Anti-cancer activity of three Arabic plants

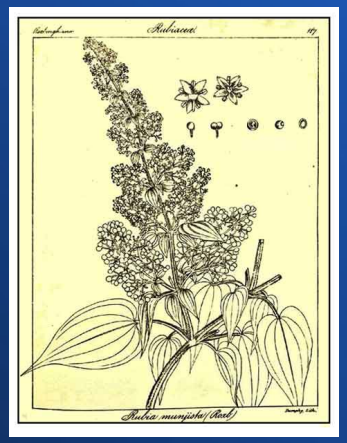
Acacia, Rubia and Alkanna

Acacia, Rubia and Alkanna :

Screening and evaluation of compounds for cancer treatment

- Recently, due to the severe side effect of using the conventional chemotherapy for cancer treatment, several studies have been carried out to find either new and safe targeted therapy against cancer cells only, or to find natural compounds that ameliorate the side effects of the conventional chemotherapy;
- Therefore, recent studies have been directed to screen, and evaluate the new compounds naturally present especially in the medicinal plants;
- Three Arabic plants (Acacia, Rubia, Alkanna) traditionally used for the treatment of several diseases were tested for their anti-cancer activity and several studies established cytotoxicity against cancer cell lines and mice-bearing human xenografts;

Arabic plants: Acacia, Rubia, Alkanna



ACACIA (bark, pods, seeds, honey, comb wax, gum, leaves)

Family: Fabacae, leguminosae, mimosoidae

Varieties: a. nilotica (gum), a. ataxacantha, a. mearnsii (leaves), a. catechu, a. hydaspica, a. leucophloea, a. colei, a. tumida, a. cyclops, a. ligulata, a. salicina, a. cyanophylla

It is estimated that there are roughly 1380 species of Acacia worldwide, about two-third of them native to Australia and rest spread around tropical and subtropical regions of the world (SAINI et al. 2008). It is a moderate size deciduous tree with rough dark grey brown bark.

RUBIA (in Sanskrit: Manjistha)

Family: Rubiaceae; it comprises of about 450 genera and 6500 species and includes trees, shrubs and herbs (Williams, 2002).

Rubia comprises about 60 species worldwide, native to the Himalayan region, north-east India, Japan, Indonesia and Sri Lanka.

R. cordifolia Linn. (Rubiaceae) is a perennial climber with very long, cylindrical, flexuose roots with a thin red bark (Kirtikar and Basu, 1980).

ALKANNA

Family: Boraginaceae

Alkanna comprises 30 to 50 species amongst them alkanna tinctoria and is widely distributed in Europe, north Africa and western Asia .

The species are hispid or pubescent herbs, with oblong, entire leaves, and bracteated racemes, rolled up before the flowers expand. The corolla is rather small, between funnel and salver-shaped; usually purplish-blue, but in some species yellow or whitish; the calyx enlarges in fruit.

Acacia : Traditional use

- Acacia has been used in traditional medicine to treat high cholesterol, diabetes, cancer, gingivitis, mouth sores, pharyngitis, indigestion in children, diarrhoea, inflammation, ophthalmia, hemorrhoid, leprosy, bleeding piles, and leucoderma problems
- The leaves are believed to possess hypotensive, CNS-depressent, antisyphilitic and antibacterial (both gram+ and gram-) principles, while the gum possesses demulscent properties (K HARE 2007)
- It is used by traditional healers of different regions of Chhattisgarh in treatment of various cancer types of mouth, bone and skin. In West Africa, the bark and gum are used against cancers and/or tumors (of ear, eye, or testicles) and indurations of liver and spleen, the root for tuberculosis, the wood for smallpox and the leaves for ulcers
- Recent scientific efforts have focused on the potential roles of extracts of traditional herbs as alternative and complementary medications for cancer treatment. In addition, Acacia has been reported to have anti-tumor activity with mouse breast cancer, human osteosarcoma and human prostate cancer (Kaur et al., 2005). In addition, Acacia showed antiproliferative effects against human colorectal, oral and lung cancer cell lines

Chemical composition of Acacia

Contents of seeds, pods and leaves

Proximate chemical composition of *Acacia leucophloea* Roxb.

Component	Seeds (%)	Pods (%)
Crude protein	25.81 ± 0.93	13.81 ± 0.93
Total lipids	5.03 ± 0.07	4.75 ± 0.09
Total carbohydrates	52.30 ± 1.16	46.75 ± 0.89
Crude fiber	6.70 ± 0.38	19.11 ± 0.44
Moisture	5.61± 0.23	9.57 ± 0.79
Ash	4.55 ± 0.56	6.01 ± 0.19

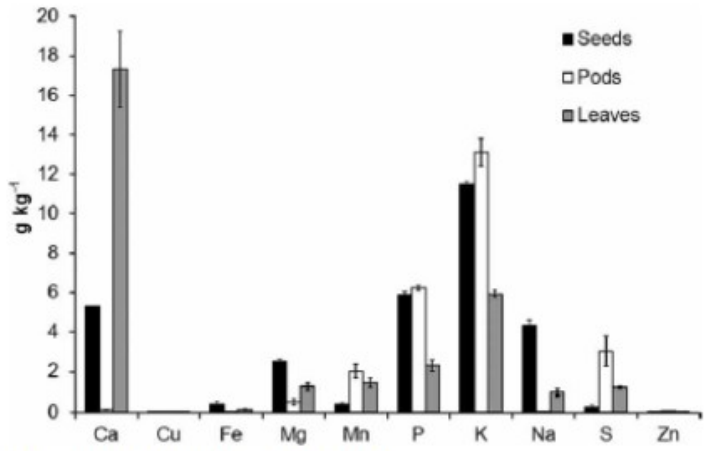


Fig. 1. Mineral contents of *Acacia leucophloea* Roxb.

- Mineral and volatile organic compound content (VOC) is very different in seeds, pods and leaves of Acacia
- The most enriched VOC profile therapy was obtained for raw honey. The latter form is mainly used in clinical trials and studies towards anti-cancer therapy

Chemical composition of Acacia:

Protein and amino-acid fractions

Tab. 3. Amino acid composition of *Acacia leucophloea* Roxb.

Amino acid	Seeds (%)	Pods (%)
Alanine	6.86 ± 0.33	8.77 ± 0.21
Arginine	6.35 ± 0.19	5.16 ± 0.62
Aspartic acid	13.07 ± 0.69	10.21 ± 0.42
Cysteine	1.21 ± 0.72	1.03 ± 0.19
Glutamic acid	17.29 ± 0.75	15.46 ± 0.89
Glycine	8.02 ± 0.05	6.91 ± 0.34
Histidine	4.36 ± 0.52	3.41 ± 0.62
Isoleucine	3.89 ± 0.41	4.13 ± 0.49
Leucine	8.01 ± 0.29	9.17 ± 0.32
Lysine	6.08 ± 0.11	8.03 ± 0.92
Methionine	0.63 ± 0.84	0.17 ± 0.23
Phenylaniline	4.62 ± 0.71	4.62 ± 0.71
Proline	5.24 ± 0.64	2.01 ± 0.44
Serine	4.37 ± 0.73	6.39 ± 0.23
Threonine	3.86 ± 0.48	5.96 ± 0.48
Tryptophan	0.32 ± 0.08	0.91 ± 0.22
Tyrosine	2.03 ± 0.55	2.03 ± 0.14
Valine	4.73 ± 0.07	5.63 ± 0.11

Tab. 2. Protein fractions of seed and pods of *Acacia leucophloea* Roxb.

Protein fractions	Seeds (%)	Pods (%)
Albumins	9.67 ± 0.39	5.18 ± 0.89
Globulins	13.01 ± 1.27	6.12 ± 0.25
Prolamines	1.21 ± 0.54	1.09 ± 0.46
Glutelins	2.32 ± 1.08	2.03 ± 0.77

Chemical composition of Acacia:
Fatty acid and phytosteroles profile

Table 1 Carotenoids and tocopherols† (mg.kg^{-1} of total lipids) of *Acacia* seed oils

Species	Oil content (dw%)	Carotenoids			Tocopherols			Total tocopherols
		Lutein	Zeaxanthin	Total carotenoids	α tocopherol	γ tocopherol	δ tocopherol	
Acacia ligulata	9.03	43.83 ^{BC}	6.28 ^{A}	50.10	315.85 ^{B}	462.14 ^{A}	30.77 ^{A}	808.76
Acacia cyclops	6.83	35.41 ^{C}	2.80 ^{B}	38.21	85.51 ^{C}	127.25 ^{D}	8.66 ^{B}	221.42
Acacia salicina	12.18	54.67 ^{B}	2.37 ^{B}	57.04	404.30 ^{B}	155.78 ^{C}	3.33 ^{C}	563.41
Acacia cyanophylla	10.05	109.55 ^{A}	4.21 ^{A}	113.76	560.14 ^{A}	185.41 ^{B}	9.15 ^{B}	754.70

† Each value is the mean of duplicate analyses.
Superscript letters with different letters in the same column of species indicate a significant difference (p <0.05) analyzed using Duncan's multiple range test.

Table 2 Sterols (g. kg^{-1} of total lipids) of *Acacia* seed oils

Phytosterols	Species			
	Acacia ligulata	*Acacia cyclops*	*Acacia salicina*	*Acacia cyanophylla*
Cholesterol	*tr*††	0.22 ^{A}	0.06 ^{C}	0.07 ^{B}
Δ^{7} cholestenol	*tr*	0.03 ^{A}	0.06 ^{A}	*tr*
Campesterol	0.08 ^{A}	0.20 ^{A}	0.13 ^{A}	0.20 ^{A}
Campestanol	0.20 ^{C}	0.42 ^{A}	0.11 ^{D}	0.27 ^{B}
Stigmasterol	0.31 ^{B}	0.36 ^{A}	0.15 ^{C}	0.29 ^{B}
Δ^{7} stigmasterol	0.28 ^{C}	0.27 ^{C}	0.57 ^{A}	0.36 ^{B}
Δ^{7} campesterol	0.35 ^{C}	*tr*	0.38 ^{B}	0.45 ^{A}
$\Delta^{5,23}$ stigmastadienol	0.19 ^{A}	0.05 ^{B}	0.15 ^{A,B}	0.22 ^{A}
β sitosterol	4.15 ^{B}	5.40 ^{A}	3.48 ^{C}	4.06 ^{B}
Δ^{5} avenasterol	0.24 ^{C}	1.13 ^{A}	0.38 ^{B}	0.33 ^{B,C}
Δ^{7} sitosterol	*tr*	*tr*	0.21 ^{A}	0.20 ^{A}
$\Delta^{5,\ 24\ (25)}$ stigmasterol	0.09 ^{C}	0.18 ^{A}	0.12 ^{B}	0.20 ^{A}
$\Delta^{5,\ 24}$ stigmastadienol	0.21 ^{B}	0.23 ^{A}	0.08 ^{C}	*tr*
Δ^{7} stigmastenol	1.11 ^{C}	2.57 ^{A}	1.22 ^{C}	1.78 ^{B}
Cycloartenol	0.45 ^{A}	0.38 ^{B}	0.15 ^{D}	0.33 ^{C}
Δ^{7} avenasterol	0.02 ^{B}	0.16 ^{A}	0.03 ^{B}	0.05 ^{B}
$^{24,\ methylene}$ cycloartanol	0.02 ^{A,B}	*tr*	0.05 ^{A}	0.06 ^{A}
Citrostadienol	0.02 ^{A}	*tr*	tr	0.05 ^{A}
Total phytosterols	**7.70**	**11.62**	**7.33**	**8.94**

† Each value is the mean of duplicate analyses.
††*tr*: traces.
Superscript letters with different letters in the same line of species indicate a significant difference (p <0.05) analyzed using Duncan's multiple range test.

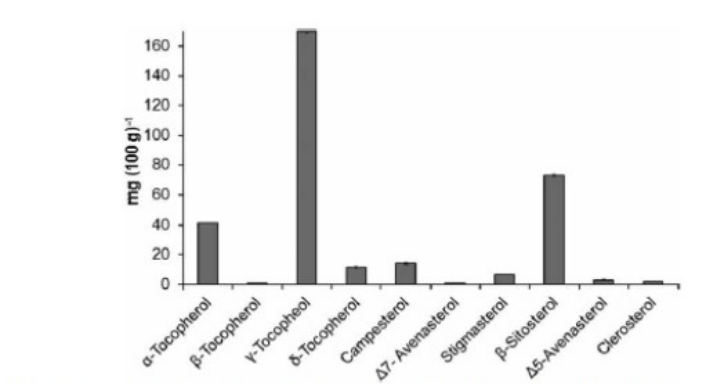


Fig. 3. Tocopherol and sterol profile of oils from seeds of *Acacia leucophloea* Roxb.

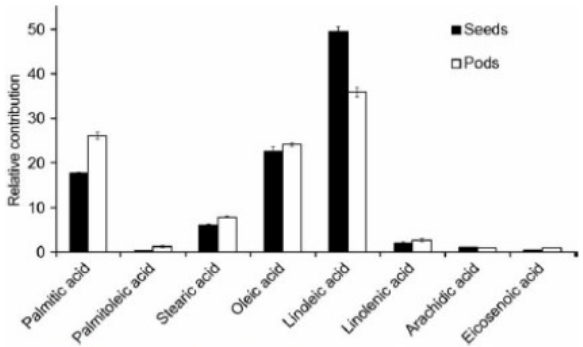


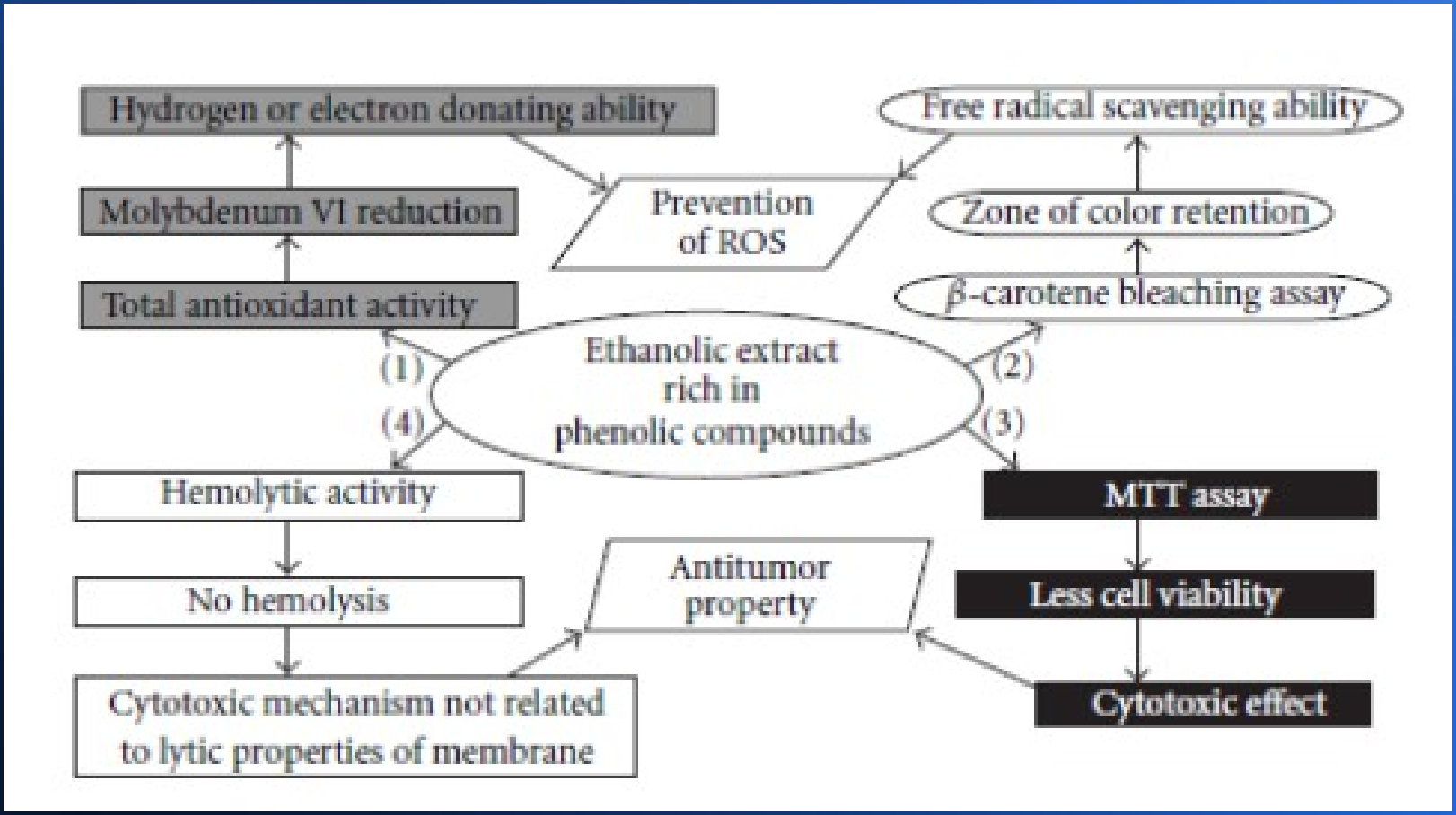
Fig. 2. Fatty acid profile of *Acacia leucophloea* Roxb.

Chemical composition of Acacia:
Volatile organic compounds (VOC)

Monoterpenes:	α-pinene, myrcene, cis-β-ocimene, ocimene (honey), 4-terpinelol, verbenone (honey), isophorone (strained honey), hexanoic acid (strained honey)
Other terpenes:	lupeol, betulinic acid, betulinic acid-3-trans-caffeate
Esters:	δ-hexalactone, γ-heptalactone, quinone, cyclopentenone
Alcohols:	1-phenylethanol, octadecyl, phytol, cis-verbenol
Polyphenols:	phenol, proanthocyanidins
Alcaloids:	dimethyltryptamine (DMT), 5-methoxydimethyltryptamine (5-MeO-DMT), N-methyl-tryptamine (NMT), kaempferol, dihydrokaempferol, taxifolin, (+)-afzelchin gum
Xanthophylls:	zeaxanthin, lutein
Flavanoids:	catechin, (-) epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, rocatechin, phloroglucinol, procatechuic acid, catecutannic acid, quercetin, quercitrin, quinolin, imidazolin, pyrrolidin
Glycosides:	poriferasterol, poriferasterol
Tannins:	gallic acid, phlobatannins

- **The most enriched VOC profile was obtained for raw honey.**
- **Number of alcohols, esters, and variety of terpenes, as well as their concentration in the honey samples decrease through ripening processes.**
- **Flavanoids are mostly present in heartwood, alkaloids in leaves and glycosides, tannins and sugars in bark.**
- **Mostly polyphenols are active against cancer and efficacy relies on the combination of various components of Acacia.**

Anti-cancer activity of Acacia species



Anti-cancer activity (1):

Evidence from *in vivo* study with testing of A. ferruginea in Dalton's lymphoma ascites (DLA) induced tumor-bearing mice

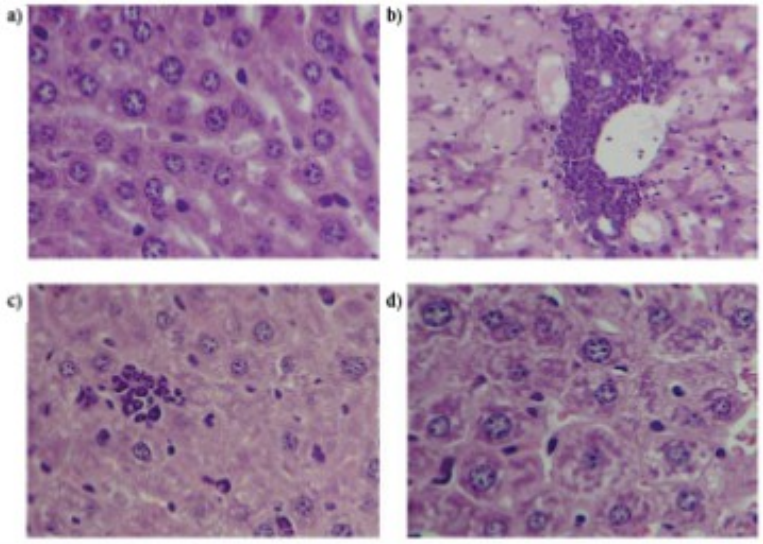


Figure 6. **Histopathological Changes in Liver of Experimental Mice.** Pictures presented are representative liver sections collected at end of experimental periods (i.e., day 15 after DLA injection). (A) Normal (no tumor, no extract); (B) tumor only; (C) tumor+*A ferruginea* extract; (D) tumor+methotrexate

- 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

- The aim of the 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.

- Treatment with extract significantly increased (63%) the life span of tumor-bearing mice to 27.2±1.5 days compared to 16.7±1.0 days for untreated mice compared to +79% for mice treated with methotrexate.

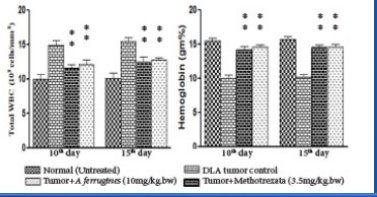
- Tumor volume decreased significantly.

- However toxicity was high: body weight loss was 17.1% in treated DLA-injected hosts (38.8% in non-treated controls) over the study period, whereas mice treated with methotrexate showed BWL of 13.6% (Table 3).

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 (*p<0.05,*p<0.01)



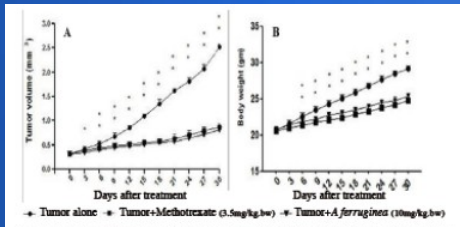


Figure 7. **Effect of *A ferruginea* Extract on Tumor Volume (A) and Body Weight (B) on Indicated Days After DLA Intramuscular Injection.** Treated animals received 14 doses of extract (10 mg/kg.b.wt). Tumor radii in extract- and non-extract-treated controls were measured at 3-d intervals using a vernier calliper. Body weights of treated and non-extract-treated controls were also measured at 3-d intervals. Values shown are means (±SD; in mm³) from 6 mice/treatment group. Values significantly different from tumor (non-extract-treated) control (*p<0.05, **p<0.01)

Anti-cancer activity (2):

Evidence from *in vivo* study with testing of A. nilotica in Dalton's lymphoma ascites (DLA) induced tumor-bearing mice

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

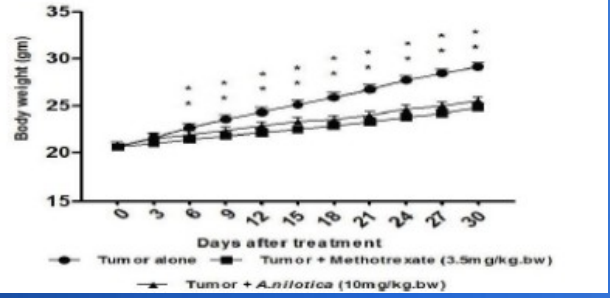
Treatment with *A.nilotica* extract for a period of 14 consecutive days in the DAL induced solid tumor bearing mice showed a significant (**p< 0.01) reduction in tumor volume (1.12±0.07 mm³) when compared to the tumor alone group (2.52±0.08 mm³) on 30th day. The animals treated with the standard drug (methotrexate 3.5 mg/kg BW) were found to be also efficient (0.94±0.09 mm³) 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).

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.

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



- The outcome of the study revealed that A. nilotica could be used as a natural anti-cancer agent for human health

- Almost normal hepatocellular architecture was observed in mice treated with A. nilotica with reduced vacuole formation and inflammation. Histological examination revealed hepatoprotective effect from A. nilotica on hepatotoxicity

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

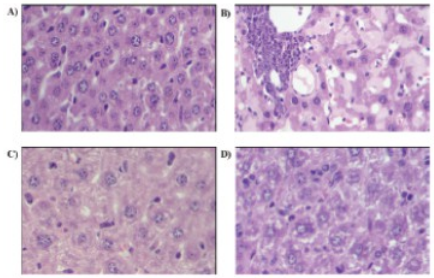


Figure 6. Histopathological Changes in Liver of Experimental Mice. Pictures shown are from representative liver sections collected at the end of the experimental periods (i.e., Day 15 after DAL cells intraperitoneal injection).

Cytotoxicity against tumor cells:

Evidence from *in vitro* studies

- Cytotoxicity of extracts from different Acacia species against cancer cell lines and vero cells was assessed in four *in vitro* studies.
- Acacia species inhibited cell proliferation significantly ($p < 0.05$) n a dose andconcentration dependent manner, with IC50 of 1.9-3.7-4.43 % (v/v) against >8% with medium.
- Extracts showed no cytotoxicity against normal vero cells.

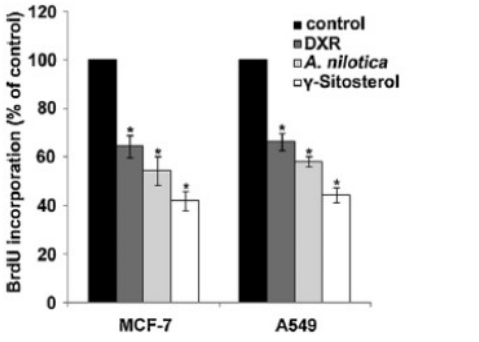


Fig. 4. Inhibition of MCF-7 and A549 proliferation by An extract and γ-Sitosterol. Quantitation of MCF-7 and A549 growth in the presence of An extract and γ-Sitosterol for 48 h ($n = 3$, ±SEM). Proliferation was determined using a colorimetric ELISA based on BrdU incorporation. *$P < 0.001$ *versus* control.

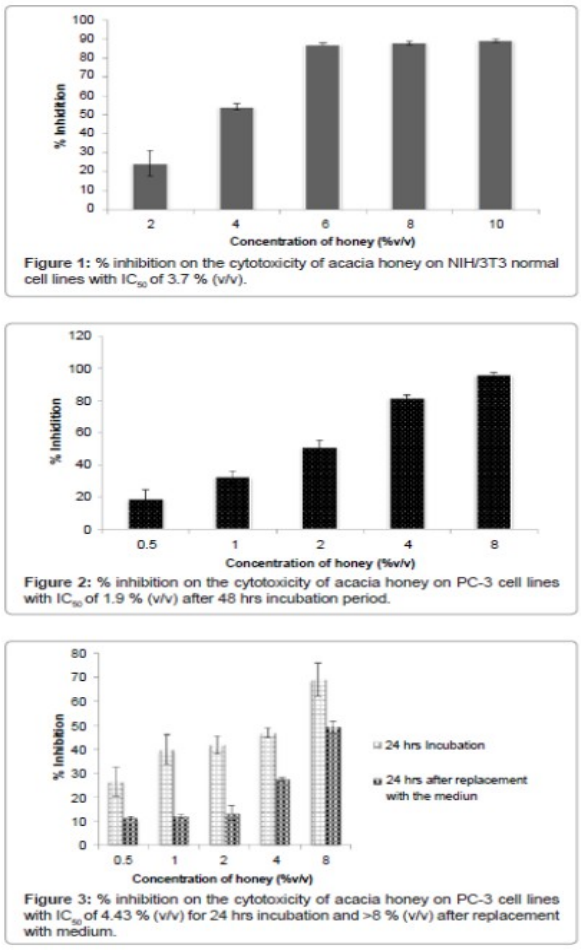
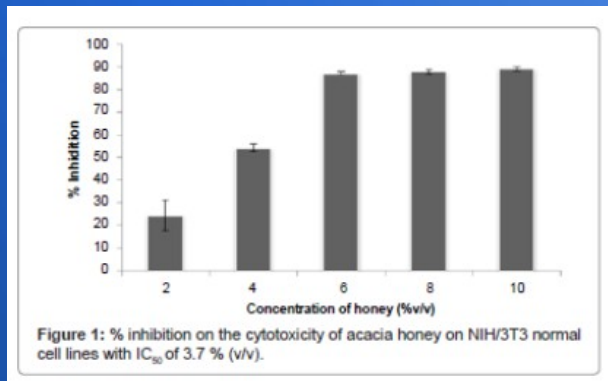


Figure 1: % inhibition on the cytotoxicity of acacia honey on NIH/3T3 normal cell lines with IC_{50} of 3.7 % (v/v).

Figure 2: % inhibition on the cytotoxicity of acacia honey on PC-3 cell lines with IC_{50} of 1.9 % (v/v) after 48 hrs incubation period.

Figure 3: % inhibition on the cytotoxicity of acacia honey on PC-3 cell lines with IC_{50} of 4.43 % (v/v) for 24 hrs incubation and >8 % (v/v) after replacement with medium.



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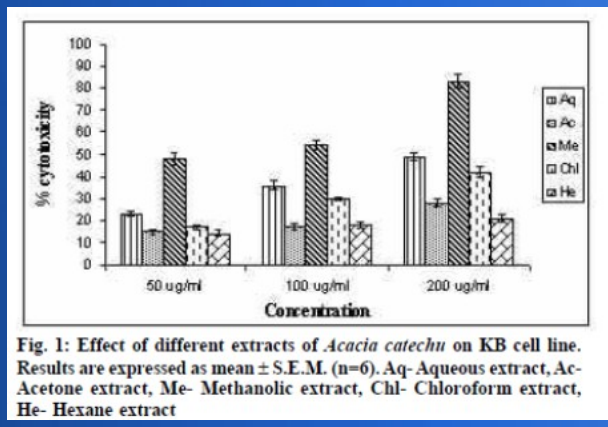


Fig. 1: Effect of different extracts of *Acacia catechu* on KB cell line. Results are expressed as mean ± S.E.M. (n=6). Aq- Aqueous extract, Ac- Acetone extract, Me- Methanolic extract, Chl- Chloroform extract, He- Hexane extract

Cytotoxicity against tumor cells:

Evidence from *in vitro* studies (2)

Table 5 Cytotoxic effect of *A. hydaspica* methanol extract and its derived fractions on MDA-MB 361, HCC-38 and Vero cells of green monkey after 48 h of treatment

Extract/Fraction	MDA361 cell line	SI	HCC38 cell line	SI	Vero cell line
	IC_{50} (µg/ml)	$IC_{50}V/IC_{50}M$	IC_{50} (µg/ml)	$IC_{50}V/IC_{50}H$	IC_{50} (µg/ml)
AHM	46.9 ± 1.31*	5.42	75.9 ± 1.32*	3.34	254 ± 1.81
AHE	29.9 ± 0.91*	9.83	39.5 ± 0.87*	7.44	294 ± 1.55
AHB	37.1 ± 1.01*	6.97	56.1 ± 0.93*	4.60	258 ± 1.68

Each value expressed as mean ± SEM ($n = 3$). Selectivity index (SI) >3 is considered to be highly selective, * shows significance at $p < 0.001$ as compared to Vero cells. M represents MDA-MB-361; V represents Vero and H represents HCC38 cell lines. (One way ANOVA followed by Tukey's multiple comparison test)

TABLE 4: Cytotoxic activity of various extracts obtained from leaves of *A. nilotica* on Vero and Hela cell lines.

Extracts	IC_{50} ($\mu g\,mL^{-1}$)	
	Vero	Hela
PE	$79.0^{(e)}$	$48.5^{(e)}$
BZ	$65.4^{(b)}$	$34.1^{(b)}$
DCM	$78.2^{(d)}$	$43.7^{(d)}$
CF	$70.5^{(c)}$	$40.9^{(c)}$
EA	$53.6^{(a)}$	$28.9^{(a)}$
AQ	$>100^{(f)}$	$>100^{(f)}$

Means within each column with different superscript letters (a)–(f) differ significantly ($P < .05$).

Anti-cancer Activity

Study was conducted to evaluate the cytotoxic effect of aqueous extract of *Acacia catechu willd* heartwood in a human epithelial carcinoma cell line (A431) and anti-tumour activity against DMBA/TPA induced squamous cell carcinoma in Balb/c mice. It was investigated that chemopreventive effect of aqueous extract of *Acacia catechu willd* heartwood maybe was due to its polyphenolic compounds that exhibit powerful antioxidant activity [3]. Study was aimed at evaluating the antiproliferative and apoptotic potentials of *Acacia catechu willd* on HeLa, COLO- 205, and fibrosarcoma HT-1080 cell lines and also to evaluate its safety on normal human lymphocytes. Different concentrations of these were evaluated for their cytotoxicity by the trypan blue dye exclusion method and MTT assay on the cancer cell lines HeLa, fi brosarcoma HT-1080, COLO-205, and a normal cell line (human peripheral lymphocytes). The apoptotic potential was analyzed by DNA fragmentation analysis, morphology observation, and fluorescence microscopical observations of the treated cells by AO/EB (acridine orange/ethidium bromide) staining. The methanol and hexane extracts of A. catechu were found to be antiproliferative and cytotoxic at lower concentrations and induced cell death in COLO-205 cells and also in HeLa cells. Their effect on HT-1080 fi brosarcoma cells was less pronounced. The methanol and hexane extracts with the same concentrations had least cytotoxicity on normal lymphocytes. The aqueous extract was less effective on the cancer cell lines[4].

Cytotoxicity against tumor cells:

Evidence from *in vitro* studies (3)

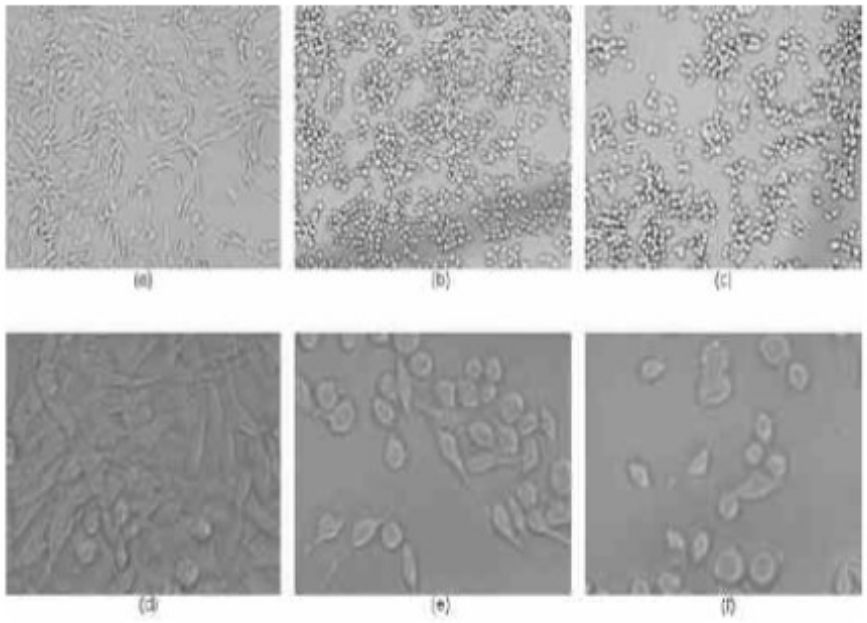


Fig. 2: Effect of methanolic extract treatment on the morphology of the KB cells at the magnification of 40X. At 0 hr (a), At 6 hr (b) and At 12 hr (c). And at the magnification of 100X. At 0 hr (d), At 6 hr (e) and At 12 hr (f).

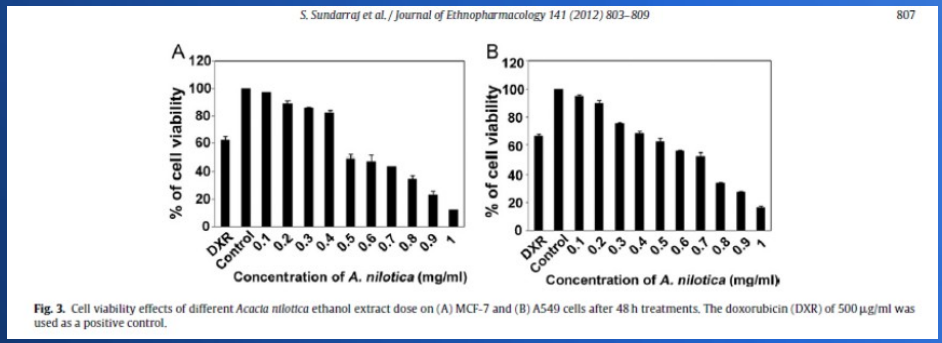


Fig. 3. Cell viability effects of different *Acacia nilotica* ethanol extract dose on (A) MCF-7 and (B) A549 cells after 48 h treatments. The doxorubicin (DXR) of 500 μg/ml was used as a positive control.

Anti-cancer activity of Acacia:
Molecular pathways (1)

- Flavanoids (amongst them catechins) may mediate the activity of acacia species on cancer cells while presenting no cytotoxic effect on healthy cells. Phenolic compounds are significant chemoprotective factors against cancer amongst them polyphenols contained in acacia species.

- Molecular pathway of acacia plant cytotoxicity might be induction of apoptosis due to arrest of cell cycle in G0/G1 phase, regulation of pro-inflammatory cytokines and TNF-gamma, extenuation of oxydative damage, diminishing of tumor angiogenesis, modulation of calcium secretion and down-regulation of prostate specific antigene (PSA).

- Immunoblot results suggested the pathway of apoptosis induction by increasing Bax/Bcl-2 ratio which results in the activation of caspase-cascade and ultimately leads to the cleavage of Poly adeno ribose polymerase (PARP).

Anti-cancer activity of Acacia:
Molecular pathways (2)

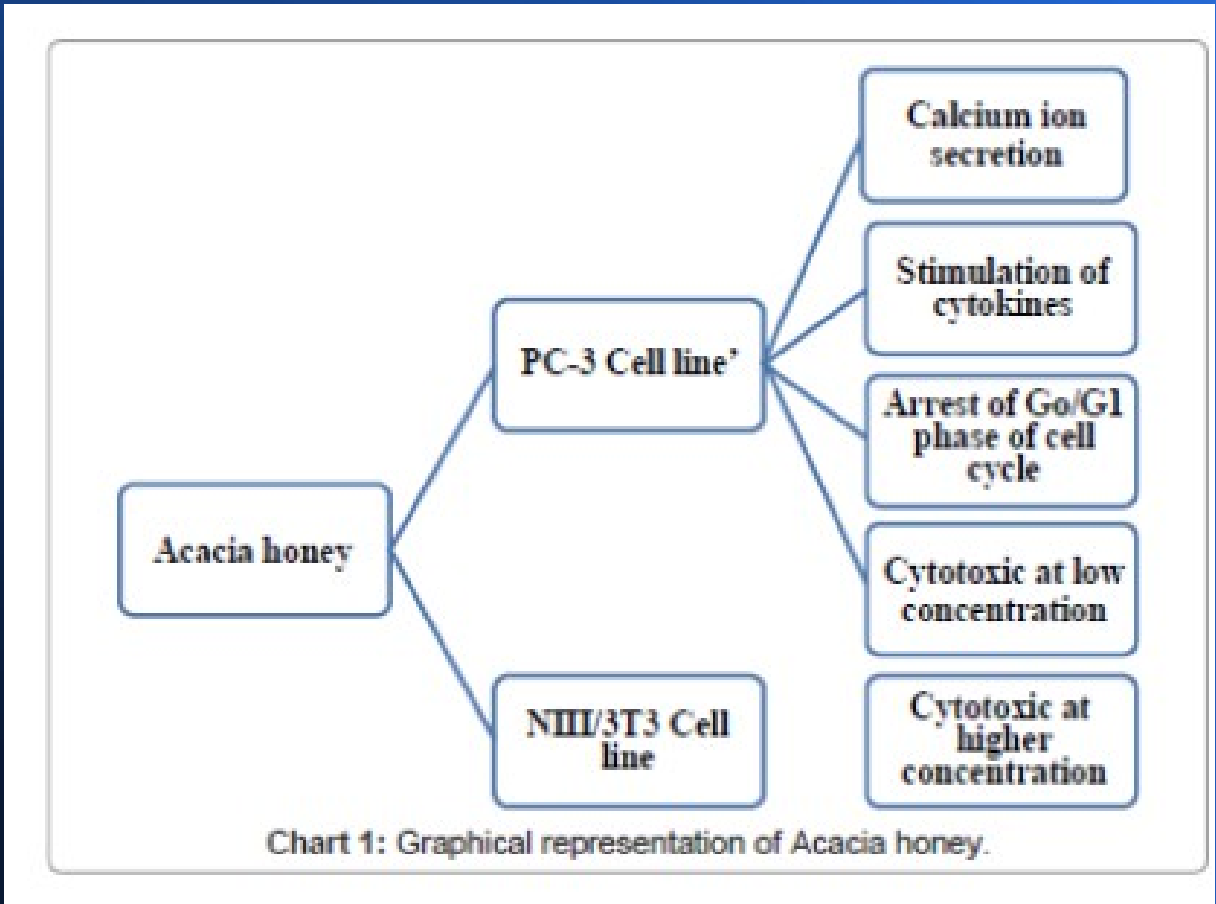


Chart 1: Graphical representation of Acacia honey.

Anti-cancer activity of Acacia:
Molecular pathways: induction of apoptosis (3)

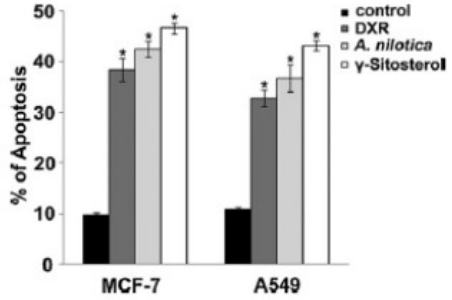


Fig. 5. An extract and γ-Sitosterol induces apoptosis of MCF-7 and A549 cells. MCF-7 and A549 cells (1×10^6 cells/ml) were treated with 0.5 mg/ml of An extract and γ-Sitosterol for 48 h staining by AO/EtBr staining. Results are mean ± SEM ($n = 3$) *$P < 0.001$ *versus* control.

3.6. Acacia nilotica extract and γ-Sitosterol induce apoptosis in cancer cells

We found that ethanolic extract of *Acacia nilotica* and γ-Sitosterol used at the concentration of 0.5 mg/ml decreased the percentage of viable cells in MCF-7 and A549 cells (Fig. 5). The percentage of apoptotic cells were increased to 42.46 and 46.68% for MCF-7 cells and 36.8 and 43.24% for A549 cells respectively, compared to untreated cells. However, treatment of *Acacia nilotica* and γ-Sitosterol significantly increased apoptosis as compared with control cells.

cycle arrest. In addition, treatment with An extract and γ-Sitosterol resulted in a significant decrease in G0/G1 phase cell number and increase in G2/M phase cell number.

Consistent with the significant accumulation of cells in G_2/M and S phases in An extract treated cells, immunoblotting analysis showed increases in expression levels of G_2/M and S phases associated cell cycle regulators such as cyclin B and cyclin E. We also observed down regulation of c-Myc levels in An extract treated cells. Since c-Myc expression is higher at late G_1 promoting G_1 to S transition, the decreased expression of c-Myc seen in An extract treated cells may reflect the partial loss of G1 cells.

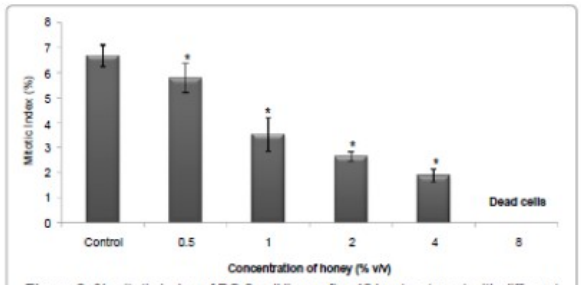


Figure 8: % mitotic index of PC-3 cell lines after 48 hrs treatment with different concentration of honey.

3.5. Changes in nuclei morphology after Acacia nilotica extract treatment

A double staining with a mixture of ethidium bromide and acridine orange was used to visualize and quantify the number of viable and apoptotic cells. Viable cells exhibit large green nuclei whereas apoptotic cells show signs of nuclear condensation or nuclear bead formation and are colored in orange.

3.3. Effect of Acacia nilotica extract on DNA and nuclear fragmentation in MCF-7 and A549 cells

One of the mechanisms by which cell growth is suppressed is apoptotic cell death. Therefore, the effect of *Acacia nilotica* extract on DNA fragmentation was examined in MCF-7 and A549 cells. The nucleosomal DNA fragmentation was observed when cells were treated with 0.5, 0.7 and 0.9 mg/ml of *Acacia nilotica* extract for 48 h. MCF-7 cells was more sensitive as even the low dose of extract induced DNA fragmentation which was further enhanced in the high dose treated group. The profile for *Acacia nilotica* extract-induced apoptosis closely correlated with its growth suppressive effects. Thus, the growth suppression induced by *Acacia nilotica* extract in MCF-7 and A549 cells may be related to the induction of apoptosis.

Anti-cancer activity of Acacia:
Molecular pathways: arrest of cell cycle (4)

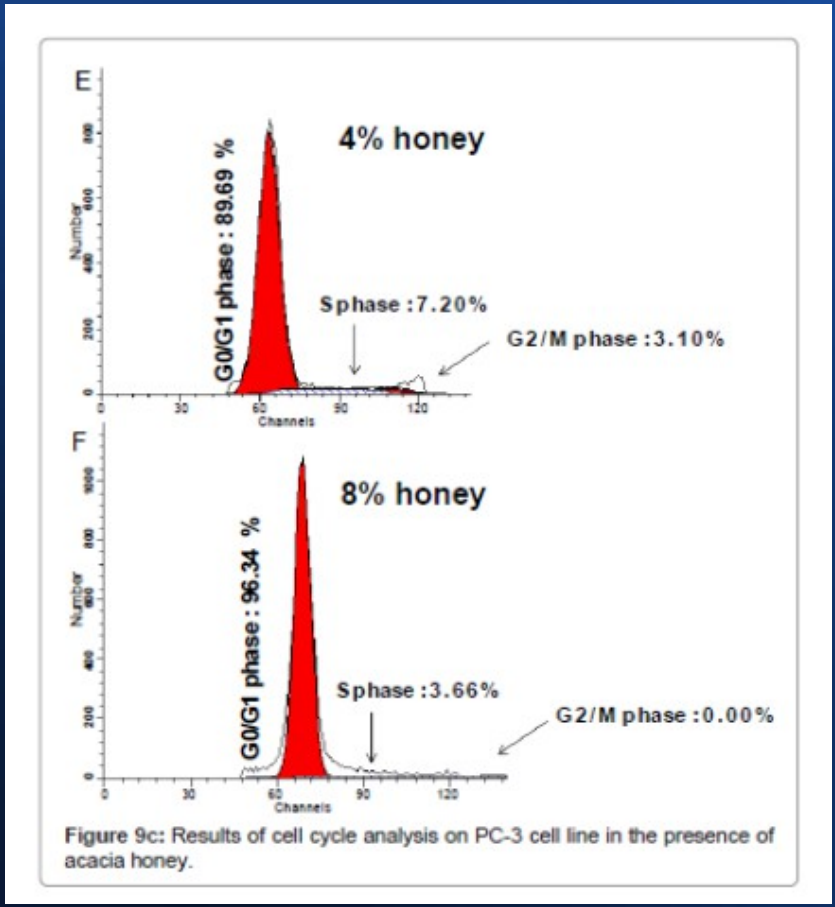


Figure 9c: Results of cell cycle analysis on PC-3 cell line in the presence of acacia honey.

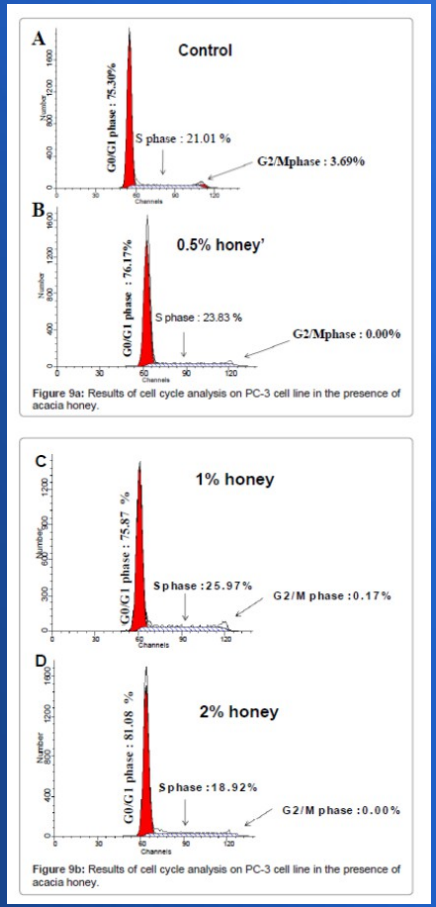


Figure 9a: Results of cell cycle analysis on PC-3 cell line in the presence of acacia honey.

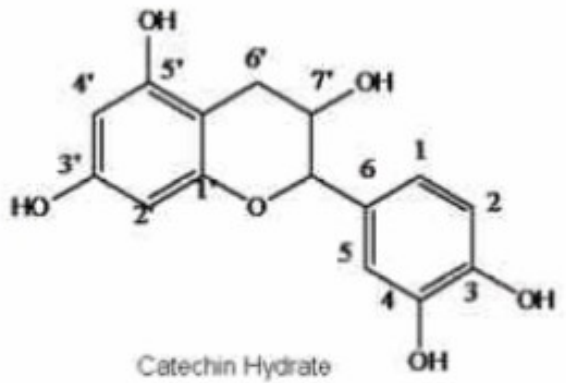
Figure 9b: Results of cell cycle analysis on PC-3 cell line in the presence of acacia honey.

Anti-cancer activity of Acacia:

Flavanoids : role of catechin hydrate (5)

cell viability in cancer cells. Our results are in agreement with the previous study of Kalaivani et al., demonstrating that distinct effect of *A. nilotica* extracts in each cell line might be due to the phyto-diversity or varied mechanisms accompanying each of the compounds [58]. Flavonoids have been presented little or no cytotoxic effect on healthy cells while being cytotoxic against various human cancer cells [59]. Flavonoids mediate their actions by various ways i.e., simply binds to the cell membrane, penetrate in vitro cultured cells or via modulation of the cellular metabolic activities. Extenuation of oxidative damage, carcinogen inactivation, inhibition of cell growth and differentiation, induction of cell cycle arrest and apoptosis, diminishing of tumor angiogenesis and restriction of metastasis are the major implications of flavonoids anti-carcinogenic activities [60, 61]. The

In conclusion, the present study has clearly demonstrated that the methanolic extract from the bark of *Acacia catechu* exhibits cytotoxic activity via inhibiting COX-2 enzyme activity and inducing apoptotic pathway as demonstrated using DNA fragmentation assay and further confirmed by morphological and structural changes. This cytotoxic effect was present because of the active component 2-(3,4-Dihydroxy-cyclohexyl)-chroman-3,5,7-triol, whose structure was found to be similar with catechin hydrate. The structure



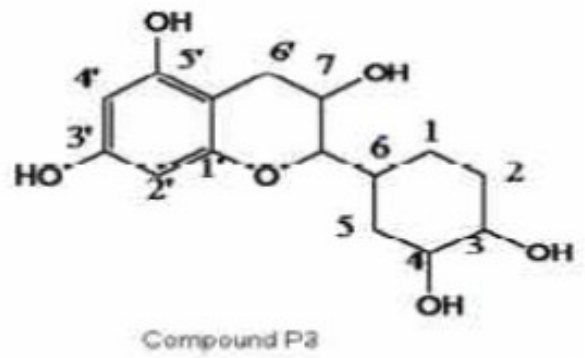
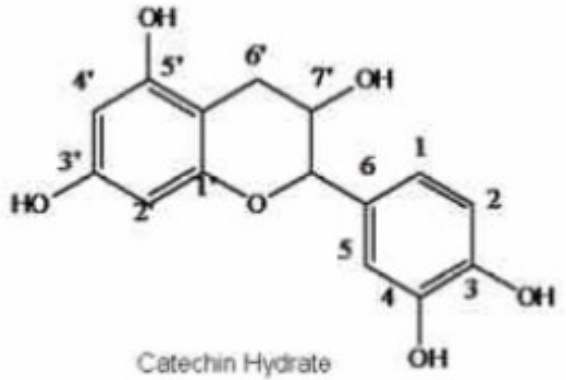


Fig. 7: Structures of Catechin Hydrate and compound P3.

Anti-cancer activity of Acacia:

Flavanoids : role of catechin hydrate (6)

In conclusion, the present study has clearly demonstrated that the methanolic extract from the bark of *Acacia catechu* exhibits cytotoxic activity via inhibiting COX-2 enzyme activity and inducing apoptotic pathway as demonstrated using DNA fragmentation assay and further confirmed by morphological and structural changes. This cytotoxic effect was present because of the active component 2-(3,4-Dihydroxy-cyclohexyl)-chroman-3,5,7-triol, whose structure was found to be similar with catechin hydrate. The structure



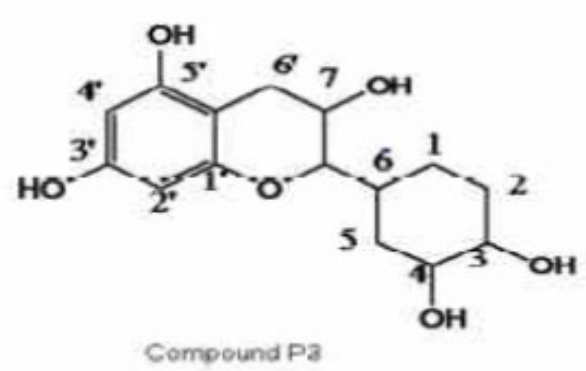


Fig. 7: Structures of Catechin Hydrate and compound P3.

Anti-cancer activity of Acacia:
Modulation of pro-inflammatory cytokines (7)

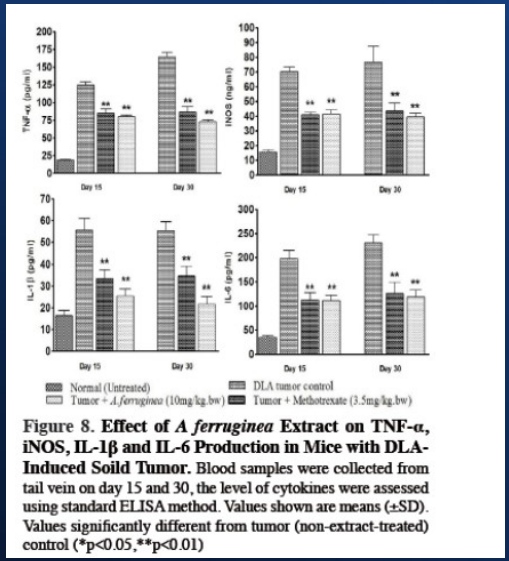


Figure 8. Effect of *A ferruginea* Extract on TNF-α, iNOS, IL-1β and IL-6 Production in Mice with DLA-Induced Soild Tumor. Blood samples were collected from tail vein on day 15 and 30, the level of cytokines were assessed using standard ELISA method. Values shown are means (±SD). Values significantly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

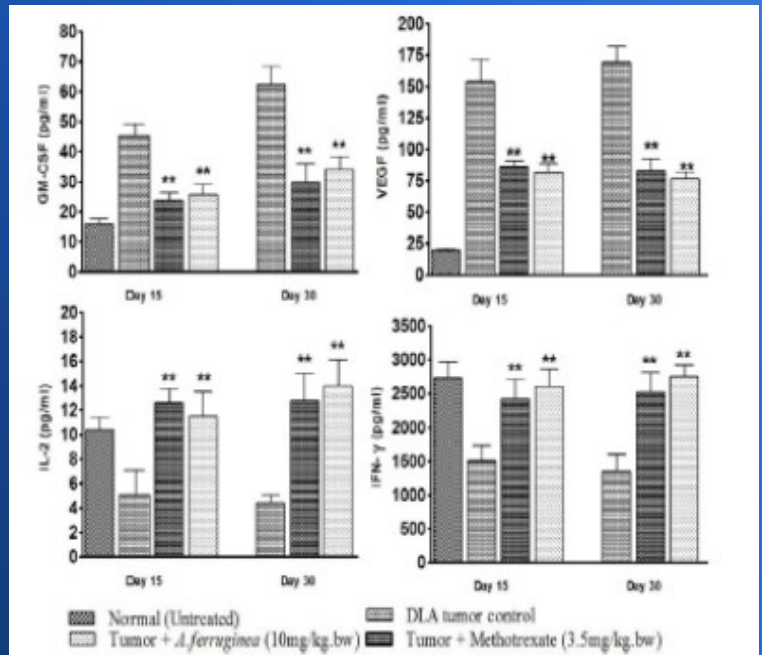


Figure 9. Effect of *A ferruginea* Extract on GM-CSF, IL-2, IFN-γ, and VEGF Level in Mice with DLA-Induced Soild Tumor. Blood samples were collected from tail vein on day 15 and 30, the level of cytokines were assessed using standard ELISA method. Values shown are means (±SD). Values significantly different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

Rubia species

Table 1: Taxonomical and morphological classification of *Rubia cordifolia* Linn.

Taxonomical classification		Morphological classification	
Kingdom	Plantae	Plant	Perennial herbaceous climber
Division	Magnoliophyta	Roots	Long, cylindrical, flexuous with a thin red bark
Class	Magnoliopsida	Stems	Very long, rough, grooved and woody base
Order	Gentianales	Branches	Scandent, quadrangular, glabrous and shining
Family	Rubiaceae	Leaves	Arranged in four whorls, ovate
Genus	Rubia L.	Fruits	4-6 mm in diameter, globose, purplish black when ripe
Species	Rubia cordifolia L.	Flowers	Small, greenish, terminal panicle-cymes

Rubia species:
Traditional use and properties

- *Rubia cordifolia (*Manjistha, Indian madder) is a plant within the *Rubiaceae* coffee family widely spread in Himalayan region, northeast India, Japan, Indonesia and Sri Lanka.
- Originally used as a red pigment, *R. cordifolia* is also used in Ayurvedic medicine to treat jaundice, joint inflammation, and cough. *R. cordifolia* is gaining popularity in western culture as alternative therapy for skin conditions such as eczema, psoriasis and dermatitis.
- In clinical biological experiments, plants of the genus *Rubia* showed antibacterial, anticancer, anticough and antiplatelet aggregation activities.
- They have also been used to enhance the number of leukocytes, and in the therapy of myocardial infraction.

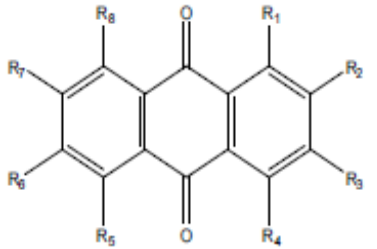
Table 3: Biomarkers of *Rubia cordifolia* responsible for therapeutical activities.

S. No.	Biomarker	Activity
1.	Rubiadin	- Hepatoprotective - Antioxidant
2.	Alizarin[25]	- Antigenotoxic
3.	Mollugin[26, 27]	- Antiadipogenesis - Antiplatelet
4.	Alizarin, mollugin, lucidin[28]	- Potent COX-2 inhibitor
5.	RA-700[29] RA-XI, XII, XII, XIV[30]	- Antitumor
6.	1-hydroxytectoquinone[31]	- Anti-inflammatory - Anticancer

Chemical composition of Rubia

Contents of stems (1)

- From the stems of *Rubia wallichiana* DECNE, thirty-four structurally related compounds were isolated and identified, amongst them three anthraquinones rubiawallin-A (1), -B (2), and -C (3) and 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone demonstrated most effective cytotoxicity towards cancer cells *in vitro.*
- Phytochemical studies have led to the isolation of anthraquinones and their glycosydes, naphthaquinones, naphthahydroquinones, cyclic hexapeptides, flavonoids, coumarins, iridoids, lignans, triterpenoids, and benzenoids, terpenes, bicyclic hexapeptides, iridoids, and carbohydrates (10 to 15 per cent), amongst them alizarin (9.6-21.8 mg/g), purpurin* (3.7-12.3 mg/g), ruberythric acid , pectin, albuminous bodies, yellow xanthine, rubichloric acid, munjistin*, purpuro-xanthine, lucidin* (1.8-5.7 mg/g), rubiawallin-A (1), -B (2), and -C (3) (*anthraquinones).
- The total anthraquinone-derivative content varied between 15.6 and 39.4 mg/g.

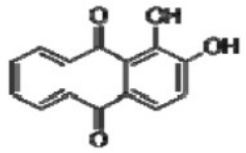


Alizarin	$R_1=R_2=OH, R_3=R_4=H$
Purpurin	$R_1=R_2=R_4=OH, R_3=H$
Pseudopurpurin	$R_1= R_2=R_4=OH, R_3=COOH$
Xanthopurpurin	$R_1= R_3=OH, R_2=R_4=H$
Munjistin	$R_1= R_3=OH, R_2=COOH, R_4=H$
Rubiadin	$R_1= R_3=OH, R_2=CH_3, R_4=H$

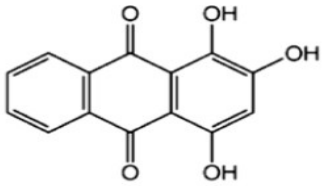
Figure A: Structure of primary chromophores found in Indian Madder.

Chemical composition
A few molecular structures of rubia compounds (1)

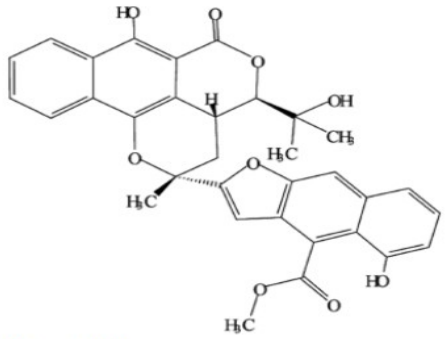
Anthraquinones from *R. cordifolia*:



Alizarin (1, 2-dihydroxy-9,10-anthracenedione)



Purpurin (1, 2, 4-trihydroxyanthracene-9, 10-Dione)



Rubicordifolin

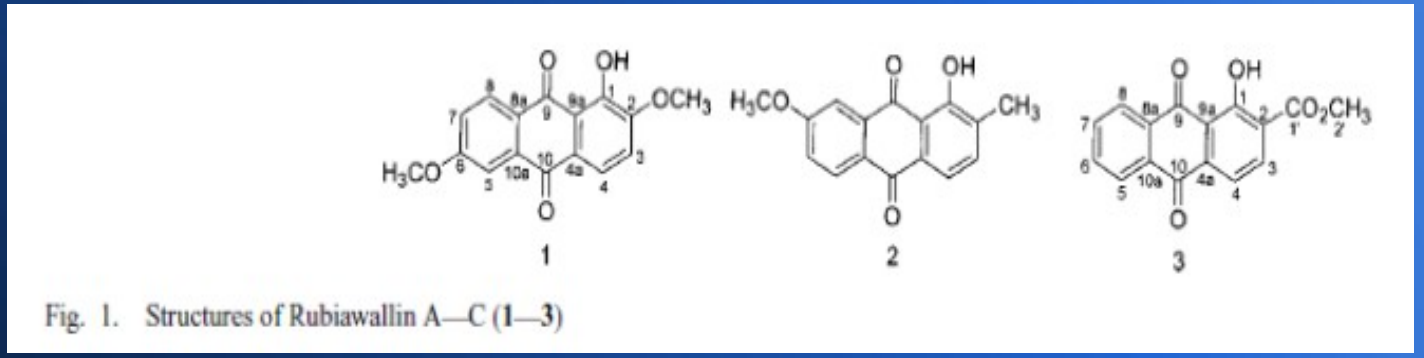


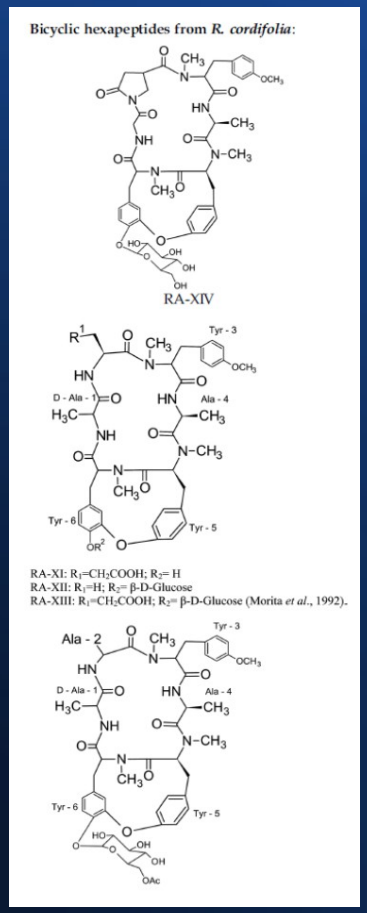
Fig. 1. Structures of Rubiawallin A—C (**1**—**3**)

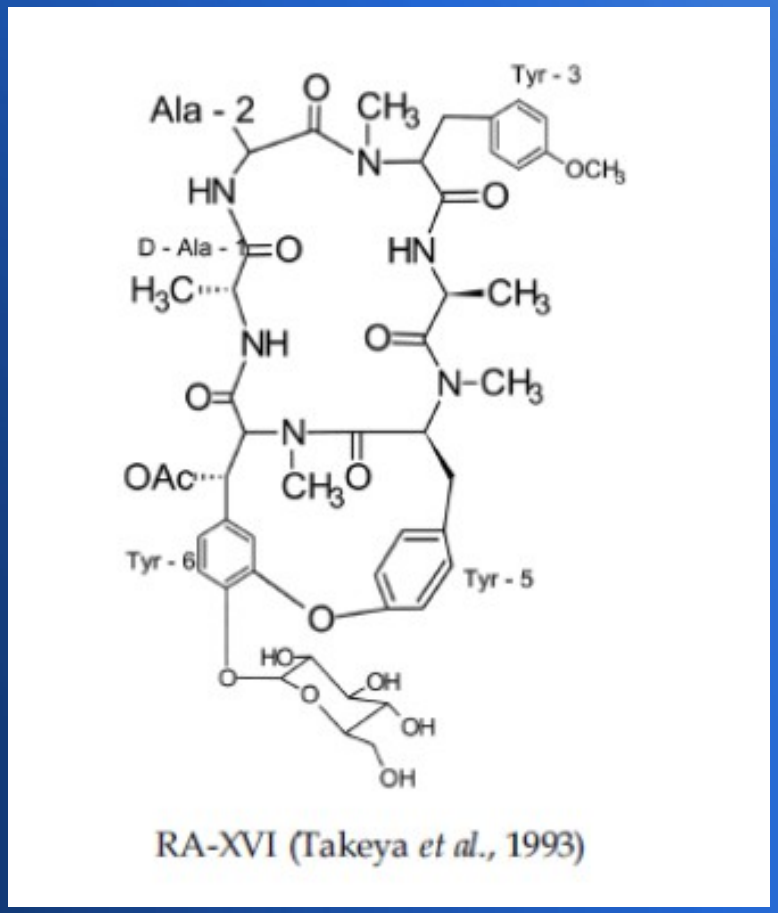
Anthraquinones

Chemical composition
A few molecular structures of rubia compounds (2)

Bicyclic

Hexapeptides





Anticancer activity of Rubia species

- Anthraquinones contained in Rubia (total: 15.6 and 39.4 mg/g) amongst them purpurin, munjistin, lucidin, rubiawallin-A (1), -B (2), and -C (3) were reported to exhibit strong antiproliferative properties on mammalian cells.
- Among the testing compounds, 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone demonstrated most effective cytotoxicity towards Hepa-3B and Colo-205 cancer cells.
- 1-Hydroxytectoquinone and oleanonic acid both also exhibited promising cytotoxicity against A375 cancer cell lines.
- Rubiarbonols A and F, rubiarbonone C, and rubianol-c showed cytotoxic effects in the MTT assay.
- The cyclic hexapeptides and quinones of Rubia exhibited strong *in vivo* anticancer activity.

Cytotoxicity (ED50) of compounds isolated from Rubia species

Table 1. Cytotoxicity ED_{50} (μg/ml) of Compounds Isolated from the Stems of *R. wallichiana* on Tumor Cell Lines

Compounds	Cell lines			
	KB	Hepa-3B	Hela	Colo-205
Doxorubicin	0.12	0.14	0.11	0.10
4	—	0.6	9.15	0.58
5	—	—	—	—
6	—	—	—	—
10	3.1	—	—	—
11	—	—	—	—
13	—	3.58	—	—
14	6.57	1.7	—	1.9
15	—	—	24.5	—
17	—	—	—	—
18	—	3.85	24.5	—
19	—	1.7	12.3	1.16
21	—	—	—	—
24	—	—	—	—
25	—	—	—	—
27	—	—	—	—
28	—	7.05	—	—
31	—	—	—	—

—: ED_{50}>25 μg/ml.

In addition, 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**4**),[12] alizarin-2-methylether (**5**),[13] lucidin (**6**),[14] lucidin primeveroside (**7**),[14] munjistin methyl ether (**8**),[15] nordamnacanthal (**9**),[16] purpurin (**10**),[16] rubiadin (**11**),[15] rubiadin primeveroside (**12**),[17] rubischumin-A (**13**),[18] xanthopurpurin (**14**),[15] 1-hydroxy-3-methoxy-2-methylanthraquinone (**15**),[19] 1,3-dimethoxy-2-hydroxyanthraquinone (**16**),[20] 1-hydroxy-2-methylanthraquinone (**17**),[21] 1-hydroxy-2-hydroxymethylanthraquinone (**18**),[20] 2-methyl-1,3,6-trihydroxyanthraquinone (**19**),[21] 2-methyl-1,3,6-trihydroxyanthraquinone 3-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside (**20**),[21] 2-methyl-1,3,6-trihydroxyanthraquinone 3-*O*-(6′-*O*-acetyl)-α-L-rhamnosyl-(1→2)-β-D-glucoside (**21**),[21] 1-hydroxy-5-methoxy-2-methylanthraquinone (**22**),[22] 2-hydroxy-1-methoxyanthraquinone (**23**),[20] 1-hydroxy-3-methoxyanthraquinone (**24**),[13] 1,8-dihydroxy-2-methylanthraquinone (**25**),[13] 1,3-dihydroxy-2-methoxymethylanthraquinone (**26**),[15] scopoletin (**27**),[23] 6,7-dimethoxycoumarin (**28**),[24] 7-hydroxy-6-methoxy-8-(3-methylbut-2-enyl)coumarin (**29**),[25] (+)-medioresinol (**30**),[26] ursolic acid (**31**),[27] the mixture of β-sitosterol (**32**) and stigmasterol (**33**),[28] and docosanoic acid (**34**)[29] were also isolated from the stems of *R. wallchiana*. They were identified by the comparison of their spectral data with those in literature.

Anticancer activity of Rubia species - *in vivo*

Antitumour activity

The cyclic hexapeptides and quinones of Rubia exhibited a significant anticancer activity against various proliferating cells. The hexapeptides showed potent antitumour activity by binding to eukaryotic 80S ribosomes resulting in inhibition of aminoacyl -tRNA binding and peptidyl – tRNA translocation, thus leading to the stoppage of protein synthesis (Morita, 1992, Morita, 1993, Itokawa, 1993). The antitumor activity of RA-700, a cyclic hexapeptide isolated from *R. cordifolia*, was evaluated in comparison with deoxy-bouvardin and vincristine (VCR). The antitumor activity of RA-700 was similar to that of deoxy-bouvardin and VCR against P388 leukemia. As with deoxy-bouvardin and VCR, the therapeutic efficacy of RA-700 depends on the time schedule. RA-700 showed marginal activity against L1210 leukemia (50% ILS), similar to that of deoxy-bouvardin but inferior to that of VCR. RA-700 inhibited Lewis tumor growth in the early stage after tumor implantation, whereas deoxy-bouvardin and VCR did not. A slight

cordifolia roots three constituents were isolated namely mollugin, furomollugin and dehydro-a-lapchone. Mollugin has shown inhibition of passive cutaneous anaphylaxis (PCA) and protection of mast cell degranulation in rats. It also exhibited considerable activity against lymphoid leukemia (P338) in mice (Gupta, 1999). The *R. cordifolia*

The cytotoxic action of *R. cordifolia* had been evaluated with DNA Topoisomerases I and II inhibition and cytotoxicity of constituents isolated from the roots was tested. Topoisomerases I and II inhibitory activities were measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. The tetrazolium-based colorimetric assay (MTT assay) was used for the cytotoxicity towards human colon carcinoma (HT-29), human breast carcinoma (MCF-7) and human liver carcinoma (HepG2) cell lines. Seven compounds were isolated possessing cytotoxic activity (Son JK, 2006). Anti-tumor activity of RC-18, a pure isolate from *R. cordifolia*, was repeatedly tested in different sets of experiments on a spectrum of experimental murine tumors, viz P388, L1210, L5178Y, B16 melanoma, Lewis lung carcinoma and sarcoma-180. RC-18 exhibited significant increase in life span of ascites leukemia P388, L1210, L5178Y and a solid tumor B16 melanoma. However, it failed to show any inhibitory effect on solid tumors, Lewis lung carcinoma and sarcoma 180. Promising results against a spectrum of experimental tumors suggested that RC-18 may lead to the development of a potential anti-cancer agent (Adwankar and Chitnis, 1982). The anticancer activity of extracts of *R. cordifolia*, tested against the P388 tumor system in BDF1 mice, compared well with that of the positive control, 5-fluorouracil (Adwankar *et al.*, 1980).

Anticancer activity of Rubia species - *in vitro*

- Dichloromethane fraction of *Rubia cordifolia* extract exhibited potent inhibition of HL60 Human Myeloid leukaemia cell lines with IC50 values of 8.57 µg/ml, 10.51 µg/ml and 16.72 µg/ml for methanol fraction, pet-ether fraction and dichloromethane fraction respectively, where moderate cytotoxicity was shown against U937 Human Histolytic lymphoma cell lines with IC50values of 27.33 µg/ml while was less active against normal HEK293 Human Epithelial Kidney cell lines, therefore displaying safety for normal cells.

- In another *in vitro* cytotoxicity assay with Hep G32 cell line, the root extract of Rubia cordifolia showed significant cytotoxic activity with IC50 values of 28.07 µg/ml, 25.13 µg/ml and 41.96 µg/ml were measured for methanol fraction, pet-ether fraction and dichloromethane fraction, respectively. No cytotoxicity against HEK293 cell line was measured in the concentration range of 0.05-100 µg/ml.

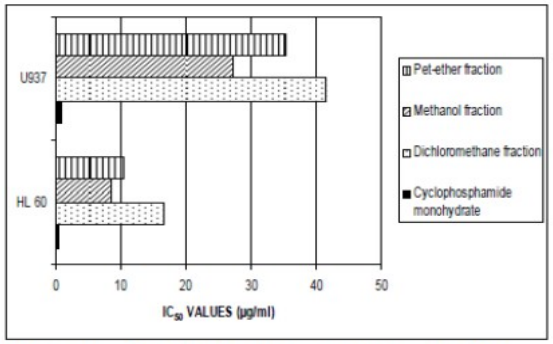


Figure 1. Graphical representation of IC_{50} values (µg/ml) of standard Cyclophosphamide monohydrate and three different extracts of *Rubia cordifolia* (Rubiaceae) against HEK 293, U937 and HL60 cell lines.

Table.1 IC_{50} values (µg/ml) of standard Cyclophosphamide monohydrate and three different extracts of *Rubia cordifolia* (Rubiaceae) against HEK 293 and Hep G 32 cell lines.

Sample	IC_{50} VALUES (µg/ml)	
	CELL LINES USED	
	HEK293	Hep G 32
Cyclophosphamide monohydrate*	>100	0.697
Dichloromethane fraction	>100	41.96
Methanol fraction	>100	28.07
Pet-ether fraction	>100	37.13

Anticancer activity of Rubia species – *in vitro* (2)

- *R. cordifolia* dry extract showed cytotoxicity against MDA-MB-231 breast cancer cell lines with IC50=44 µg/ml or 5.1µM GAE.

- From Fig. 2, the concentration of extract leading to 50% inhibition of cells (IC50) was 286 µg/ ml and 43 µg/ml for 24 hrs or 72 hrs treatment with freshly prepared *R. cordifolia* extract. Therefore, *R. cordifolia* was more cytotoxic after 72 hrs treatment as compared to a 24 hr treatment. 72 hr tests suggest an average IC50 value of 44 µg/ ml for *R.* cordifolia extract; the corresponding total phenols concentration is 5.1x10-6M GAE.

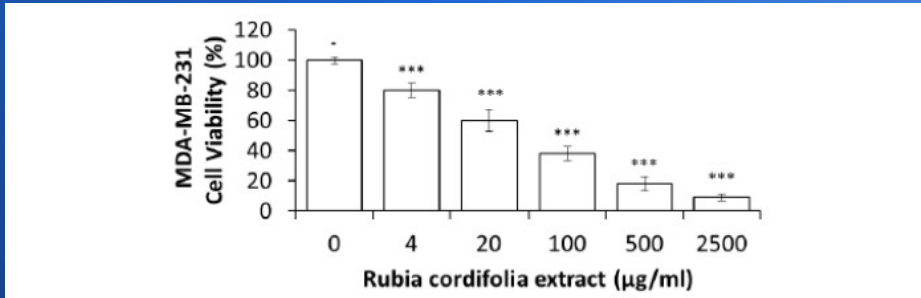


Fig. 3. Anticancer activity of freeze dried *R. cordifolia root extract* measured by the Sulforhodamine B assay

*Anticancer activity evaluated using MDA-MB-231 breast cancer cells. Treatment time was 72 hours. Viability was determined by Sulforhodamine B staining. Data shows mean results from three experiments (n=18) for each treatment with ± SEM. ***Show significant difference between treatments and vehicle control (P =0.05). The 50% inhibitory concentration (IC50) occurs at 45 µg/ml*

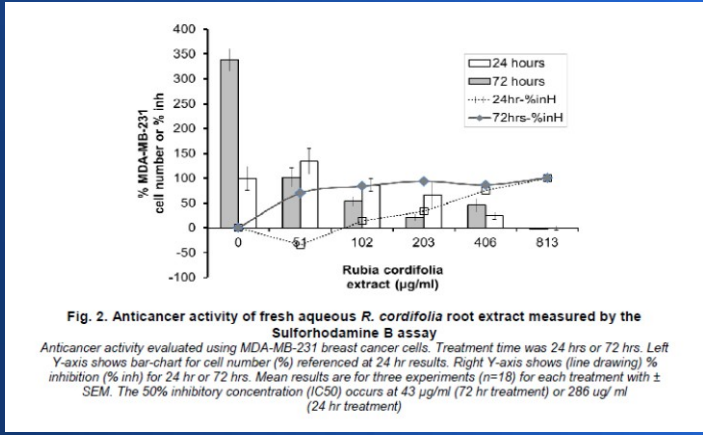


Fig. 2. Anticancer activity of fresh aqueous *R. cordifolia* root extract measured by the Sulforhodamine B assay

Anticancer activity evaluated using MDA-MB-231 breast cancer cells. Treatment time was 24 hrs or 72 hrs. Left Y-axis shows bar-chart for cell number (%) referenced at 24 hr results. Right Y-axis shows (line drawing) % inhibition (% inh) for 24 hr or 72 hrs. Mean results are for three experiments (n=18) for each treatment with ± SEM. The 50% inhibitory concentration (IC50) occurs at 43 µg/ml (72 hr treatment) or 286 ug/ ml (24 hr treatment)

Anticancer activity of Rubia species - *in vivo*

- After treatment with alkannin (**1**) or angelylalkannin (**2**) for 48 h, the proliferation of HCT-116 and SW-480 cells in human colon cancer cells HCT-116 and SW-480 was suppressed dose dependently.
- For the HCT-116 cells, growth was inhibited by 2.7%, 40.7% and 78.1%, respectively. On SW-480 cells, the antiproliferative effect was 20.1%, 35.5% and 99.2%, respectively; while results with angelylalkannin (**2**) were 2.9%, 3.3% and 83.3%, respectively.
- 5-FU data can be considered a positive control for the present study. In this study, alkannin (**1**) and angelylalkannin (**2**) possessed significantly stronger antiproliferative activity than 5-FU at the same concentration.

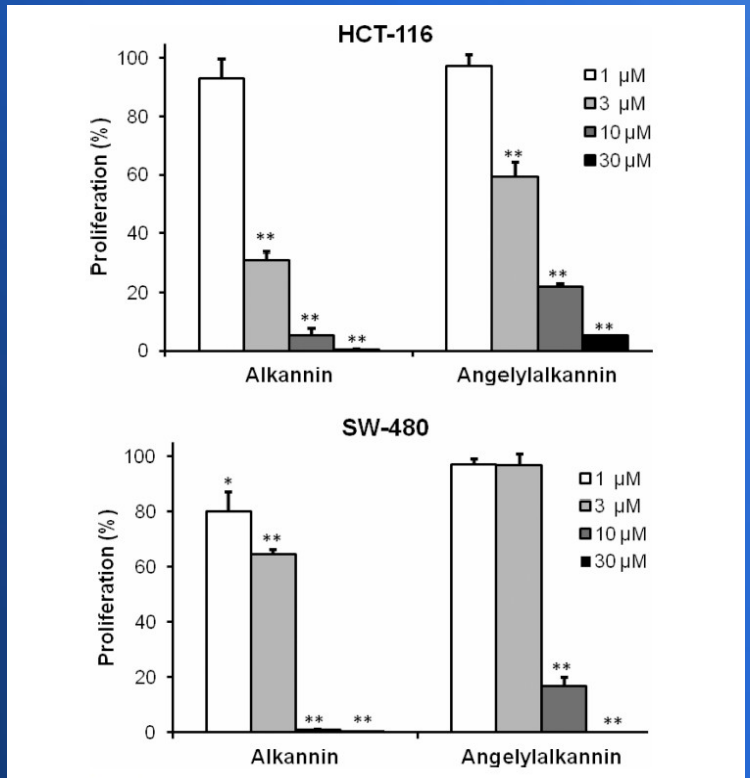


Figure 2.
Antiproliferative effects of alkannin (**1**) and angelylalkannin (**2**) on human colorectal cancer cells. HCT-116 and SW-480 cells were treated with the compounds at various concentrations for 48 h. Cell proliferation was determined using MTS method. Data are presented as mean ± standard error. *, $P<0.05$; **, $P<0.01$ vs. control.

Anti-cancer activity of Rubia:
Molecular pathways (1)

- **Induction of apoptosic pathways:**

topoisomerases I and II [24]. Oleanolic acid had selective inhibitory activity against DNA topoisomerase II compared with DNA topoisomerase I but weak cytotoxicity against HT-29, MCF-7 and HepG2 [26]. Maslinic acid was able to induce caspase-dependent apoptosis in human colon-cancer cells via the intrinsic mitochondrial pathway [73,74]; it could also potentiate anti-tumor activities of TNF-α and inhibit pancreatic tumor growth and invasion by activating caspase-dependent apoptotic pathway and by suppressing NF-κB activation and its downstream gene expression [75]. Inhibition of Protein kinase C (PKC) that was related to the tumor development might lead to inhibition of cells growth and spreading of cancer cells, while maslinic acid acted as a PKC inhibitor. Ursolic acid potentiated TRAIL-induced apoptosis through activation of reactive oxygen species and

JNK-mediated up-regulation of death receptors and down-regulation of decoy receptor 2 and cell survival proteins [76,77]; it was also considered as a novel blocker of STAT3 activation that might have a potential in prevention and treatment of multiple myeloma and other cancers [78]. Ursolic acid and oleanolic acid possessed markedly apoptotic effects on four cell lines via increasing DNA fragmentation, decreasing mitochondrial membrane potential, lowering Na^+-K^+-ATPase activity and elevating caspase-3 and caspase-8 activities; they could suppress cell adhesion and reduce the production of VEGF and ICAM-1 in these cell lines [79].

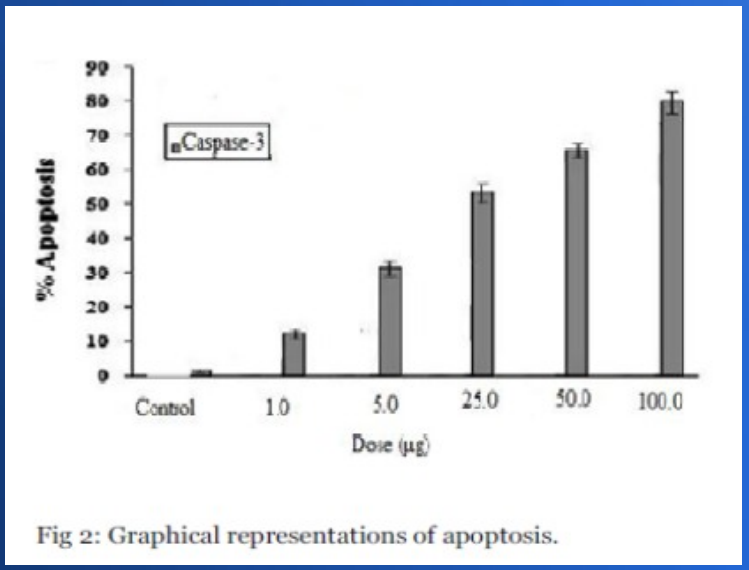


Fig 2: Graphical representations of apoptosis.

Anti-cancer activity of Rubia:
Molecular pathways (2)

- DNA-Topoisomerase I and II inhibitory activities:

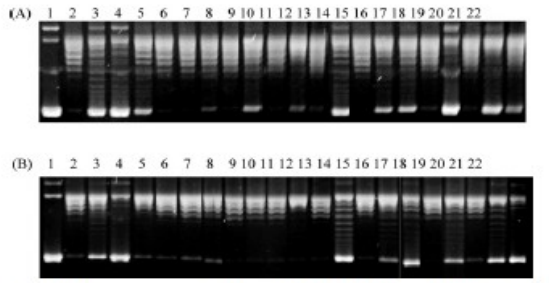


Fig. 2. DNA Topoisomerase I (A) and II (B) Inhibitory Activities of Compounds **1**—**9**

(A) 1: supercoiled DNA alone; 2: supercoiled DNA+topoisomerase I (calf thymus); 3: supercoiled DNA+topoisomerase I (calf thymus)+camptothecin (20 μM, positive control); 4: supercoiled DNA+topoisomerase I (calf thymus)+camptothecin (100 μM, positive control); 5—13: compounds **1**—**9** (20 μM); 14—22: compounds **1**—**9** (100 μM); (B) 1: supercoiled DNA alone; 2: supercoiled DNA+topoisomerase II (human); 3: supercoiled DNA+topoisomerase II (human)+etoposide (20 μM, positive control); 4: supercoiled DNA+topoisomerase II (human)+etoposide (100 μM, positive control); 5—13: compounds **1**—**9** (20 μM); 14—22: compounds **1**—**9** (100 μM).

Topoisomerase I and II inhibitory activities were measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. As shown in Table 2, **1**, **6** and **8** strongly inhibited DNA topoisomerase I at 100 μM, and when these three compounds were assayed at 20 μM, **1** and **6** showed 42 and 33% inhibition, respectively. In DNA topoisomerase II assays, **1**, **4** and **9** showed 65, 54 and 70% inhibition at 100 μM, respectively. The new compound, **3**, showed 42 and 23% inhibition for topoisomerases I and II at 100 μM, respectively. Camptothecin and etoposide for the positive control assays of topoisomerase I and II inhibited 81 and 86% at 100 μM and 70 and 58% at 20 μM, respectively.

Table 2. Inhibitory Effects of Compounds **1**—**9** on DNA Topoisomerases I and II (%, Inhibition Ratio of Relaxation) and Their IC_{50} Values against HT-29, MCF-7 and HepG2 Cell Lines

Compd.	Topo I (%) (μM)		Topo II (%) (μM)		IC_{50} (μM)		
	100	20	100	20	HT-29[a]	MCF-7[b]	HepG2[c]
1	58	42	65	5	66.9	>100	>100
2	0	2	0	0	>100	>100	60.2
3	42	1	23	7	>100	41.3	76.3
4	51	11	54	7	>100	>100	>100
5	6	1	0	0	14.3	25.0	40.5
6	96	33	30	0	81.8	81.8	69.3
7	1	1	3	0	>100	>100	>100
8	75	17	49	0	51.6	>100	>100
9	37	4	70	0	61.4	>100	>100
CPT[d]	81	70	NA[f]				
VP-16[e]	NA		86	58			

a) HT-29: human colon carcinoma. *b*) MCF-7: human breast carcinoma. *c*) HepG2: human liver carcinoma. *d*) Camptothecin: positive control for topoisomerase I. *e*) Etoposide: positive control for topoisomerase II. *f*) NA: not applicable.

Protective effects of rubia plant against neuropathic pain induced by anticancer drugs:

This study concludes that AERC significantly inhibited paclitaxel-induced neuropathic pain after sciatic nerve ligation. The combination of *R. cordifolia* with paclitaxel may help to reduce side effects and a dose of paclitaxel.

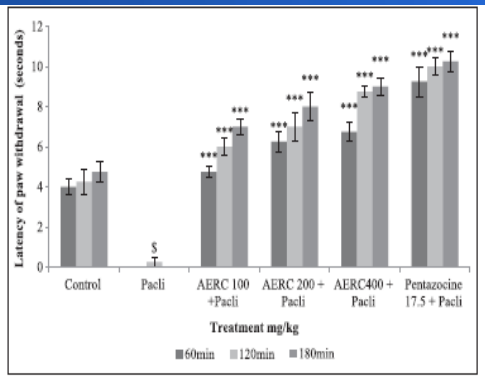


Figure 5: Effect of alcoholic extract of *Rubia cordifolia* on paclitaxel (pacli)-induced neuropathic pain in hot plate method on 10th day $n = 5$. The observations are mean ± standard error of the mean, $\$P < 0.001$ as compared to control. $^{***}P < 0.001$ as compared to paclitaxel (one-way ANOVA followed by Dunnett's test)

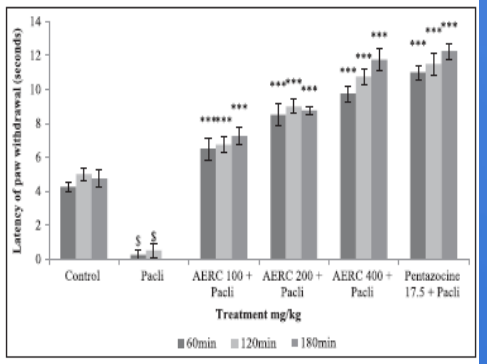


Figure 6: Effect of alcoholic extract of *Rubia cordifolia* on paclitaxel (pacli)-induced neuropathic pain in hot plate method on 21st day $n = 5$. The observations are mean ± standard error of the mean, $\$P < 0.001$ as compared to control. $^{***}P < 0.001$ as compared to paclitaxel (one-way ANOVA followed by Dunnett's test)

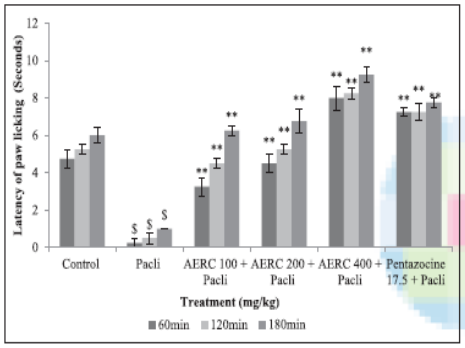


Figure 3: Effect of alcoholic extract of *Rubia cordifolia* on paclitaxel (pacli)-induced neuropathic pain in cold allodynia on 21st day $n = 5$. The observations are mean ± standard error of the mean, $\$P < 0.01$ as compared to control. $^{**}P < 0.01$ as compared to paclitaxel (one-way ANOVA followed by Dunnett's test)

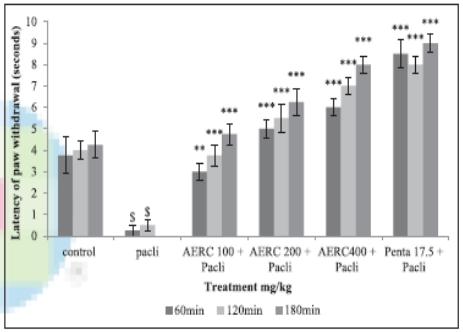


Figure 4: Effect of alcoholic extract of *Rubia cordifolia* on paclitaxel (pacli)-induced neuropathic pain in hot plate method on 7th day $n = 5$. The observations are mean ± standard error of the mean, $\$P < 0.001$ as compared to control. $^{***}P < 0.001$ as compared to paclitaxel (one-way ANOVA followed by Dunnett's test)

Alkanna species

Alkanna species:
Traditional use and properties

- Alkanna tinctoria (Boraginaceae) is widely distributed in Europe and western Asia.
- Its root has been used as a dye for fabrics, cosmetics, and food and as botanical drugs for ulcers, inflammation, and wounds (Papageorgiou et al., 1977).
- Previous phytochemical studies on the plant have resulted in the isolation of a series of naphthoquinone pigments, alkannin and its derivatives (Papageorgiou et al., 1977; 1980) .
- Some of these compounds have been found to exhibit biological activities, including cytotoxic, antimicrobial, anti-Leishmanial, and anti-inflammatory activities (Papageorgiou et al., 1977;1979; 1980; Plyta et al., 1998; Assimopoulou and Papageorgiou, 2005; Ali et al., 2011).

Chemical composition of Alkanna

- **Alkannin** (**1**) and **angelylalkannin** (**2**) are principle components of the red pigments from the roots of A. tinctoria along with some minor derivatives reported to date.
- In a continuing program to discover new anticancer agents from plants, especially naphthoquinones from the Alkanna genus, Alkanna cappadocica was investigated. Bioassay-guided fractionation of a dichloromethane/methanol (1:1) extract of the roots led to the isolation of four new and four known naphthoquinones. The known compounds are 11-deoxyalkannin (1), β,β-dimethylacrylalkannin (2), 11-O-acetylalkannin (3), and alkannin (4).
- Compound 7 showed remarkable cytotoxicity with IC50 values between 0.09 and 14.07 μM. It was more potent than the other compounds in six out of 12 cancer cell lines and the positive controls doxorubicin and etoposide.

Anticancer activity of Alkanna species - *in vitro*

- Previous studies have shown that alkannin derivatives possess antiproliferative effects on several cancer cell lines, e.g. K562, GLC-82, CNE2, and Bel-7402 (Wu et al., 2005; Deng, 2010; Bogurcu et al., 2011).
- Recently, there have been reports on molecular mechanisms of their cytotoxic actions regarding apoptosis induction in K562 leukemia, GLC-82 adenocarcinoma, and Hep3B hepatocarcinoma cell lines, respectively (Deng, 2010; Bogurcu et al., 2011).
- Compounds showed remarkable cytotoxic activity against HT-29, MDA-MB-231 and PC-3 cell lines, comparable or stronger than positive control CPT-11 (CAMPTOSTAR®, irinotecan) with IC_{50} values in between 0.1μM and 1μM.
- These studies revealed that *β,β-dimethylacrylalkannin* (*2*) and *acetylalkannin* (*3*) have great potential as topo-I-inhibitors compared to other compounds and CPT-11, with 2–3μM inhibition value

Anti-cancer activity of Alkanna:
Molecular pathways – cell cycle arrest

- Treatment with alkannin (**1**) gradually changed the cell cycle profile of HCT-116 cells.
- After treatment with alkannin (**1**) at 1 μM, the distribution was 69.2% in G1 phase, 14.5% in S phase, and 14.1% in G2/M phase. At an alkannin concentration of 3 μM, the fractions of cells were 70.3% (G1 phase), 12.9% (S phase) and 14.8% (G2/M phase).
- After treatment with angelylalkannin (**2**) at 1 μM, the cell cycle profile was 69.5% (G1 phase), 15.6% (S phase) and 14.0% (G2/M phase), while with angelylalkannin (**2**) at 3μM, the fractions of cells were 72.2% (G1 phase), 9.4% (S phase) and 16.2% (G2/M phase).
- Notably, both alkannin (**1**) and angelylalkannin (**2**) significantly arrested HCT-116 cells at the G1 phase at the tested concentrations.

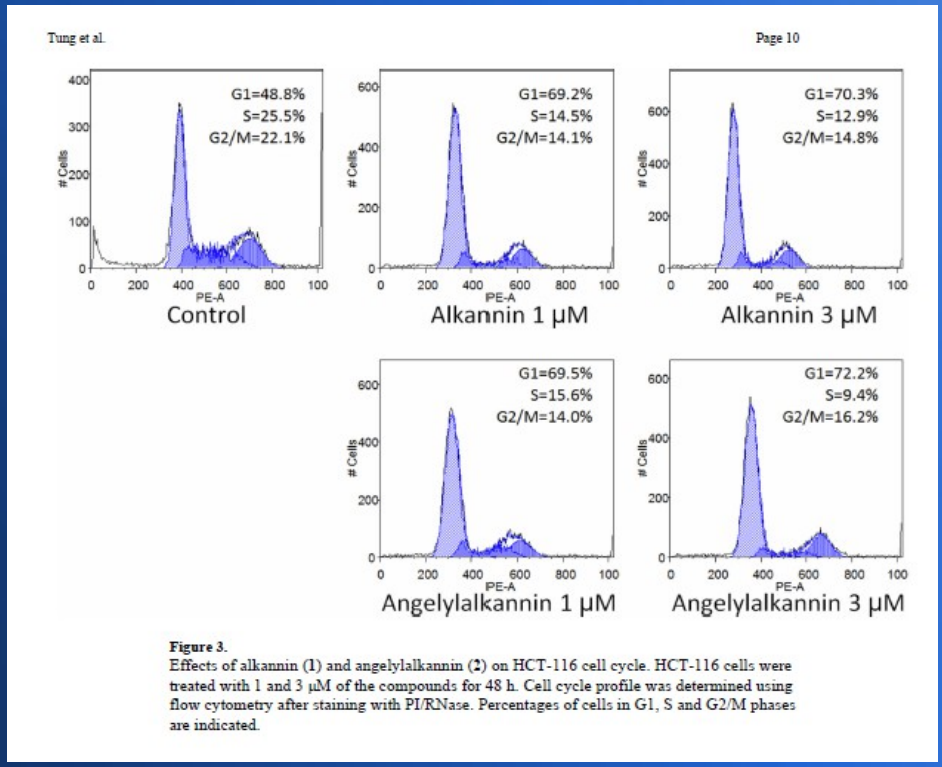


Figure 3.
Effects of alkannin (1) and angelylalkannin (2) on HCT-116 cell cycle. HCT-116 cells were treated with 1 and 3 μM of the compounds for 48 h. Cell cycle profile was determined using flow cytometry after staining with PI/RNase. Percentages of cells in G1, S and G2/M phases are indicated.

Anti-cancer activity of Alkanna:
Molecular pathways – induction of apoptosis

- Antiproliferative effects of alkannin (**1**) and angelylalkannin(**2**) could be mediated by the induction of apoptosis.
- After treatment for 48 h, the percentage of apoptotic cells (early and late apoptosis) induced by alkannin (**1**) at 1, 3, and 10 μM were 7.5%, 56.2% and 63.5%. The percentage of apoptotic cells induced by angelylalkannin (**2**) at concentrations of 1, 3, and 10 μM were 8.8%, 28.8 to 55.7%, respectively.
- Compared to control (4.0%), treatment with 3 μM or 10 μM of the two compounds significantly induced cancer cell apoptosis.

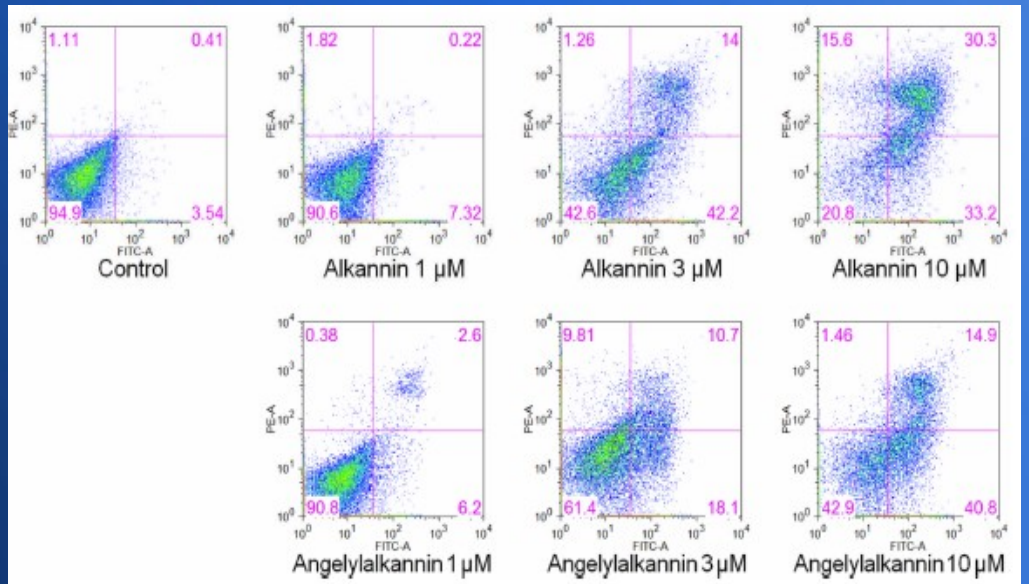


Figure 4.
Effects of alkannin (1) and angelylalkannin (2) on HCT-116 cell apoptosis. HCT-116 cells were treated with 1, 3, and 10 μM of the compounds for 48 h. Apoptosis was quantified using flow cytometry after staining with annexin V/PI.