

# Evaluation of *in vitro* Antioxidant and Anticancer Activity of *Simarouba glauca* Leaf Extracts on T-24 Bladder Cancer Cell Line

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## History

- Submission Date: 24-06-2017;
- Review completed: 18-07-2017;
- Accepted Date: 31-08-2017.

DOI : 10.5530/pj.2017.6.142

## Article Available online

<http://www.phcogj.com/v9/i6>

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## ABSTRACT

**Objective:** Screening of preliminary phytochemicals, evaluation of *in vitro* antioxidant and *in vitro* anticancer activities of *Simarouba glauca* leaf extracts on T-24 Bladder cancer cell line.

**Materials and Methods:** Herbal extraction was carried out by Soxhlet method using chloroform, ethylacetate, methanol, ethanol, aqueous and hydroalcohol. Phytochemical investigation was done using biochemical tests. Total phenolic content was estimated by Folin-Ciocalteu reagent (FCR) method. Antioxidant potential of leaf extracts was analyzed by Ferric ion reducing antioxidant power (FRAP) assay, Phosphomolybdenum (PM) assay and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. *In vitro* anticancer activity on T-24 bladder cancer cell line was assessed by MTT assay. **Statistical analysis used:** Statistical analysis of data was performed by analysis of variance (one-way ANOVA) and level of statistical significance between groups was carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). **Results:** Phytochemical analysis revealed the presence of rich secondary metabolite present in all the solvent extracts. Hydroalcoholic extract showed highest presence of phenolic content (92.38±0.29 mg/g) GAE. Ethanol and methanol extract showed highest antioxidant capacity in DPPH, FRAP and PM assay as compared to the other extracts based on the test performed. The results confirmed that ethanol extract significantly (p<0.05) inhibited T-24 cell line with IC<sub>50</sub> value (533.55±25.02 µg/mL) as compared to standard drug doxorubicin (0.16µM/mL). **Conclusions:** The results of the present findings strengthen the potential property of *Simarouba glauca* as a resource for the discovery of novel antioxidant and anticancer agents.

**Key words:** Antioxidant, Anticancer, Bladder Cancer, Phytochemical, *Simarouba glauca*.

**Key Messages:** The use of medicinal plants have been practiced to treat cancer ailments across the world. Our study demonstrates the implication of herbal extracts to inhibit cancer cell lines at *in vitro* levels. However, the further approaches at *in vivo* level using lead compounds will give core insights of *S. glauca* extracts on Bladder cancer.

## INTRODUCTION

Bladder cancer is a kind of urological tumor with relatively high mortality, morbidity and recurrence metastasis rate.<sup>1</sup> Although endoscopic transurethral resection of bladder (TURB) is often performed as the primary therapy, 50-70 percent of patients have a recurrence within five years and approximately 20 percent progress to invasive disease.<sup>2</sup> The current treatment options like hormone therapy, radiotherapy, chemotherapy are into practice, but severe side effects limit its clinical use and underscore the need for unconventional therapies using less toxic substances. Many natural substances are touted for their medicinal aspects and side effect profiles, and some of these have been well characterized for their biological and medicinal properties.<sup>3</sup> Despite the availability of several therapeutic options, a safer and more effective modality is urgently needed for treatment of bladder cancer.<sup>4</sup> Thus, the primary therapeutic objectives are to prevent

multiple recurrences, as well as progression to a more advanced, invasive disease.

In recent years, there are constant researches on *Simarouba glauca* (*S. glauca*) in inhibiting the metastasis and invasion of breast cancer tumors according to local Ayurvedic practitioners, but there are untouched studies on *S. glauca* inhibiting bladder cancer cells. *Simarouba glauca* belonging to family *Simaroubaceae* is commonly known as paradise-tree, dysentery-bark, and bitter wood.<sup>5</sup> It is native of El Salvador that has been introduced in India during 1960.<sup>6</sup> It is known for its high medicinal value and have strong anti-plasmodial, antibacterial, antifungal activity.<sup>7,8</sup> Extracts of *S. glauca* have been used for treatment of gastrointestinal disorders.<sup>9</sup> Thus, in order to deeply comprehend, we evaluated *S. glauca* leaf extract for preliminary phytochemical screening, total phenolic content, and *in vitro* antioxidant

**Cite this article:** Puranik SI, Ghagane SC, Nerli RB, Jalalpure SS, Hiremath MB. Evaluation of *in vitro* Antioxidant and Anticancer activity of *Simarouba glauca* leaf extracts on T-24 Bladder cancer cell line. Pharmacogn J. 2017;9(6):906-12.

potential was analyzed by ferric ion reducing antioxidant power assay, phosphomolybdenum assay, DPPH assay and *in vitro* anticancer activity was evaluated on T-24 bladder cancer cells by MTT assay.

## Subjects and Methods

### Collection of plant and authentication

The plant *Simarouba glauca* was collected from Indian Council of Medical Research-National Institute of Traditional Medicine (ICMR-NITM) Campus, Belagavi in the month of February 2015. It was authenticated by Dr. Harsh Hegde, Taxonomist, Scientist 'D' (ICMR-NITM), Belagavi, India. The herbarium of the plant was prepared. The leaves of the plants were removed cleaned for unwanted dust particle and shade dried for 20 days. The shade dried leaves were crushed to fine powder and stored in air tight container until further used.

### Preparation of plant extract

About 100 g of powdered leaves were subjected to soxhlet extraction following the method of Jensen,<sup>10</sup> using different solvents in their increasing polarity viz. chloroform, ethyl acetate, methanol, ethanol, aqueous and hydro alcohol followed by concentration of extractions by rotary vacuum evaporator and drying by using water bath.

### Phytochemical analysis

Preliminary phytochemical screening was carried out for all six solvent extracts. The crude extracts were tested for presence of phytochemicals namely alkaloids, flavonoids, glycosides, phenols, lignin's, saponins, sterols, tannins, anthraquinone and reducing sugar by following the protocol of Deepti *et al.*<sup>11</sup>

### Determination of total phenolic content

The total phenolic content of *S. glauca* was determined by using Folin-Ciocalteu method described by Pardhi *et al.*, with slight modification.<sup>12</sup> A volume of 0.125 mL of extract is mixed with equal volume of Folin-Ciocalteu reagent and incubated for 10 min. Then 1.25 mL of aqueous sodium carbonate and 1mL distilled water is added and the reaction mixture is incubated for 90 min. at 37° C. The absorbance was read at 760 nm spectrophotometrically. Gallic acid is used as standard and total phenolic content was expressed as mg/g Gallic acid equivalent (GAE).

### *In vitro* antioxidant assay

#### 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging effect of *S. glauca* extract was assessed with the stable scavenger DPPH with slight modifications of the method described by Brand-Williams *et al.*<sup>13</sup> Briefly, the concentrations (100 µL to 500 µL) of extracts were prepared in ethanol. DPPH solution (0.004%) was prepared in ethanol and 1 mL of this solution was mixed with the same volume of extract and standard solution separately. The mixture was incubated for 30 min. in the dark at room temperature and the absorbance was measured at 517 nm. Ascorbic acid was used as a standard. The degree of DPPH-purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity.

The scavenging activity against DPPH was calculated using the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

Where  $A_c$  is the absorbance of the control reaction (1mL of ethanol with 1mL of DPPH solution), and  $A_t$  is the absorbance of the test sample. The results were analyzed in triplicate. The  $IC_{50}$  value is the concentration of sample required to inhibit 50% of the DPPH free radical.

### Ferric ion reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay was used to measure the antioxidant power of the extracts. Antioxidant activity assays were performed by the method described by Oyaizu *et al.* with slightest modification.<sup>14</sup> Ethyl acetate, methanol, ethanol, aqueous and hydro alcohol extracts of *S. glauca* in different concentrations ranging from 100 µL to 500 µL were mixed with 2.5 mL of 0.2 mM phosphate buffer (pH 7.4) and 2.5 mL of potassium ferricyanide, (1% W/V). The resulting mixture is incubated at 50° C for 20 min. followed by the addition of 2.5 mL of trichloroacetic acid (10% W/V) and centrifuged at 3000 rpm for 10 min. 2.5 mL of distilled water is added and later 0.5 mL of ferrous chloride (0.1% W/V). Finally, the absorbance was measured at 700 nm. Ascorbic acid was used as positive reference standard.

### Phosphomolybdenum (PM) assay

Total antioxidant activity was estimated by PM assay using standard procedure of Prieto *et al.*<sup>15</sup> Ethyl acetate, methanol, ethanol, aqueous and hydro alcohol extracts of *S. glauca* in different concentration ranging from 100 µL to 500 µL were added to each test tube individually containing 3 mL of distilled water and 1 mL of molybdate reagent solution. These tubes were kept incubated at 95°C for 90 min. After incubation, they are kept in room temperature for 20-30 min. and the absorbance is measured at 695 nm. Ascorbic acid is used as reference standard.

### Culturing of cell lines

T-24 bladder cancer cell line and Mouse embryo fibroblast (MEF-L929) normal cell line were procured from National Centre for Cell Science, Pune, India. The cells were subcultured in dulbecco modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids in tissue culture flasks and incubated in a CO<sub>2</sub> incubator in a 5% CO<sub>2</sub> and 95% humidity atmosphere. After trypsinization the cell count was done and the cell viability was tested by trypan blue dye exclusion method using haemocytometer. A known number of cells (4 X 10<sup>3</sup> cell/well in 100 µL of medium) were seeded into 96-well plates respectively for carrying out MTT assay by following the method of Ghagane *et al.*<sup>16</sup>

### Treatment groups

T-24 and MEF-L929 cell lines were treated with *S. glauca* leaf methanol, ethanol and aqueous extract (5 mg/mL). Desired concentrations of test compounds were prepared in 0.1% di-methyl sulfoxide (DMSO) prior to the experiment.<sup>17</sup> The reactant mixtures were diluted with media and cells were treated with different concentration ranges of the extract (3.125-200 µg/mL) and incubated for 72 h respectively which was the optimal treatment time of the extracts in each of the cell lines. The effect induced was also compared to the standard drug used viz. doxorubicin T-24 bladder cancer cell line. The following treatment groups were set up of the study. Negative control: cells alone. Positive control: cells + doxorubicin. Test groups: cells+ methanol extract of *S. glauca* leaves; cells+ ethanol extract of *S. glauca* leaves; and cells+ aqueous extract of *S. glauca* leaves, same treatment group was followed for mice embryo fibroblast (MEF-L929) normal cell lines.

### MTT Cell Viability assays

After 72 h the media of treated cells (100 µL), were removed and the cell culture were incubated with 50 µL of MTT at 37° C for 4 h. After incubation, the formazan produced were then solubilized by the addition of 100 µL DMSO. The suspension was placed on a microvibrator for 5 min and then absorbance was recorded at 470 nm by ELISA reader and the results were analyzed in triplicate and percentage was calculated.

## Statistical analysis

All results were expressed as mean  $\pm$  SD for three replications. Statistical analysis of data was performed by analysis of variance (one-way ANOVA) and level of statistical significance between groups was carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). At least three independent analyses were carried out per sample.

## RESULTS AND DISCUSSION

In recent years ethno-medicine plants have played an important role in prevention and treatment of various diseases, there are several known and unknown medicinal compounds obtained from plant origin.<sup>18</sup> Many species of the *Simaroubaceae* family display prominent anticancer activity.<sup>19</sup> The plant collected from (ICMR-NITM) Campus, was identified according to their taxonomical characters as *Simarouba glauca* and analysed for the presences of phytoconstituents with five solvent extracts. In the present study 100 g of *S. glauca* leaves powder gave total yield of 6.8 g in chloroform, 9.6 g in ethyl acetate, 24.64 g in methanol, 16.52 g in ethanol, 20.80 g in Aqueous and 31.2 g in hydroalcoholic solvent w/w respectively with reference to the air dried plant material. All six different extracts of *S. glauca* subjected for preliminary phytochemical analysis showed presence of rich phytoconstituents. Phytochemical analysis revealed the presence of rich secondary metabolites synthesized in the plant that includes phenols, flavonoids, alkaloids, saponins, anthraquinone, glycosides, lignins and tannins. The result of phytochemical test has been summarized in (Table 1). These bioactive compounds are employed in the treatment of cancer, malaria, worms, viruses, gastritis, ulcer, inflammation, diarrhoea and diabetes, in addition to their insecticide, healing and tonic activities.<sup>20</sup>

The total phenolic content of different extracts of *S. glauca* leaves was determined by Folin-Ciocalteu reagent method. Phenolic content of aqueous and hydroalcoholic extract, calculated from the calibration curve ( $R^2 = 0.9867$ ) was  $92.38 \pm 0.29$  mg/g GAE and ( $R^2 = 0.9837$ ) was  $90.55 \pm 0.66$  mg/g GAE showing the highest phenolic content as compared to ethanol ( $R^2 = 0.9807$ )  $84.55 \pm 0.59$  mg/g GAE, ethyl acetate and methanol extracts  $82.41 \pm 0.54$  mg/g GAE and  $76.69 \pm 0.14$  mg/g GAE. The phenolic content is responsible for the bioactivity of these crude extracts. The presences of phenolic compounds in the *S. glauca* extracts holds redox properties, which allow them to act as antioxidants and scavenge the free radical activity facilitated by their hydroxyl groups, the total phenolic concentration could be used as a base for rapid screening of antioxidant properties.<sup>21,22</sup>

Different concentrations of ethyl acetate, methanol, ethanol, aqueous and hydroalcoholic extract of *S. glauca* leaf extracts were subjected to DPPH free radical scavenging assay. The antioxidant capacity of the extract was compared with ascorbic acid as standard antioxidant. Ethanol, methanol aqueous and hydro alcohol extract presented higher antioxidant activity than the ethylacetate extract. Reactive oxygen species play an important role in the oxidative damage of biological systems.<sup>23</sup> Herbal extracts rich in secondary metabolites, including phenolics, flavonoids and tannins, have antioxidant activity due to their redox properties and chemical structures.<sup>24</sup> The DPPH radical scavenging method is widely used in assessing free radical scavenging activity because of the ease of the reaction.<sup>16</sup> The ethanol leaf extract ( $90.35 \pm 0.51\%$ ) of *S. glauca* had strong antioxidant activity against all the free radicals investigated at concentration of 500 mg slightly lower to that of standard ascorbic acid, ( $92.15 \pm 0.88\%$ ) compared to other solvent extracts (Table 2).

The reducing power of the crude extracts was studied as a function of their concentration. Antioxidant potential of *S. glauca* was examined

**Table 1: Preliminary phytochemical screening of *S. glauca* leaves extracts**

Constituent	Test	Chloroform	Ethyl acetate	Methanol	Ethanol	Aqueous	Hydro alcohol
Alkaloids	Iodine	-	-	-	-	-	-
	Wagner's	++	-	++	++	+	+
	Dragendroff's	+	-	++	++	-	+
Flavonoids	Pew's	-	-	+	+	-	-
	Shinoda	-	-	+	++	+	-
	NaOH	-	+	-	++	++	+
Glycosides	Keller-Killani	+	++	++	++	+	-
	Glycosides	-	++	-	++	++	+
	Conc. H <sub>2</sub> SO <sub>4</sub>	++	++	++	++	++	++
	Molisch's	++	++	++	++	++	++
Phenols	Ellagic acid	-	++	++	++	++	++
	Phenol	-	++	++	++	++	++
Lignins	Labat	+	-	+	+	-	-
Saponins	Foam test	-	-	+	-	+	+
Sterols	Salkowski's	+	+	++	++	+	+
Tannins	Gelatine	+	-	-	+	+	+
	Lead acetate	-	-	++	++	++	++
Anthraquinone	Bomtrager's	-	-	-	++	+	-
Phlobatannins		-	-	++	+	-	-
Reducing sugar		++	+	+	++	+	-
Volatile oil		++	-	-	+	-	+

: - Absent; +: Moderately present; ++: High presence

**Table 2: Determination of percentage inhibition of DPPH radical scavenging activity of *S. glauca***

Concentration	Percentage of Inhibition					
	Ethyl acetate	Methanol	Ethanol	Aqueous	Hydroalcohol	Ascorbic acid
100 mg	41.52±0.54	64.96±0.42	59.64±0.80	31.68±0.20	38.53±0.19	78.20±0.56
200 mg	55.30±0.19	74.87±0.58	64.76±0.63	56.05±0.10	55.46±0.31	83.23±0.12
300 mg	68.24±0.19	81.42±0.45	78.12±0.86	72.85±0.24	64.58±0.98	84.85±0.12
400 mg	71.54±0.41	85.48±0.44	84.81±0.51	81.62±0.67	76.20±0.01	86.63±0.21
500 mg	73.60±0.78	88.43±0.18	90.35±0.51	83.68±0.77	88.93±0.32	92.15±0.88

Values are mean ± SD, n = 3. Results were analyzed using One-way ANOVA.

**Table 3: IC<sub>50</sub> values of cell proliferation inhibition of *S. glauca* extracts (µg/ml)**

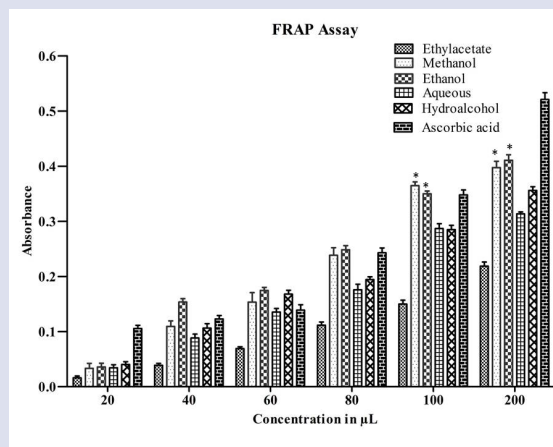
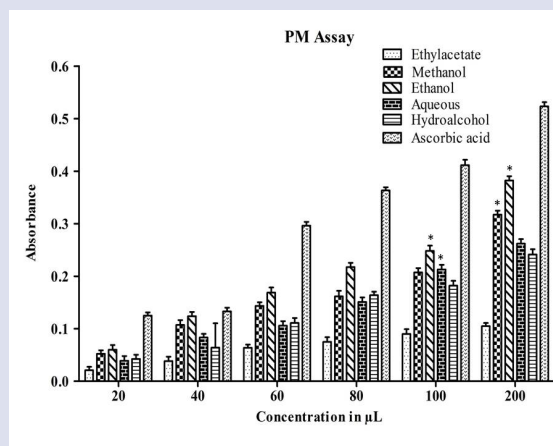
Cells	Ethyl acetate	Methanol	Ethanol	Aqueous	Hydroalcohol
T-24	2650.11±12.05	842.27±14.04	533.55±25.02	1019.10±11.05	1642.21±18.03
Doxorubicin	0.16 µM/mL				

for their ability to reduce ferri cyanide Fe<sup>3+</sup> to ferro cyanide Fe<sup>2+</sup> by donating an electron. In the present study the FRAP assay was estimated by using the leaf extracts of *S. glauca*. Out of five extracts, ethanol extract exerted the highest reductive capacity followed by subsequent extracts. The presence of antioxidants in any extract causes the reduction of the ferri cyanide Fe<sup>3+</sup> (complex) to ferro cyanide Fe<sup>2+</sup> (ferrous form).<sup>25</sup> The ferrous ion can be measured by FRAP assay at 700 nm which give the quantifiable estimation of phenols involved in redox reactions. The *S. glauca* leaf extracts showed quantitatively similar reducing ability with ethanol and methanol extract being far superior to aqueous and hydro alcohol extracts at higher concentrations. However, the reducing ability was slightly lower than that of ascorbic acid tested (Figure 1).

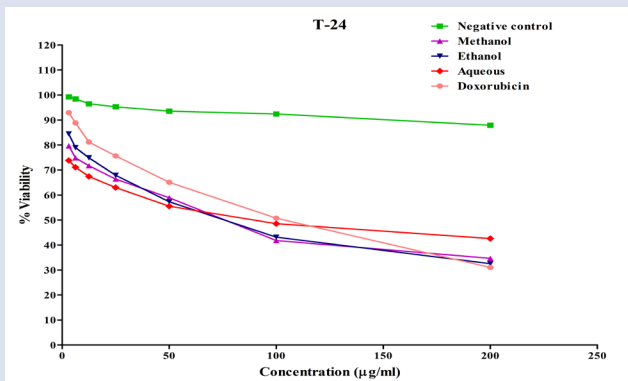
The quantitative phosphomolybdenum method was developed to evaluate the total antioxidant capacity of the *S. glauca* extracts. The reducing power of a compound is associated with electron donating capacity and serves as an indicator of antioxidant activity.<sup>26</sup> The total antioxidant capacity was evaluated by phosphomolybdenum assay, which measures the reduction of Phosphate-Mo (VI) to Phosphate-Mo (V) by the sample and subsequent formation of a bluish green colored Phosphate-Mo (V) complex at acidic pH. Basically it helps to investigate the reduction rate among antioxidant and molybdenum ligand<sup>15</sup>. In the present investigation ethanol (0.383±0.002) and methanol (0.317±0.005) extracts exhibited higher absorbance than the aqueous and hydro alcohol extracts in various solvents exhibiting different degrees of activity as shown in. (Figure 2). Absorbance is directly proportional to the antioxidant activity and gives reducing potential of plant extracts.<sup>27</sup>

Cytotoxicity, commonly found within the Simaroubaceae family, is primarily attributed to the dense chemical diversity, due to the presence of quassinoids, alkaloids, phenols, steroids, flavonoids, anthraquinone, coumarins and saponins, which have pharmacological properties such as cytotoxicity, antimalarial, insecticidal, antitumor, hypoglycemic and antiulcer activities characterize the species of this particular family.<sup>28,29</sup>

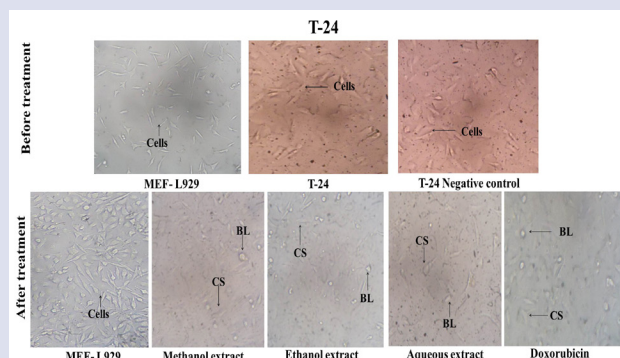
*In vitro* cytotoxicity test using T-24 bladder cancer cell line was executed to evaluate potentially toxic compounds that affect basic cellular functions and morphology.<sup>30</sup> Cellular proliferation was estimated by the MTT assay with varying concentration (3.125, 6.25, 12.5, 25, 50, 100, 200 µg/mL) of *S. glauca* leaf extracts and positive control (doxorubicin) for 72 h. Each extract were evaluated in triplicates by serial dilution. The absorbance at 490 nm was detected. Ethanol and methanol extracts showed effective growth inhibition at higher concentration 200 µg/mL compared

**Figure 1: FRAP assay for extracts of *S. glauca*.****Figure 2: PM assay for extracts of *S. glauca*.**





**Figure 3:** Cytotoxicity cell viability of *S. glauca* Negative control, Ethyl acetate, Methanol, Ethanol, Aqueous, Hydro alcohol leaf extracts and Doxorubicin drug on T-24 Bladder cancer cell lines.



**Figure 4:** Morphological changes of T-24 bladder cancer cell lines and MEF-L929 normal cell line for 72 h. **CS:** Cellular shrinkage; **BL:** Membrane blebbing (Magnification for T-24 was 20X and MEF-L929 was 20X).

to other extracts and lower than standard drug doxorubicin (Figure 3). All extracts showed increasing cytotoxicity in dose and incubation time period dependent mode from lower to higher concentration. Cell morphological changes in T-24 bladder cancer cells treated with *S. glauca* leaf extracts were observed microscopically. Treatment with *S. glauca* leaf extracts and doxorubicin drug significantly decreased the number of viable cells and produced microscopic changes in morphology compared with those of the controls after 72 h incubation at 200 µg/mL. The cells showed shrinkage, membrane blebbing and cytoplasmic vacuolation (Figure 4). However, there was no inhibition and morphological changes observed in normal (MEF-L929) cell line. The  $IC_{50}$  values for the *in vitro* cytotoxic activity was investigated for ethyl acetate, methanol, ethanol and aqueous extracts of *S. glauca* and standard drug doxorubicin against T-24 bladder cancer cell lines and noncancerous (MEF-L929) cell line. The results showed that ethanol extract significantly ( $p < 0.05$ ) inhibited T-24 cell lines and was the most potent extract with  $IC_{50}$  value ( $533.55 \pm 25.02$  µg/mL) as compared to standard drug doxorubicin (0.16 µM/mL). However, methanol, aqueous, hydroalcohol and ethyl acetate extracts showed moderate cytotoxicity (Table 3). Moreover, a higher degree of correlation was observed between antioxidant and anticancer of *S. glauca* extracts this might be due to the presence of rich secondary metabolites present in the plant.<sup>31,32</sup> The result of MTT assays

revealed that most effective cell growth inhibition activity were observed in ethanol and methanol extract of *S. glauca* leaves that decreased the percent viability of all the cells but to different extent towards T-24 cancer cell lines. No cytotoxic effect towards noncancerous normal mice embryo fibroblast (MEF-L929) cell line was observed among the extracts used. Standard chemotherapeutic drugs doxorubicin which is commonly used in the treatment of bladder cancer was found to a certain degree of similarity to the effect induced by the extracts. The cells were visually and microscopically inspected each time they were handled. Correspondingly morphological changes were observed in the cells enduring membrane blebbing and cell shrinkage by the combination of extracts and the standard drug induced in cancerous cells.

## CONCLUSION

In conclusion the overall results of the present study provided evidence of antioxidant and anticancer activities of *S. glauca*. The selectivity of the plant proved to be a promising anticancer potential by inhibiting T-24 bladder cancer cell line. However, the search for new lead compounds from natural sources with more effective and less toxic compounds constitute interesting alternatives for the development of anticancer drugs in bladder cancer treatment. Thus, further studies are in process to evaluate the potent lead fraction of the active plant.

## ACKNOWLEDGEMENT

The authors are grateful to Dr. Harsh Hegde, Taxonomist, Scientist 'D', "(ICMR-NITM)", Belagavi, India, for identifying the plant. The authors express their sincere gratitude to the authorities of Dr. Prabhakar Kore Basic Science Research Centre, KLE University, Belagavi, India, for providing the laboratory facilities to execute the soxhlet extraction and cell culture experiments. The authors also wish to thank the authorities of Department of Biotechnology and Principle K.L.E'S R. L. Science Institute, (Autonomous), Belagavi, India, for providing infrastructure to execute phytochemical analysis and Antioxidant assays.

## CONFLICT OF INTEREST

The authors declare conflicts of interest as None.

## ABBREVIATION USED

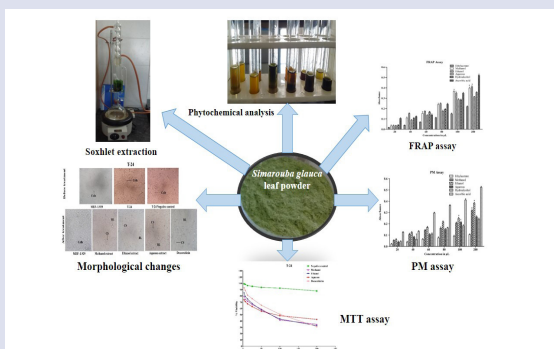
**TURBT:** Transurethral resection of bladder; **GAE:** Gallic acid equivalent; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **FRAP:** Ferric ion reducing antioxidant power assay; **PM:** Phosphomolybdenum assay; **MEF:** Mouse embryo fibroblast; **DMEM:** Dulbecco modified eagle medium; **DMSO:** Di-methyl sulfoxide; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **ELISA:** Enzyme-linked immunosorbent assay.

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## GRAPHICAL ABSTRACT



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## SUMMARY

- The plant *Simarouba glauca* was collected from (ICMR-NITM), campus, Belagavi.
- Preliminary phytochemical analysis revealed high presence of phenolic content in methanol, ethanol and hydro-alcohol leaf extracts of *S. glauca*.
- Methanol, ethanol proved to be high potential of antioxidant properties in the leaf extracts analyzed by FRAP assay, PM assay and DPPH assay.
- *In vitro* anticancer activity on T-24 bladder cancer cell line was assessed by MTT assay which proved to be a promising anticancer potential by inhibiting T-24 bladder cancer cell line.
- The search for new natural sources with more effective and less toxic compounds constitute interesting alternatives for the development of anticancer drugs in bladder cancer treatment.



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**Cite this article:** Puranik SI, Ghagane SC, Nerli RB, Jalalpure SS, Hiremath MB. Evaluation of *in vitro* Antioxidant and Anticancer activity of *Simarouba glauca* leaf extracts on T-24 Bladder cancer cell line. Pharmacog J. 2017;9(6):906-12.