

Original Research Article

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Qualitative and Quantitative Estimation of Crown Gall Inhibitory Activity of Curcumin, Hydroalcoholic Extract of *Curcuma aromatica* Salisb and *Curcuma zedoaria* (Christm.) Roscoe

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ABSTRACT

Keywords

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Crown gall is a neoplastic disease characterized by the transformation of normal plant cells into autonomous tumor cells in a short period of time in more than 60 families of dicots and many gymnosperms. This paper deals with the comparison of crown gall inhibitory activity of Curcumin, a hydroalcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* both qualitative and quantitative. Qualitative assay results showed that the hydroalcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria*, and Curcumin found to possess crown gall inhibitory activity which was found to increase the concentration of the drug. Among the three tested compound curcumin showed good crown gall inhibitory activity from the concentration 500 µg/ml to 1000 µg/ml. In the quantitative assay, all the three tested compounds showed a good percentage of inhibition which was found to be dose-dependent. Among the three compound curcumin showed 56.13% of tumor inhibition at the concentration of 1000 µg/ml, whereas the hydroalcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* showed 29.59 % and 23.46% inhibition of tumor. The less crown gall inhibitory activity of a hydroalcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* might be due to the lower concentration of curcumin.

Introduction

Based on folklore and anecdotal information about traditional medicines paved the way for the production of new antitumor and antibiotic drugs. Huge amounts and the long period of research is required for the development of pharmaceuticals of ethnobotanical anecdotal or folkloric origin. Biological assays and

ultimate clinical testing are required for the verification of claimed biological activity. Information about the biological activity of plant extract could be obtained by bioassays which could also be used to identify the active components by direct fractionation of herbal extract. In assessing the antitumor activity of plant extracts bioassay is used over many years. Vincristine, Vinblastine,

Podophyllotoxin derivatives, Taxol are the example of phytoconstituents which has been discovered by using bioassays (Coker *et al.*, 2003).

In both developing and developed countries cancer is one of the most life-threatening diseases and serious public health problem. Synthetic drugs, as well as conventional treatment, are being failed to fulfill tumor control which is the main objective of cancer treatment due to their toxic effects and adverse side effects.

To achieve the fulfillment in the treatment of cancer, herbal medicines play an important role (Soriful Islam *et al.*, 2009).

Owing to the versatile applications, plant-derived substances have become a great interest recently. In the traditional system of medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entitles to synthetic drugs, medicinal plants are found to be the richest bio-source of drugs. Due to the problem of cytotoxicity to normal cells, there is an increasing need to search new compounds with cytotoxic activity for the treatment of cancer. Based on medicinal properties of *Curcuma aromatica*, *Curcuma zedoaria* and Curcumin were selected to study their role as efficient antitumor agents.

Crown gall is a neoplastic disease characterized by the transformation of normal plant cells into autonomous tumor cells in a short period of time in more than 60 families of dicots and many gymnosperms. Before performing the *in vivo* 3PS leukemic mouse assay antitumor activity, crown gall tumor inhibition assay could be used to prescreen the compounds for their antitumor activity because this assay is comparable, rapid, safe, inexpensive and statistically reliable (Tulsi Naik *et al.*, 2014)

To evaluate the crown gall tumor inhibitory activity of Curcumin, the hydro alcoholic activity of *Curcuma aromatica* Salib and *Curcuma zedoaria*, potato disc assay is used. In assessing the antitumor activity of plant extracts bioassays methods are used. Cell type selective toxicity is assessed by bioassays. A potato disc tissue is an assay based on antimiotic activity which can detect a broad range of known and novel antitumor effect by inhibition of *Agrobacterium tumifaciens* induced tumors. Because of the tumorigenic mechanisms that are similar in plants and animals, the validity of this assay is predicted. Initiation of crown gall tumor inhibition on potato disc and subsequent growth showed good correlation with compound and extracts active in 3PS leukemic mouse assay.

Agrobacterium tumifaciens, a gram-negative bacterium, is the causative agent of crown gall disease in which a mass of tissue bulging from stems and roots of the woody and herbaceous plant. On plant, these tumors may be spongy or hard, and may be or may not have a deleterious effect. *A. tumifaciens* produce tumors produced are histologically similar to those found in humans and animals. *A. tumifaciens* contain a tumor producing plasmid (Ti-Plasmid) during the infection of plant material, the plasmid gets incorporated into plant's chromosomal DNA. Phenols will be released when the plant tissue is wounded, which activate the Ti- Plasmid causes the plant's cells to multiply rapidly without going through apoptosis resulting in tumor formation similar in nucleic acid content similar to humans and animal cancer. The potato disc assay is a fairly rapid, inexpensive and reliable method for screening antitumor activity (Coker *et al.*, 2003).

Materials and Methods

The rhizomes of *Curcuma aromatica* salisb and *Curcuma zedoaria* (Christm.) Roscoe is

purchased from PSS Herbs Pvt. Ltd, Kerala, India in the month of July 2015 and authenticated by Dr. Palani, University of Madras, Chennai Curcumin was sponsored by Sami Labs Pvt. Ltd, Bangalore, sponsored Curcumin. This research was carried out at Department of Pharmacy, SRM University Kattankulathur. Kancheepuram District, Tamilnadu, South India.

Preparation of the powder

The rhizomes of *Curcuma aromatica* Salisb, *Curcuma zedoaria* (Christm.) Roscoe was shade dried, milled, and the coarse powder was separated.

Preparation of extracts

50% hydroalcoholic extracts are prepared for *Curcuma aromatica* salisb, *Curcuma zedoaria* (Christm.) Roscoe rhizomes and these prepared extracts are used for crown gall inhibitory activity studies.

Solubility test and pH test

With various solvents, Solubility of a hydroalcoholic extract of *Curcuma aromatica*, Curcumin, a hydroalcoholic extract of *Curcuma zedoaria* was checked and dimethyl sulfoxide (DMSO) was selected as a solvent.

Qualitative crown gall inhibition

Qualitative crown gall inhibition was carried out as per the method prescribed by Coker *et al.*, 2003 with modification. Inoculums were prepared by inoculating *Agrobacterium tumifaciens* in yeast extract media after incubating at 28⁰ C for 48 hours. *Solanum tuberosum* were scrubbed under running water with a brush and disinfected by immersing in 10 % sodium hypochlorite solution for 20 minutes. Potatoes were blotted on sterile paper towels and a flat surface was

prepared without skin. Trimmed section of potatoes was placed in a sodium hypochlorite solution (20 %) for 15 minutes. From the disinfected sections, cylinders were cut using the sterile borer(10mm) and then placed in a sterile distilled water for 2 times. From the cylinder, a disc about 0.5 cm thick was cut aseptically. In a 24- well culture plates containing 15 % water agar, the potato disc was placed.

Standardized suspension in the range of 1×10^9 CFU was prepared for *A. tumifaciens*. Various concentrations of the drug were prepared by dissolving in the suitable solvent DMSO. DMSO with phosphate buffer saline, DMSO without the bacterium and DMSO with the bacterium were used as the other controls.

400 µl of drug or control solution, 100 µl of water and 400 µl of standardized bacterial suspensions constitute the test solution.

50 µl of plant extract/ water/ bacteria mix was overlaid on each disc in the 24 well culture plate and incubated at room temperature for 12 days. The discs were stained with Lugol's reagent on the 12th day.

Starch in the potato tissue stains dark blue to dark brown color by the Lugol's reagent whereas tumors produced by *A. tumifaciens* will appear creamy to orange by not taking up the stain. Under the Motic microscope, stained potato disc was observed.

For each sample, twelve replicates were analyzed and the experiments were repeated three times. Either the activity of bacterial or the tumor induction was interfered by the solvent DMSO. Potential anticancer agents that interfere with neoplastic growth can be detected by potato disc assay because crown gall is a neoplastic disease of the plants induced by the specific strain which was first demonstrated in 1980 by Galsky.

Quantitative estimation of crown gall inhibitory activity

Quantitative estimation of crown gall inhibitory activity was carried out as per the protocol described by Rahman *et al.*, 2001 with some modifications. For 20 minutes fresh potato tubers of moderate size were surface sterilized by immersion in liquid bleach. With the help of sterilized (ethanol and flame) cork borer (6 mm diameter) a core cylinder of tissue was removed from the potato. In each potato cylinder, 2 cm ends were discarded and the remaining portion of the cylinder was cut into discs with uniform thickness. The discs were then transferred to 1.5% agar plate. Each plate contained 4 discs and the experiment was conducted in triplicate for each tested compound. From 48 hour culture containing 5×10^9 cells, 2 ml of *Agrobacterium tumifaciens* was added aseptically to each tested compound. Sterile water for injection was used in the place of testing compounds for the control. 0.05ml of each sample as well as control tubes were used to inoculate the respective potato discs and was spread over the disc surface with the help of disposable micro tips fitted with a micropipette. The plates were incubated at room temperature for twelve days, the lid was sealed to minimize the moisture loss. After twelve days of inoculation, the tumors were counted after staining with Lugol's solution, under a dissecting microscope. The tumor cells were lacking starch. The number of tumors in the control was used as a reference for determining the activity.

Evaluation

$$\text{Percentage of tumor} = \frac{\text{Mean No of tumor (test sample)}}{\text{Mean No of tumor (control)}} \times 100$$

$$\text{Percentage of tumor inhibition} = 100 - \text{Percentage tumor}$$

Results and Discussion

Positive control without extract

The potato disc, which showed the mutation that is caused by the *Agrobacterium tumifaciens* and considered as the positive control without the extract.

Negative control

Potato disc treated with distilled water is kept as negative control. When it treated with Iodine solution appeared blue in color which indicated the presence of unmutated cells.

Curcuma aromatica

The potato disc treated with the hydro alcoholic extract of *Curcuma aromatica* at the concentration of 250 µg/ml showed white cells due to the mutations that are caused by *Agrobacterium tumifaciens*. This clearly stated that the 250 µg/ml concentration of the extract was not sufficient to prevent crown gall tumors.

The potato disc treated with 500 µg/ml concentration of hydroalcoholic extract of *Curcuma aromatica* showed the mutations of cells to some extent. Here the proportion of blue color increased when compared to 250 µg/ml concentration (Fig. 14–16).

The potato disc treated with 1000 µg/ml concentration of hydroalcoholic extract of *Curcuma aromatica*. Protected to a maximum extent against mutation which was indicated by the appearance of blue color on the disc.

Curcuma zedoaria

The potato disc treated with 250 µg/ml concentration of hydro alcoholic extract of *Curcuma zedoaria* showed slight protection against the mutation caused by *Agrobacterium*

tumifaciens. The potato disc treated with 500 µg/ml concentration of hydro alcoholic extract of *Curcuma zedoaria* showed protection which was found to be better when compared to the disc treated with 250 µg/ml concentration.

The potato disc treated with 1000 µg/ml concentration of hydro alcoholic extract of *Curcuma zedoaria* showed good protection to the cells when compared to other two concentrations of extract (Fig. 17–19).

Curcumin

The potato disc treated with 250 µg/ml concentration of Curcumin showed slight protection against the grown gall formation that is caused by *Agrobacterium tumifaciens*

The potato disc treated with Curcumin at the concentration of 500 µg/ml, protection for the cells was found to be good when compared to 250 µg/ml concentration.

The potato disc treated with Curcumin at the concentration of 1000 µg/ml which showed good protection against the mutation caused by *Agrobacterium tumifaciens* (Fig. 20–22).

In the quantitative assay, all the three tested compounds such as hydroalcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria*, and curcumin showed a good response in potato disc assay and percentage of inhibition of tumors found to increase in a dose-dependent manner. Among the three compound curcumin showed 56.13 % of tumor inhibition at the concentration of 1000 µg/ml/ whereas the hydro alcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* showed 29.59% and 23.46 % inhibition of tumor.

The lesser activity of a hydroalcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* might be due to the lower concentration of curcumin, which is the chief compounds that are responsible for crown gall inhibitory activity.

Fig:1 Positive control without extract



Fig:2 Negative control

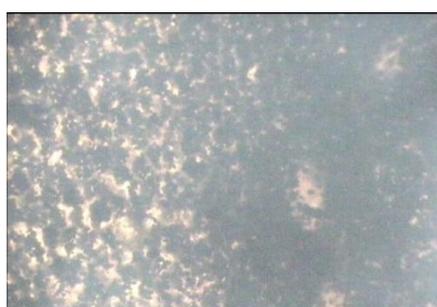


Fig:3 250 µg/ml



Fig: 4 500 µg/ml

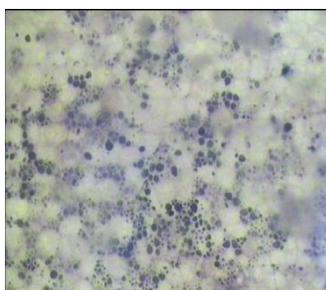


Fig:5 1000 µg/ml

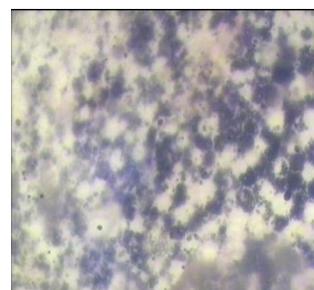


Fig:6 250 µg/ml

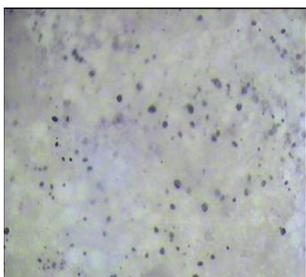


Fig:7 500 µg/ml

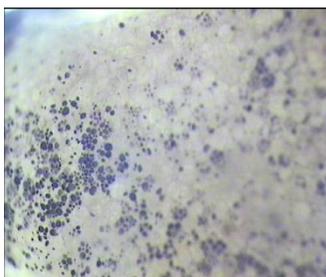


Fig:8 1000 µg/ml

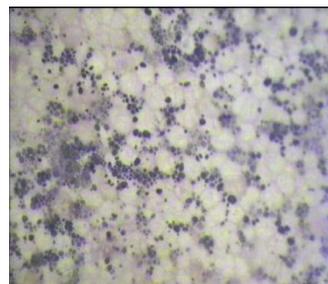


Fig: 9 250 µg/ml

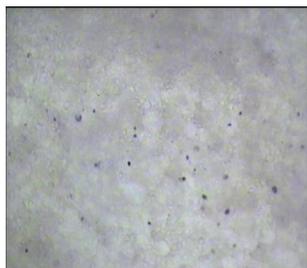


Fig: 10 500 µg/ml

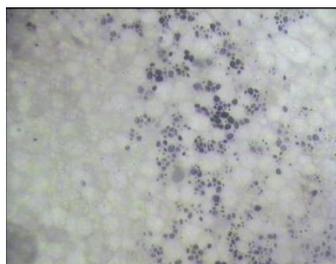


Fig: 11 1000 µg/ml

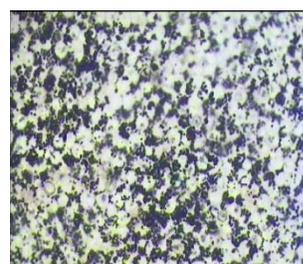


Fig: 12 Negative control



Fig:13 Positive control



Curcuma aromatica extract

Fig:14 250 µg/ml



Fig:15 500 µg/ml



Fig:16 1000 µg/ml



Curcuma zedoaria extract

Fig: 17 250 µg/ml



Fig: 18 500 µg/ml



Fig:19 1000 µg/ml



Curcumin

Fig:20 250 µg/ml



Fig:21 500 µg/ml



Fig: 22 1000 µg/ml



Quantitative estimation of crown gall tumor inhibition activity

Sample	Mean No of tumor cells \pm S.D	Percentage of tumors	Percentage inhibition of tumors
Control	98.47 \pm 6.32	-	-
Hydroalcoholic extract of <i>Curcuma aromatica</i>			
250 μ g/ml	92.22 \pm 3.8	93.87	6.12
500 μ g/ml	80.42 \pm 0.75	81.63	18.36
1000 μ g/ml	69.14 \pm 3.9	70.40	29.59
Hydroalcoholic extract of <i>Curcuma zedoaria</i>			
250 μ g/ml	94.24 \pm 2. 87	95.91	4.09
500 μ g/ml	86.73 \pm 10.45	87.75	12.24
1000 μ g/ml	75.81 \pm 4.23	76.53	23.46
Curcumin			
250 μ g/ml	89.95 \pm 4.92	90.81	9.18
500 μ g/ml	71.33 \pm 5.49	72.44	27.56
1000 μ g/ml	43.38 \pm 7.28	43.87	56.13

In establishing the biological purpose, such as antitumor, antibacterial, antioxidant and phytotoxic properties special advantages, are offered by biases which are the preliminary step in drug discovery. Bioassays are useful for screening of biological and synthetic bioactive compounds (Islam *et al.*, 2013). For checking known and novel antitumor molecule potato essay which is based on *Agrobacterium tumifaciens* on potato disc was shown to be useful (Islam *et al.*, 2009). The tumorigenic mechanism initiated in plant tissue by *A. tumefaciens* in many ways similar to that of animals (Srirama *et al.*, 2007).

Even *Bartonella henselae*, a bacterium causing tumor in humans have the similar pathogenic characters such as common toxins, secretion system, adhesion mechanism, invasion and regulations that are similar to *A. tumifaciens* (Kempf *et al.*, 2002; David *et al.*, 2004). This potato disc assay appears to be adapted for the purpose of standardization and quality control of bioactive compounds from past 15 years (Jerry *et al.*, 1998). The synthetic compounds and extracts which showed the inhibition capacity of the crown

gall formation by *A. tumifaciens* on potato disc showed good correlations which are statistically much more productive in both *in vitro* and *In vivo* antileukemic activity (Islam *et al.*, 2013; Galsky *et al.*, 1980; Ferigni *et al.*, 1982; Coker *et al.*, 2003)

For various chemicals that interfere with cell cycle, potato disc assay seems to be sensitive (Coker *et al.*, 2003; Islam *et al.*, 2010) for the detection and isolation of many antitumor compounds from plant, microbial or biomolecules potato disc assay, which needs a complete aseptic condition was found to be very much useful which is confirmed by *in vivo* tumor inhibition in animals (Jerry *et al.*, 1998) to increase the screening system, other vegetables such as bet, radish, carrot disc were also used to evaluate the antitumor activity of various compounds

Genotoxicity occurs in cells due to the potential consequences of genetic damage. A damage response mechanism is available in all the cells and the organism. In cells, genotoxicity is caused by the substance called genotoxins, for the treatment of various

diseases; a majority of the population in India uses traditional natural preparations. So it is becoming essential to estimate the Clastogenic potential of traditional plant extract and phytoconstituents derived from the plant source. When genotoxicity increases, it increases the risk of developing cancer. Most effective procedures for preventing cancer and genetic disorders in humans are to use antimutagenic and anticarcinogenic agents in everyday life. Bioactive components present in medicinal plants can block or reverse carcinogenic activity at early stages. Medicinal plants were considered to be effective and inexpensive in the treatment of cancer. In inhibiting the carcinogenic activity of some chemical mutagens, herbal plants play an important role (Srividya *et al.*, 2012).

Stored information's in the cells could be transferred from one generation to next generations by means of genetic material called deoxyribonucleic acid (DNA). DNA, RNA, and proteins that are present in the cells get damaged when they are continuously exposed to various chemicals and environments, in turn, produces serious consequences in the organism which increases the chance for developing cancer and Alzheimer's diseases. All cells have a variety of DNA repair system of their own, but DNA repair may not be always perfect. With age and the age-related decrease in DNA repair efficiency, certain forms of DNA damage accumulates in the cells which lead to larger inter-individual differences.

The studies which have been carried out by Srividya *et al.*, (2013c) by SOS assay showed that a hydroalcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria* was found to be non genotoxic at the concentrations of 250, 500 and 1000 µg/ml whereas curcumin was found to be genotoxic at the concentration of 500 and 1000 µg/ml concentration.

In the DNA sugar damage test, among the test compounds from 250 µg/ml concentrations, both hydroalcoholic extract of *Curcuma aromatica* and curcumin protected the sugar moiety in DNA whereas the hydro alcoholic extract of *Curcuma zedoaria* only at the 1000 µg/ml concentration protected the sugar moiety in the DNA.

In the plasmid nicking assay or DNA damage protective activity, hydroalcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* protected the DNA from their lowest concentrations such as 250 µg/ml whereas curcumin caused slight damage to the DNA in all the tested concentrations such as 250, 500 and 1000 µg/ml (Srividya *et al.*, 2013c).

In the comet assay, at the concentration of 50 µg/ml concentrations curcumin was found to be genotoxic when compared to the hydro alcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria*. In the micronucleus test at the concentration of 50 µg/ml concentrations, all the tested compounds showed less effective in micronucleus formations in HEp-2 cells. In the Ames reversion assay, all the tested compounds showed less revertant colonies which confirm the nongenotoxic activity of *Curcuma aromatica*, *Curcuma zedoaria* and curcumin (Srividya *et al.*, 2013b).

In the chromosomal aberration assay, both in the presence and absence of S9 factor, hydroalcoholic extract of *Curcuma aromatic* has not shown aberration in the culture of human lymphocytes. Curcumin showed Abression both in the presence and absence of S9 factor. Whereas hydroalcoholic extract of *Curcuma zedoaria* showed the Abression in the absence of S9 factor (Srividya *et al.*, 2013a)

All the studies which have been carried out by Srividya *et al.*, (2013a–c) showed good

Genoprotective activity in all the models. This crown gall inhibition assay also showed the similar results that of previous studies which confirmed that potato disc assay is the suitable model for screening of antitumor activity.

Curcumin showed the maximum crown gall inhibitory activity when compared to the hydro alcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria*. The potato disc assay was found to be the suitable, cheap, easy and less time-consuming convenient method

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