

Original Article

POTENTIAL CYTOTOXIC DRUG EFFECTS OF SECONDARY METABOLITES DERIVED FROM
SELECTED MEDICINAL PLANTS OF SAVANADURGA FOREST IN KARNATAKA

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ABSTRACT

Objective: To evaluate the potential cytotoxic and antitumor activities of secondary metabolites of selected medicinal plants of Savanadurga forest, Karnataka.

Methods: The soxhlet extracted crude methanolic leaf extracts of nineteen medicinal plants were assessed for their potential cytotoxic and antitumor activities by brine shrimp lethality assay and potato tumor inhibition assay at 100µg/ml respectively.

Results: *Kirganelia reticulata* and *Cissua quadrangularis* showed highest cytotoxicity while *Flacourtia indica* failed to show any inhibitory activity in brine shrimp lethality assay. *Kirganelia reticulata* exhibited 100% antitumor activity while *Albizia amara* failed to show any antitumor activity as tested by crown gall tumor inhibition assay.

Conclusion: Both brine shrimp lethality assay and potato tumor inhibition assay indicated that *Kirganelia reticulata* seems to be the best anticancer plant.

Keywords: Brine Shrimp Lethality Assay, Potato Crown Gall Tumor Inhibition Assay, *Kirganelia reticulata*, Camptothecin, *Catharanthus roseus*, *Withania somnifera*, cytotoxicity.

INTRODUCTION

Plants have been the major source of drugs in traditional as well as modern medicine throughout the world. The tropical rich forests which serve as bio-resource are believed to be among the most potential source of bioactive lead compounds [1, 2]. Today about 80% of the world population residing in third world countries still rely almost entirely on plant products for their primary health care [3]. The remaining 20% of individuals living in the first world use more than 25% of the drugs which have been directly derived from plant products. These range from common drugs to prescription drugs such as the analgesic morphine and the cardiac glycoside digitonin isolated from *Papaver somniferum* and *Digitalis purpurea* respectively. Bio prospecting plants for anticancer activity has been a major focus in the search for plant based cures and resulted in the development of drugs such as *Vinca* alkaloids (Vincristine, vinblastine and vinorelbine), the epipodophyllotoxins (etoposide and teniposide), the taxanes (paclitaxel and docetaxil) and the camptothecin derivatives (irinotecan and topotecan).

Generally, plant extracts are prepared and screened against panels of mechanism based assays and human cancer cells. Although, the systematic drug discovery has helped to identify few plant derived chemotherapeutic agents, the need for identifying more effective antineoplastic agents remains. Most common tumors of the adult are resistant to presently available antineoplastic drugs and majority of the drugs have limited anti-solid tumor activity. So there is inadequacy in current chemotherapeutic agents for the treatment of abnormal malignancies. This led to the appreciation of the significance of natural products as sources for structurally novel and mechanistically unique drugs.

The need for effective neoplastic drugs and appreciation of natural products as sources coupled with rich vegetation of India provides a unique opportunity for discovering newer and potent cytotoxic and neoplastic drugs. The Savanadurga forest is situated in Ramanagara District, Karnataka, India, between latitudes 12.847° and 12.945°N

and longitudes 77.275° and 77.326°E, covering an area of 27 km² of dry deciduous forest which is considered as shrub and tree savannah of *Anogeissus-Chloroxylon-Acacia* series recording over 59 trees and 119 shrub species [4]. This forest area is rich in medicinal plants and has been identified as one of the most important medicinal plant conservation area of Karnataka. The objective of our present work was to screen the medicinal plants collected from Savanadurga forest for cytotoxicity and antitumor activity using brine shrimp lethality assay and potato crown gall tumor inhibition assay. These assays act as excellent preliminary assays for assessment of cytotoxicity and anticancer properties of plant derived secondary metabolites.

MATERIALS AND METHODS

Plant Collection

Fresh healthy leaves of nineteen medicinal plants belonging to sixteen families were collected from Savanadurga forest based on their availability and used for extraction of secondary metabolites (Table 1). Out of nineteen plants, two known anticancer plants *Catharanthus roseus* and *Withania somnifera* were used as standard reference plants. *C. roseus* is the main source of vinca alkaloids such as vincristine and vinblastine which are used as common drugs to treat cancers. *Withania somnifera* also contains a steroidal lactone withaferin A which acts as an anticancer compound. The collected plant materials were identified and authenticated by a researcher from Department of Forestry & Environmental Sciences, UAS, GKVK, Bangalore. The collected leaves of each plant were washed with deionized water and shade dried at room temperature for ten days, chopped and mechanically ground to coarse powder.

Preparation of plant extract

A thimble was prepared by packing ten grams of ground leaf powder from each plant in a Whatman filter paper No.1 and extracted with 150 ml of 70% methanol in soxhlet extractor at 70°C for 4 hours.

After filtration, the solvent was removed by evaporation using a rotary vacuum evaporator under reduced pressure at temperature below 50°C. The dried methanolic extracts were stored in a refrigerator for future phytochemical analysis.

Cytotoxic Brine Shrimp Bioassay

The brine shrimp lethality assay has been used as bench-top bioassay for cytotoxicity assessment of plant derived secondary metabolites. The cytotoxic activities of methanolic crude leaf extract of all the nineteen plants were carried out according to the standard procedure [5]. Brine shrimps were hatched using brine shrimp (*Artemia salina*) eggs in sterile artificial sea water [6, 7].

After hatching, active naupulii were attracted to one side of the chamber with a light source. Twenty naupulii were transferred into 5 ml brine solution in 20-ml tubes. The dried methanolic extracts of plants dissolved in DMSO were tested in quadruplicate at 100µg/ml along with control (vehicle) and positive control camptothecin. The camptothecin is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I and shows remarkable anticancer activity. The treated naupulii containing tubes were incubated at 28°C for 24 h. After 24 h of incubation, survived naupulii were counted with the help of 3× magnifying glass and calculation was done using Abbot's formula: % Death = (Sample - Control / Control) × 100

Table 1: Selected Indian plants collected from Savanadurga forest

S. No.	Botanical Name	English Name	Vernacular Name	Family
1	<i>Alangium salvifolium</i>	Sage leaved alangium	Ankola	Alangiaceae
2	<i>Albizia amara</i>	Bitter albizia	Chujjalu	Mimosaceae
3	<i>Canthium parviflorum</i>	Carray cheddie	Kaare gida	Rubiaceae
4	<i>Cassia auriculata</i>	Indian senna	Avarike	Caesalpinaceae
5	<i>Cissus quadrangularis</i>	Edible-stemmed vine	Asthisamharaka	Vitaceae
6	<i>Catharanthus roseus</i>	Madagascar periwinkle	Smashana Kanigale	Apocynaceae
7	<i>Flacourtia indica</i>	Indian plum	Bilehuli	Salicaceae
8	<i>Ficus hispida</i>	Hairy fig	Kadu hatti	Moraceae
9	<i>Gymnema sylvestre</i>	Australian cowplant	Madhunashini	Asclepiadaceae
10	<i>Justica adhatoda</i>	Malabar nut	Adusogae	Acanthaceae
11	<i>Kirganelia reticulata</i>		Kari hooli	Euphorbiaceae
12	<i>Limonia acidissima</i>	Wood apple	Belada mara	Rutaceae
13	<i>Passiflora subpeltata</i>	White passion flower	Vishnu Chakra hambu	Passifloraceae
14	<i>Parthenium hysterophorus</i>	Ragweed	Parthenium	Asteraceae
15	<i>Secamone emetica</i>	Common secamone	Siranige Hambu	Asclepiadaceae
16	<i>Tarenna asiatica</i>	Asiatic Tarenna	<i>Kumnigida</i>	Rubiaceae
17	<i>Ventilago maderaspatana</i>	Red creeper	Aithala beelu	Rhamnaceae
18	<i>Withania somnifera</i>	Winter cherry	Ashwagandha	Solanaceae
19	<i>Wrightia tinctoria</i>	Dyer's Oleander	Beppaale	Apocynaceae

Potato Crown Gall Tumor Inhibition Assay

The crown gall tumor inhibition assay is simple, safe, rapid, inexpensive and generally used to evaluate and prescreen the antitumor/cytotoxic properties of natural products. In the present study, the potato disc antitumor bioassay was performed according to the procedure described by Jamil and others [8]. A sensitivity test was performed against *Agrobacterium tumefaciens* strain to check out its viability against plant extracts at 100 µg/ml. After confirming their inability to inhibit the *Agrobacterium* at that concentration, the strain was used for inducing tumors in potato. The potato discs with 3 cm diameter and a thickness of 3 mm were surface sterilized with 70% ethanol, sodium hypochlorite and finally washed with sterile distilled water. Three potato discs were placed in each petri plate containing 3% sterile agar. Fifty microlitre of 48 h grown *Agrobacterium* culture was added and incubated for 48 hrs at 30°C. Two hundred microlitre (100µg/ml) of methanolic plant extracts, positive control camptothecin and DMSO as vehicle were added on each disc and incubated further at 30°C for 21 days. After incubation, the discs were stained with 5% Lugol's solution for 30 min and observed for the number of tumors formed. The percentage of tumor inhibition was calculated using the formula: % inhibition = (100 - Avg. number of tumors on sample / Avg. number of tumors on control) × 100.

RESULTS AND DISCUSSION

The search for new drugs derived from plants has been receiving renewed interest among researchers throughout the world in view of discovering new drugs that possesses potency to combat the menace of drug resistant pathogenic microorganisms, antitumor and anticancer agents [9, 10].

The brine shrimp lethality assay is a rapid, simple and inexpensive bioassay for the assessment of cytotoxicity which found to correlate positively with anti-tumor properties [11]. In this study, nineteen

medicinal plants collected from the medicinal plant conservation area at Savanadurga were used for extraction of secondary metabolites and then subjected to brine shrimp lethality assay to assess their cytotoxic & anti-tumor activities at 100 µg/ml for 24h (Table 2). Eleven out of nineteen plants have shown 80% of inhibition after 24 h. *Kirganelia reticulata* and *Cissus quadrangularis* showed highest percent inhibition, 98% and 94% respectively as compared to the standard anticancer drug camptothecin (100%) and the reference plants *Catharanthus roseus* (99%) and *Withania somnifera* (99%). Five plants have shown <50% inhibition while *Flacourtia indica* has failed to show any inhibitory activity. The significant lethality shown by *Kirganelia reticulata* indicates the presence of potent cytotoxic components which needs to be related with other antitumor assay results.

Potato Crown Gall assay

Potato crown gall is a neoplastic disease of plants induced by *Agrobacterium tumefaciens*. Since the mechanism of tumor induction is similar to that in animals, this test has been used to evaluate the extracts of nineteen Indian medicinal plants for their cytotoxic & anti-tumor activities along with the antitumor drug camptothecin. We successfully induced tumor formation on potato discs using *Agrobacterium tumefaciens*. Among the plants tested, *Kirganelia reticulata* exhibited 100% antitumor activity which is similar to that of reference plants, *Catharanthus roseus*, *Withania somnifera* and the standard drug camptothecin.

This result reveals the presence of potent cytotoxic components in *Kirganelia reticulata* which needs to be tested further for the nature of metabolites involved in anti-tumor activities (Table 2). In addition, this study also brings about potential antitumor ability of many other plants tested which could be exploited by further testing with cancer cell lines. The use of this bioassay by many other authors has helped to identify many plants with anticancer activities and in turn to identify novel compounds from such plants [12, 13].

When brine shrimp lethality assay and crown gall tumor inhibition assay results were compared with each other (Table 2), as expected the standard anticancer drug camptothecin has shown 100% cytotoxic and antitumor activity in both the assays. Both the reference plants, *Catharanthus roseus* and *Withania somnifera* have shown 99% and 100% cytotoxic and antitumor activity with respect to brine shrimp lethality assay and crown gall tumor inhibition assay respectively. Among the remaining seventeen plants, *Kirganelia reticulata* recorded the highest cytotoxic and antitumor activity of 98 and 100% in brine shrimp lethality assay and crown gall tumor

inhibition assay respectively. As both the assays were positive for cytotoxic and antitumor activity of *Kirganelia reticulata*, it indicates that this could be a potential plant for exploitation. The presence of tannins, flavanoids, glycosides, anthraquinone glycosides has been reported in *Kirganelia reticulata* [14] and these metabolites from other plants have been reported to have anticancer properties [15,16,17]. However, this needs further tests to identify the nature and mechanism of bioactive metabolites present in this plant. For the first time, we have reported the anticancer nature of *Kirganelia reticulata* using two separate bioassay systems.

Table 2: Relative toxicity of extracts and standard drug against brine shrimp larvae and antitumor activity

S. No.	Species Name	% death as measured by brine shrimp lethality assay ^a	% tumor inhibition as measured by potato tumor assay ^b
1	Camptothecin ^c	100.00	100.00
2	Alangium salvifolium	17.00	95.20
3	Albizia amara	17.00	00.00
4	Canthium parviflorum	40.00	90.48
5	Cassia auriculata	80.00	95.24
6	Cissus quadrangularis	94.00	95.24
7	Flacourtia indica	00.00	90.48
8	Ficus hispida	44.00	85.72
9	Gymnema sylvestre	60.00	85.72
10	Justica adhatoda	60.00	76.12
11	Kirganelia reticulata	98.00	100.00
12	Limonia acidissima	84.00	76.12
13	Passiflora subpeltata	84.00	95.24
14	Parthenium hysterophorus	84.00	85.72
15	Secamone emetica	84.00	76.12
16	Tarenna asiatica	87.00	85.72
17	Ventilago maderaspatana	37.00	76.12
18	Catharanthus roseus ^d	99.00	100.00
19	Withania somnifera ^e	99.00	100.00
20	Wrightia tinctoria	80.00	76.12

a = % death of brine shrimps, after 24 hours at the concentration of 100 µg/ml, **b** = % tumor inhibition on potato discs at 100 µg/ml concentration, **c** = Positive control, **d** and **e** = Reference standards

CONCLUSION

The present study screened the extracts of nineteen plants from dry deciduous forest for cytotoxic and antitumor activities using brine shrimp lethality assay and crown gall tumor inhibition assay and was successful in identifying *Kirganelia reticulata* as a potent anticancer plant for the first time. Further tests are required for confirmation of our results to exploit this plant as a source of anticancer compounds.

CONFLICT OF INTERESTS

Declared None

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