



CYTOTOXIC AND ANTITUMOUR ACTIVITY OF METHANOLIC EXTRACTS OF MEDICINALLY IMPORTANT PLANTS.

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ABSTRACT

Traditional knowledge reveals that most of the plants are medicinally important, especially against cancer and tumors. In the present study, plants resistant and susceptible to crown gall were analysed at laboratory level for cytotoxic and antitumor activity to know whether the plants resistant to crown gall has any constituents or compounds that are cytotoxic or anti-tumour against human tumours. Methanolic extract of *Simaruba glauca* (crown gall susceptible) showed highest cytotoxic activity against brine shrimps at an LC50 of 166.4ppm followed by *Erythroxylum monogynum* and *Alianthus excelsa* (crown gall resistant) extracts at LC50 of 172.3ppm and 238.3ppm respectively. We further assessed antitumor activity against the tumors induced by *Agrobacterium tumefaciens* using carrot disc anti-tumour bioassay. Results indicated that the extract of *Simaruba glauca* inhibited tumor induction on carrot discs at a lowest concentration of 100ug/ml as compared to the other two plant extract which inhibited the tumour at a concentration of 800ug/ml and above. The overall results indicate a strong cytotoxic and anti-tumour activity in a crown gall susceptible plant, while crown gall resistant plants are comparatively less cytotoxic as well as anti-tumour activity.

KEY WORDS: Bioassays, Cytotoxicity, *Agrobacterium tumefaciens*, Crown Gall tumour Assay.



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INTRODUCTION

Cancer has been known ever since human societies first recorded their activities; it was well known to the ancient Egyptians and the succeeding civilizations¹. In India, around 555,000 people died of cancer in 2010, according to an estimate published in The Lancet today ((March 28, 2012). Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs². There is an increasing need for search of new compounds with cytotoxic activity as the treatment of cancer with the available anticancer drugs is often unsatisfactory due to the problem of cytotoxicity to the normal cells. For the last few decades, phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of plausible anticancer compounds. Plants were selected based on their medicinal properties as well as the main criteria was to study the cytotoxicity and anti-tumour activity of crown gall resistant plants and their role as efficient anti-tumour agents³. Three plants were selected for the study, two plants were crown gall resistant and one plant was crown gall susceptible, *Alianthus excelsa*, (Simaroubaceae) and *Erythroxylum monogynum* (Erythroxylaceae) both crown gall resistant plants and *Simaruba glauca* (Simaroubaceae) a crown gall susceptible plant. All the three plants are reputed to be medicinal plants in scientific and folkloric literature, and its medicinal values are well documented. An aqueous decoction of these plants is a popular remedy for many diseases, including cancer in the indigenous system of medicine, but very less scientific attempt has been made to evaluate the effects of its extracts. Considering the medicinal activity of these plants based on traditional information, the present study was conducted to evaluate the extracts of all three plants for their anti-

tumorous potential by utilizing cytotoxicity and antitumor bioassays.

Screening programs for biologically active natural products require the right bioassays. Detection of compounds with the desired activity in complex plant extracts depends on the reliability and sensitivity of the test systems used. Thus, bioassays are essential for monitoring the required effects throughout activity guided fractionation and purification until the active mono-substances are obtained.⁴ Brine Shrimp (BS) (*Artemia salina* L.) bioassay is considered as a preliminary screening for the presence of antitumor compounds and is used to determine the toxicity of the plant extract^{5,6}. Using BS larvae, pharmacognosists and natural product chemists were able to detect and isolate plant constituents and active compounds with a variety of pharmaceutical activities. Thus it is possible to detect and then monitor the fractionation of cytotoxic as well as 3PS (P388)(in-vivo murine leukemia) active extracts using Brine Shrimp Toxicity (BST) assay rather than more tedious and expensive in-vitro and in-vivo anti-tumour assays⁷. This assay is considered as rapid and inexpensive in-house bioassay for screening and fractionation monitoring of physiologically active plant extract assays while it has positive correlation with 9KB (human nasopharyngeal carcinoma) cytotoxicity and 3PS (P388)(in vivo murine Leukemia) and can substitute both of them^{5,7}. BST assay has advantages of being simple and rapid (24 hours). It easily utilises a large number organisms for statistical validation and requires no special equipment and relatively small amount of samples. Furthermore it does not require animal serum as is needed for other cytotoxicity tests. Animal right advocates have not yet objected to the use of these invertebrates in experimental work⁷, the aim of this study is to provide a front line screen that can be backed up by more specific and more expensive bioassays once the active compound have been isolated.

Crown gall is a neoplastic diseases of plants which occur in more than 60 families of dicots and many gymnosperms the disease⁸ is characterized by the transformation of normal plants cells into autonomous tumor cells in a short period of time⁹. The causative agent for this disease are specific strains of gram negative bacterium *Agrobacterium tumefaciens*. During infection of plant material with *A.tumefaciens*, a tumour producing plasmid (ti-plasmid) is incorporated into the plants chromosomal DNA. When plant tissue is wounded it releases phenols which will activate the ti- plasmid in *A. tumefaciens*. The ti- plasmid causes the plant cells to multiply rapidly without going through apoptosis resulting in tumour formation similar in nucleic acid content and histology to human and animal cancers¹⁰. The rationale for use of bioassay is that the tumorigenic mechanism initiated in plant tissue by *Agrobacterium tumefaciens* is similar to that of animals; the relevance of crown gall tumour system to the general cancer problem has been thoroughly understood. Crown gall tumours could routinely used as a comparative, rapid, safe and inexpensive, and statistically reliable prescreen for *invivo* 3PS antitumor activity. The authors have since used this assay to detect and isolate several novel, anti-tumor compounds from various plants.¹¹

MATERIALS AND METHODS

Plant material

Leaves of all three plants viz *Alianthus excelsa*, (Simaroubaceae), *Erythroxylum monogynum* (Erythroxylaceae) and *Simaruba glauca* (Simaroubaceae) was collected from the Lead Botanical garden, UAS, GKVK, Bangalore - 560065 Karnataka INDIA during July 2012. Fresh aerial parts of these plants were rinsed with distilled water and kept under shade till dry.

Preparation of extract

Extraction was carried out by simple maceration process. Aerial parts (250 g) were ground and submerged in 1.25 L methanol. Homogenates were kept for 3 days at room temperature (25 ± 2^oC) in extraction bottles. After 3 days when the tissue becomes colourless the mixture was filtered twice, first using ordinary filter paper and then Whatman-41 filter paper. Methanol was completely evaporated using the freeze drier. In total, 25-30g dried methanolic extract of aerial parts was obtained. Each dried extract was then transferred to a fresh clean glass vial and weight was determined. These extracts were dissolved in 1%DMSO and stored at 4^oC for future work (Table 1).

Table 1

List of plants selected, their family and the parts used and solvent used for extraction

Sl no	Name of the plant	Family	Parts used	Selection criteria	Solvent used for extraction
1	<i>Alianthus excelsa</i>	Simarubaceae	Leaves	Crown gall resistant	methanol
2	<i>Erythroxylum monogynum</i>	Erythroxylaceae	Leaves	Crown gall resistant	methanol
3	<i>Simaruba glauca</i>	Simarubaceae	Leaves	Crown gall sensitive	methanol

Alianthus excelsa, *Erythroxylum monogynum*, *Simaruba glauca* leaves collected and authenticated from Lead Botanical Garden, University of Agricultural Sciences, GKVK, Bangalore-560065, Karnataka, India

Brine shrimp Cytotoxicity assay (cytotoxic activity)

For the brine shrimp assay, the procedure described by⁵ was followed. In brief, a rectangular dish (22 x 32 cm) was compartmentalized into two unequal halves with plastic divider of 2 mm with several holes

and filled with artificial seawater (28 g sea salt/L, Sigma). Approximately 15 mg eggs (*Artemia salina*) were sprinkled in the larger compartment, which was darkened, while the smaller compartment was illuminated. After 24 hours, phototropic nauplii (brine shrimp larvae) were collected using a Pasteur pipette from the

lightened side. 10 shrimps were transferred to each vial containing 2ml of sea water and 0.2 ml of 100, 200, 400 and 800 ppm concentration of the extract prepared in 1% DMSO. The control vials were treated with 1 % DMSO (negative control) instead of extract. Each concentration replicates was performed in triplicates. All the vials were placed under illumination at room temp, and after 24 hours the number of survivors was counted. The number of death if occurred in the control was also counted and, the data was corrected using the following formula. LC50 was calculated using Probit analysis¹².

$$\text{Percentage of death (\%)} = \frac{[\text{dead test} - \text{dead control}]}{10} \times 100$$

Carrot Disc tumour assay (antitumor activity)

For antitumor activity, the procedure describe by MonMMet.al and Galasky AB et.al^{13,14} with slight modification was followed. In brief, *Agrobacterium tumefaciens* virulent strain (Ach13) (provided kindly by Prof Veluthambi, Emeritus scientist, School of Botany MKU Chennai INDIA) were grown for 48 hours in Luria Bertanin (LB) medium containing rifampicin (10 µg/ml). 1% inoculum from the 48hrs culture was inoculated to 25 ml broth for 18hrs and this culture was used for infection. *Daucus carota* was surface sterilized using 70% isopropanol and then the outer peel was removed and soaked in 10% sodium hypochlorite solution for 10 mins and thoroughly washed/soaked in autoclaved distilled water. Carrot discs (5 mm x 8 mm) were made with sterile blade or cork borer and placed on agar (1.5%) plates (10 discs per plate). *Agrobacterium* culture (1.0 OD cells) mixed with 50 µl each of 100, 200, 400 and 800µg/ml of extract (prepared in DMSO) was applied on the surface of each disc of respective concentration. All the concentrations were replicated in triplicates, while Camptothecin (CPT) served as positive inhibitory control⁷. Other controls included DMSO without the *Agrobacterium* and DMSO with the *Agrobacterium*. Petri plates were then

placed at 28°C for 10-14 days. Numbers of tumors per disc were counted and percent inhibition for each concentration was determined by the formula given below.

$$\text{Percent Inhibition} = 100 - \frac{\text{Average No of tumours in sample}}{\text{Average No of tumours in controls}} \times 100$$

Antibacterial assay

Antibacterial assay of higher concentrations of the extract of all the plants was performed against *Agrobacterium* strain, using agar well diffusion method¹⁵.

RESULTS AND DISCUSSION

Bioassays offer special advantages for identification of medicinal botanical extracts. Most often, a desired biological response is not due to one component but rather to a mixture of bioactive plant components. Therefore, crude extracts must be screened for biological activity and then any active extract should be fractionated and directed with bioassays to exploit the bioactive compounds¹⁴. In the present study, the extracts of aerial parts were prepared from three plants using methanol as a solvent. This study was divided into two major parts, i.e. Brine shrimp cytotoxicity assay, Carrot disc tumour assay for anti-tumour activity and antibacterial assay. According to¹⁶, the search for antitumor agents can be simplified by coupling one or two cytotoxic assays associated with an in vivo murine lymphocytic model. Cytotoxicity screening models provide important preliminary data to help select, plant extracts with potential antineoplastic properties for future work¹⁷. Brine shrimp cytotoxicity assay results (Table 2) (Table3) (Fig1) clearly indicated the toxic effects of the extracts. *Ailanthus* showed LC50 of 238.3ppm with average death of 1.33±0.57 in 100ppm, 3±1 in 200ppm, 6.33±0.57 in 400ppm, 8.66±0.57 in 800ppm with 13.3%, 30%, 63.3% and 86.65% mortality rate respectively, *Erythroxylum* showed LC50 172.9ppm 2.33±0.57 in 100ppm, 4.66±1.1 in 200ppm, 6.66±1.1 in 400ppm, 8.66±1.1 in 800ppm with 23.3%,

46.6% ,66.6%, 86.6% mortality rate respectively and *Simaruba* showed least LC₅₀ of 166.4ppm and average death of 3.33±0.57 in

100ppm, 5±1 in 200ppm, 8±1 in 400ppm, 8.33±0.57 in 800ppm with 33.3%, 50%, 80%, 83.3% mortality rate respectively.

Table 2

Effect of three different extracts at four different concentrations of extract on Shrimps

Alianthus			Erythroxyllum			Simaruba		
A	B	C	A	B	C	A	B	C
100	1.33±0.57	13.3	100	2.33±0.57	23.3	100	3.33±0.57	33.3
200	3±1	30	200	4.66±1.1	46.6	200	5±1	50
400	6.33±0.57	63.3	400	6.66±1.1	66.6	400	8±1	80
800	8.66±0.57	86.6	800	8.66±1.1	86.6	800	8.33±0.57	83.3

A – Concentration in ppm, B – Average Death ± SD, C - % Mortality

Table 3

LC₅₀ values of the three extracts using Brine Shrimp cytotoxicity bioassay values were obtained from Probit analysis using Stat plus Software2009

SI no	Name of the plant	LC ₅₀ ppm
1	Alianthus excelsa	238.3
2	Erythroxyllum monogynum	172.9
3	Simaruba gluaca	166.4

Fig 1: Graphical representation of cytotoxicity of all the three plant extracts at different concentrations of the extracts

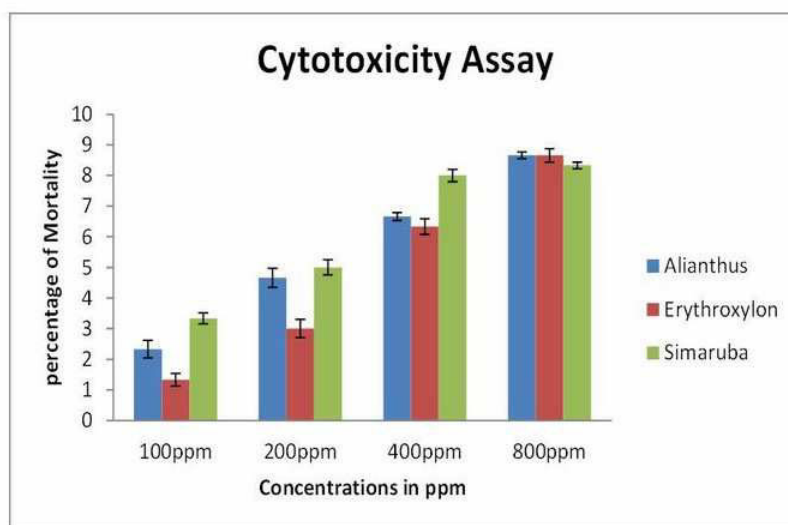


Figure 1

Graphical representation of cytotoxicity of all the three plant extracts at different concentrations of the extracts

Toxic effects on brine shrimps by the plant extracts indicated the anti-tumour potential of plant extracts as suggested by ^{18,19}. From the above results we were able to observe least LC50 in *Simaruba* extract followed by *Erythroxylum* and *Ailanthus* respectively therefore since all the extracts showed cytotoxicity they were further checked for anti-tumour potential. The extracts showing significant activities in the cytotoxic assay are usually subjected to carrot disc assay to confirm the anti-tumour potential of medicinal plants. This assay can be routinely employed as a comparatively rapid, inexpensive, safe and statistically reliable pre-screen for 3PS (in-

vivo murine leukemia) antitumor activity. The mechanism by which *Agrobacterium* inserts material into plant materials is by type IV secretion, which is very similar to mechanisms used by animal pathogens to insert materials (usually proteins) into human cells also by type IV secretion¹³. Carrot disc assay was carried out using *Agrobacterium tumefaciens* (AcH13) for tumor induction against four different concentrations (100,200,400,800ug/ml) of the extracts. All three extracts showed significant difference in tumor inhibition at different concentrations. (Fig2A)

Fig 2A: Varied inhibition of the tumour formation at different concentration of the extracts.

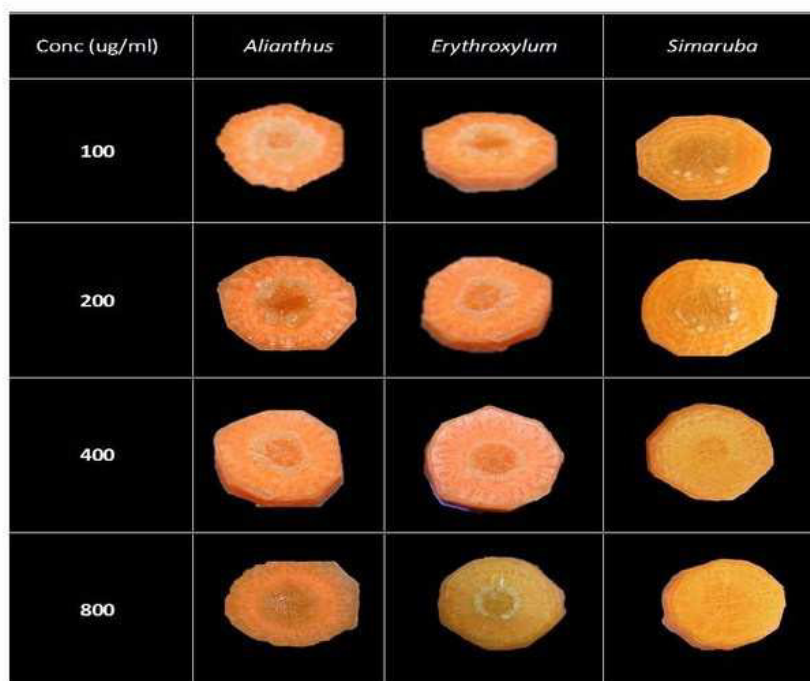


Figure 2A

Varied inhibition of the tumour formation at different concentration of the extract. Our results showed on an average 30 tumours on the control disc (*Agrobacterium* infection alone), whereas the positive control Camptothecin (CPT) ⁷ inhibited the tumours completely at a concentration of 30ug/ml. (Fig 2B).

Fig 2B: plates showing tumour growth in negative controls and tumour inhibition in positive controls

Average Number of Tumours in Control=30

PlateA: -^{ve} Controls

- C1: *Agrobacterium* Alone
- C2: Methanol+: *Agrobacterium*
- C3: 1%DMSO+: *Agrobacterium*
- C4: PBS+: *Agrobacterium*
- C5: No Infection
- C6 & C7: *Agrobacterium* 0.5 OD cells

PlateB: +^{ve} controls CPT (Camptothecin)

- PC: *Agrobacterium* alone
- PC1&PC2: *Agrobacterium* +30ug/ml CPT
- PC3&PC4: *Agrobacterium* +60ug/ml CPT
- PC5&PC6: *Agrobacterium*+120ug/ml CPT
- PC7&PC8: *Agrobacterium* +240ug/ml CPT

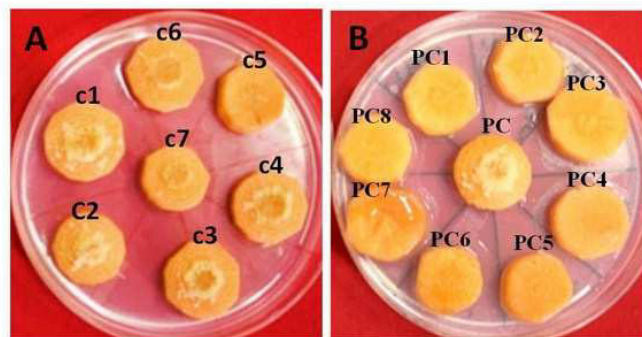


Figure 2B

plates showing tumour growth in -^{ve} controls and tumour inhibition in +^{ve} controls PlateA: -^{ve} Controls, Average Number of Tumours in Control=30

- C1: *Agrobacterium* Alone,
- C2: Methanol+: *Agrobacterium*,
- C3: 1%DMSO+: *Agrobacterium*,
- C4: PBS+: *Agrobacterium*,
- C5: No Infection,
- C6 & C7: *Agrobacterium* 0.5 OD cells

- PlateB: +^{ve} controls CPT (Camptothecin)
- PC: *Agrobacterium* alone
- PC1&PC2: *Agrobacterium*+30ug/ml CPT
- PC3&PC4: *Agrobacterium*+60ug/ml CPT
- PC5&PC6: *Agrobacterium*+120ug/ml CPT
- PC7&PC8: *Agrobacterium*+240ug/ml CPT

The extract of *Ailanthus* showed 13.3% inhibition at 100ug/ml and 60% inhibition at 800ug/ml, *Erythroxyllum* showed 30% inhibition at 100ug/ml and 76.6% inhibition at 800ug/ml, but significant results were observed with the extract of *Simaruba glauca* which showed as much as 95% tumour inhibition at a lowest

concentration of 100ug/ml (Table 4) (Fig 3). Usually, 20% inhibition of tumor is considered as a significant value for plant extracts for anti-tumour activity²⁰. Hence the plant extracts used in the present study have a significant anti-tumour activity. These results are significant at P < 0.05 for concentrations used.

Table 4

Different concentration of the extracts showing average no of tumour on the disc and percentage of tumour inhibition on carrot discs

Alianthus			Erythroxyllum			Simaruba		
A	B	C	A	B	C	A	B	C
100	26.6±2.7	13.3	100	21.6±1.52	30	100	1.66±0.57	94.6
200	21±3.1	30	200	17±1.0	43.3	200	1.66±1.1	96.6
400	18±1.8	40	400	12±0.57	40	400	0	100
800	12±1.8	60	800	7±2	76.6	800	0	100

A – Concentration of extracts in µg/ml,
 B – Average number of tumours±SD,
 C - % of tumour Inhibition
 Level of significance P < 0.05

Fig 3: Graphical representation of tumour inhibition of all the three plant extracts at different concentrations

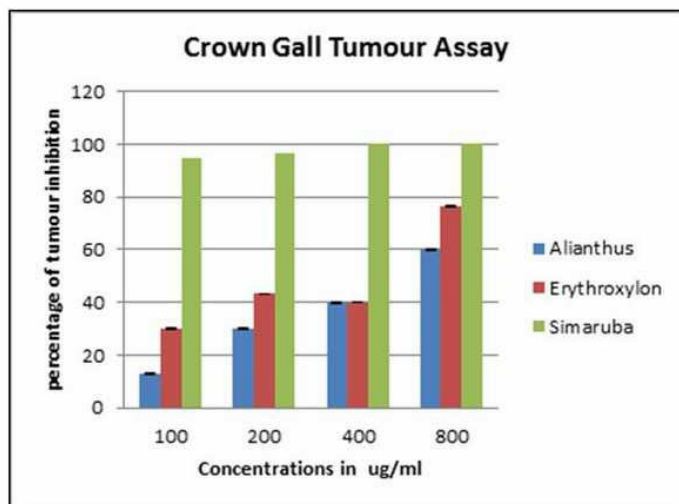


Figure 3

Graphical representation of tumour inhibition of all the three plant extracts at different concentrations. On the basis of the above results antibacterial assay was performed with different concentrations of the three plant extracts against the *Agrobacterium* strain that was used for both bioassays. This was done to check if the extracts have any antibacterial activity at any level that is necessary for the genetic transformation mechanism and finally induction of tumours. The antibacterial assay (Fig 4) showed no effect on the general growth and viability of the *Agrobacterium tumefaciens*

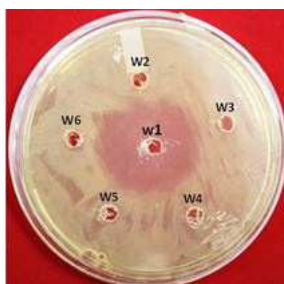


Fig 4: Effect of extracts on the viability of *Agrobacterium tumefaciens* (AcH13) strain.

- W1: Cefotaxim 50µg/ml (positive control)
- W2: Alianthus extract 1mg/ml
- W3: Erythroxyllum extract 1mg/ml
- W4: Simaruba extract 1mg/ml
- W5: SEA salt water
- W6: 1% DMSO

Hence the cytotoxic and the anti-tumour activities of the three plant extracts used in the present study might be due to the effect of the compounds present in extract.

CONCLUSION

According to traditional knowledge, all the three plants that were selected no doubt has medicinal potential, and in the present study this information was confirmed at laboratory level by performing different biological assays including cytotoxic, anti-tumour activity, and antibacterial assays. Significant cytotoxic and antitumor activity was found, which varied between three plant extracts, but all values fall in the acceptable range mentioned for specific assay by different authors. In the present study our one of the objective was also to look into the criteria based on which the plants were selected i.e weather the crown gall resistant plant constituents has any positive effect that can contribute to the cytotoxicity and anti-tumour activity of the extracts when compared to the crown gall susceptible plants. But our results suggest that this particular property of the plant in our study did not play any key role in contributing towards the cytotoxicity or anti-tumour activity. As crown gall susceptible plant *Simaruba glauca* showed higher and significant cytotoxic as well as anti-tumour activity compared to the other two plant extracts that are crown gall resistant. Another major conclusion is that both Brine Shrimp cytotoxicity assay and Carrot Disc Tumour assay are of great help in evaluating a vast

number of botanicals without having to use expensive and time consuming methods for screening and selection of botanicals for cytotoxic as well as anti-tumour activity. Based on the above conclusions, our further work will now aim at studying different parts of the *Simaruba glauca* plant and isolation of bioactive compounds and their identification and validation using analytical and cell line studies.

CONFLICT OF INTEREST

Conflict of interest declared none.

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