that it stimulates ROS formation/release by immune cells (lymphocytes, monocytes) (Herman et al., 2005). At this point, it is uncerain what impact the extract has on ROS formation by macrophages. Once this information is in hand, it will be easier to establish if the significant reductions in ascites (as well as solid tumor growth/size) seen with the extract treatments was a product of a two-pronged 'attack', i.e., increased susceptibility of tumor cells to the ROS that are now being produced at even greater levels by local macrophages, etc.

γ-Glutamyl transferase (GGT), directly involved in GSH metabolism (catalyzing transfer of γ-glutamyl moieties between glutamate and cysteine), is often significantly increased under tumor-bearing conditions and its role in tumor progression and invasion has been reported. The studies here clearly showed that increases in levels of GGT triggered by the injection of the DLA cells were mitigated by the extract treatment. As indicated in the expansive review by (Zhang et al., 2009), the regulation of GGT genes under various conditions such as oxidative stress, though established in rodent models, is still vaguely understood. Knowledge about which GGT genes are regulated, what signaling pathways are involved, and what is the expression profile of different GGT transcripts in responsive to oxidative stress would help in understanding how GGT is involved in normal physiology as well as in diseases like cancer. Thus, whether the outcomes here (i.e., reductions in GGT) are simply a useful marker of extract anti-tumor activity or if the changes in GGT activity themselves play any critical run in the anti-tumor activity itself are important points that remain to be clarified.

Lastly, nitric oxide (NO), released during various pathophysiological processes (includ-ing inflammation and carcinogenesis) and an important mediator of tumor growth was reduced in in tumor cells recovered from *A ferruginea* extract-treated hosts. As was noted above with regard to the GSH and ROS parameters, whether there is a concomitant change in the formation of NO by local macrophages (used to kill tumor cells) remains a critical piece of data to obtain in order to understand how the extract might impart an anti-tumor activity (Ruttimann, 2007).

To ascertain whether this effect of *A ferruginea* extract on DLA cells was local (cytotoxic effect) or systemic, a second experimental system, i.e., a DLA-induced solid tumor model, was employed. The results showing tumor growth inhibition and a normalization of host body weight (relative to that in non-extract/non-methotrexate-treated hosts) confirmed that the anti-cancer effect was systemic. Immune cells execute many of their functions through production of numerous cytokines. Cytokines (large family of soluble proteins) serve as mediators of immune response and have been linked with tumorigenesis process. Extensive studies have indicated that tumor cells exhibit an elevation in constitutive production of several proinflammatory cytokines such as TNF-α, GM-CSF, IL-1β and IL-6 (Dinarello, 1996). Studies on murine models suggests that TNF-α is a key mediator of cancer cachexia, in addition with IL-1β and IL-6. Release of TNF-α causes polymorphonuclear neutrophil influx and release of various inflammatory mediators from multiple cell types (Chen et al., 1999; Song et al., 2003).

The pleiotropic cytokine IL-1β induces immunosuppression in different experimental conditions *in vivo*, also it potentiates tumor invasiveness and metastasis by elevating the level of various growth factors and angiogenesis-promoting factors (VEGF). Increased expression of proinflammatory cytokines IL-1β and IL-6 have been shown in patients with head and squamous carcinoma (Suzuki et al., 1992). GM-CSF, a hematopoietic

growth factor belongs to glycoproteins family and plays a pivotal role in regulation of bone marrow progenitor cells proliferation. It is mainly produced by T lymphocytes or non-hematopoietic cells and highly expressed in solid tumors and enhances tumor cell proliferation as well as angiogenesis (Gasson, 1991). Moreover, the results obtained in this study indicates that *A ferruginea* extract could inhibit the production of IL-1β and IL-6 in tumor bearing mice and also exerts its regulatory effect on TNF-α, GM-CSF and iNOS, moreover inhibition of VEGF level also shows prevention of tumor-directed new blood vessel formation by downregulating these molecules. The lymphokine, IL-2 stimulates Natural Killer (NK) cell and T cell proliferation, further activated NK cells secretes IFN-γ exerts direct antitumor activity by interfering with killing of tumor cells by upregulating class I major histocompatibility complex molecules and inhibiting angiogenesis (Theze et al., 1996). Significant increase in level of both IL-2 and IFN- γ in serum after treatment with *A ferruginea* extract in tumor bearing animals indicate its stimulatory effect on immune system. The different subpopulations of T-cells are predictable largely by their expression of surface proteins (CD markers). Overall T-cells express a hetero-oligomeric protein CD3 (part of T-cell receptor complex), and could further subdivided in to those cells that express $CD4^+$ and $CD8^+$ populations which include helper and cytotoxic cells were increased by *A ferruginea* treatment in DLA-injected hosts. The increase in CD4/CD8 ratio suggested a strong predominance of TH1 cytokine producing T-cells on treatment with *A ferruginea*.

Compounds (quinone, quinoline, imidazolidine, pyrrolidine, pyrazole, thiazole, cyclopentenone, catechin and coumarin derivatives) identified by the GC/MS and LC/MS analysis *A ferruginea* methanolic extract have been reported to possess various biological and pharmacological activities, that could result in other forms of immune stimulation and antitumor activity against DLA tumor cells. For example, it was reported that anthraquinone derivatives (group of polyphenolic constituents) has been shown to exhibit antitumorigenic activity by activation of ERK pathway and increased expression of enhancer binding protein β (C/EBPβ), followed by Nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1) expression and subsequently induces apoptosis in human colorectal cancer (Nualsanit et al., 2012). Recently a report shown imidazolidine derivatives are involved in modulation of immune system and exhibits anti-inflammatory and antinociceptive activities (Guerra et al., 2011). It is well known; medicinally important coumarins and catechins comprise a large class of compounds found throughout the plant kingdom. Coumarin derivatives are able to arrest cell cycle at G2/M stage and in addition induce apoptosis of human cancer cells (Kim et al., 2009). For example, Eryciboside, a coumarin derivative was isolated from the plant *Laguncularia racemose* showed significant antioxidative activity and potent inhibition of human tumor related protein kinases FLT3 and SAK (Shi et al., 2010). Interestingly, a recent report evidenced that catechin rich fractions from *Acacia catechu* inhibited the 7,12-Dimethylbenz[a] anthracene-Induced Mammary Carcinoma in murine models (Monga et al., 2012). It is possible that the coumarin and catechin derivatives present in the *A ferruginea* extract may have afforded protection to the treated mice towards DLA tumor cells.

In conclusion, the present investigation clearly indicates that treatment with *A ferruginea* extract was effective in inhibiting inflammation and tumor progression *in vivo*. This is most likely due to high content and synergistic activity of specific constituents such as flavonoids, phenolics, steroids, terpenoids, alkaloids, saponins, quinones and tannins. Nevertheless, the precise molecular mechanism by which *A ferruginea* extract mediates anti-tumor activitiy remains to be determined. Further investigations are in progress in our laboratory to isolate the specific bioactive agents in the extract with potential for use in anti-cancer therapy, and to elucidate their associated mechanisms of therapeutic action.

Acknowledgements

The authors wish to thank, South Indian Textile Research Association, Coimbatore and SGS Laboratories, Chennai, India for GC/MS and LC/MS analysis respectively. The valuable suggestions from Dr. J. Jannet Vennila, Head, Department of Biotechnology and Dr. M. Patrick Gomez, Director, School of Biotechnology and Health Sciences, Karunya University, is gratefully acknowledged. The authors also wish to thank, Services Team, Qube Bioscience Pvt Ltd, Hyderabad, India for Flow Cytometry analysis

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