CHAPTER SEVEN

MISCELLANEOUS BIOACTIVITIES
INTRODUCTION

In addition to Anti-inflammatory, Antiviral, Anticancer, Antifungal, and Antibacterial activities, *G. glabra* exhibits other bioactivities like Anti-tumor, Anti-mutagenic, Immunostimulant, Antiulcer, Estrogenic, Expectorant, Antidiabetic and Anti-thrombic activities (1-61). We have tested the anti-tumor activity of *T. cuneifolia* using *Agrobacterium tumefaciens* induced tumors on potato disc (which is used as a model bioassay for screening of anti-tumor agents) and compared with that of *G. glabra*. The model systems are based on the different mechanisms of tumorigenesis e.g. Astrocytoma for antimitotic agents, Phage induction for DNA damage, *Agrobacterium tumefaciens* for plasmid transfer and tumor inhibition, *Candida* for cell membrane activity, *Xanthomonas* for glycosylation inhibitors etc. (62). *Agrobacterium tumefaciens*, a gram-negative bacterium is a well-known agent inducing crown galls/tumors (a neoplastic disease) in a wide range of plants (62). It exhibits a large Ti (Tumor inducing) plasmid, which transforms wounded as well as normal plant cells into tumor cells (62). Mechanisms of Tumorigenesis like incorporation of extraneous DNA and tumor initiation are common in both plant and animals (62) and inhibition of *Agrobacterium tumefaciens* induced tumors in plants showed considerable correlation with the *in vivo* mouse leukemia antitumor assay (62). This method was developed and standardized by Ferrigni *et al.* (1982) as an *in-vitro* model system for the screening of natural anticancer agents wherein inhibition of *Agrobacterium tumefaciens* induced tumors (Crown galls) in potato disc was tested.

Antimutagenic activity of both the plant extracts was tested by the inhibition of the toxicity induced by Ethyl methane sulfonate (a direct acting mutagen) in *Salmonella*
typhimurium strains. Maron et al., (1983) has developed this bioassay method for screening of antimutagenic activity (63).

_Candida albicans_ is a well-known human pathogenic, dimorphic fungus. Germ tube formation is one of the factors associated with its pathogenicity. We have tested anti-Germ tube induction activity of _T. cuneifolia_ and _G. glabra_, using serum induced germ tube formation assay method (64).

Inhibition of browning in apple juice by the different extracts of _T.cuneifolia_ and _G.glabra_ was studied _in vitro_. Browning is an oxidative process mediated by the enzymes like Polyphenol oxidase (catecholase/cresolase etc.) wherein phenols present in juice get converted in to quinone (65). Thus inhibition of fruit juice browning by _G.glabra_ extract can be adopted for the preservation of fruit juice, as it is being used as a flavoring agent. This activity of the extracts could be ascribed to the antioxidant compounds like phenolics, flavonoids tannins etc. which is involved in the inhibition of Polyphenol oxidase enzyme activity (62).

**MATERIALS AND METHODS:**

**Extraction of plant materials:**

Powdered plant materials were extracted using a soxhlet extractor for six to eight hrs with 70% Ethyl alcohol. Twenty grams of plant materials were extracted in 200 ml of 70% ethanol. The extracts were then filtered through Whatmann filter paper No 1, evaporated under reduced pressure to yield brown residues. The residues were resuspended in distilled Dimethyl sulfoxide (DMSO) and used as crude extract. Powder of _T. cuneifolia_ was extracted sequentially with Petroleum ether, Chloroform and Ethyl alcohol. Extracts were filtered, evaporated under reduced pressure and the resulting residues were
resuspended in dimethyl sulphoxide. These extracts were used for testing Anti-tumor activity, inhibition of mutagen induced toxicity using Salmonella typhimurium, inhibition of serum induced germ tube formation in Candida albicans and inhibition of fruit juice browning.

Cultures and Chemicals

A standard strain of Agrobacterium tumefaciens ATCC 33970 an inducer of tumors (Crown gall) in plants was purchased from Bangalore Genei Pvt. Ltd., Bangalore, India. Standard strains of Salmonella typhimurium NCIM 2501, and Candida albicans MTCC 227 were purchased from The Institute of Microbial Technology (IMTECH) Chandigarh, India. Bacterial cultures were maintained on Nutrient broth slants at 37°C and Yeast culture on Yeast Extract Peptone Dextrose agar slant at 28°C. Nutrient broth, Yeast Extract Peptone Dextrose Broth and Ethyl Methane Sulfonate (EMS) were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India.

I. Toxicity of the extracts against Agrobacterium tumefaciens (ATCC 33970) Salmonella typhimurium (NCIM 2501) and Candida albicans (MTCC 227)

Toxicity of the extracts of T.cuneifolia and G.glabra were tested against Agrobacterium tumefaciens, Salmonella typhimurium and Candida albicans (MTCC 227) by agar dilution assay method (66). Nutrient agar and YPD agar media were autoclaved and different concentrations of the extracts (0, 0.5, 1.0, 1.5 mg/ml) of both the plants were added separately to it at 45°C - 50°C temperature, aseptically. Twenty ml of these media was poured in each sterile petriplates. After solidification, the plates were inoculated with 50 μl of 24 old cultures of Agrobacterium tumefaciens, Salmonella typhimurium culture in Nutrient broth and Candida albicans culture grown in YPD broth, containing approximately
2x10^4 cfu/ml. Three plates were used for each concentration. Bacterial plates were incubated at 37°C for 24 hrs and *Candida albicans* plates at 28°C for 48 hrs. Number of colonies appeared were counted and compared with that of solvent control, lacking the extract.

**II. Antitumor Activity**

Anti-tumor activity of the extracts of *T.cuneifolia* and *G.glabra* was tested by Potato Discs, known as Potato Disc Assay method which is used for screening of anti-tumor constituents from herbal samples (62).

**Potato Disc Assay:**

Medium sized potatoes were purchased from the local market and kept in sodium hypochlorite solution to disinfect for one hour. Twenty ml of autoclaved agar (1.5%) was poured in each sterile petriplates aseptically and allowed to solidify. After solidification, five wells of 1.5-cm diameter were made in each petriplate. Potato discs of 0.5-cm height and 1.5 cm diameter were collected from the core of the disinfected potato tubers using sterile cork borer and scalpel. Discs were transferred in each well i.e. five discs/petriplate. 8 mg of extracts were dissolved in minimum quantity of DMSO and volume was adjusted to two ml with sterile distilled water. These extracts were filter sterilized using Millipore filter (0.22 µm). 0.5 ml of the sterilized and diluted extracts were then added in test tubes containing 1.5 ml distilled water. In these test tubes 2-ml of 48-hr old broth culture of *Agrobacterium tumefaciens* containing approximately 5x10^8 cells/ml were added and mixed gently. 50 µl of this inoculum/disc was used for inoculation. Mixture without extract was used as control. Three plates were kept for each concentration. All the plates were
incubated at 29±1°C and number of tumors on the discs were counted on 12th day and compared with control (62).

III. Protective effect of Extracts against a Mutagen Induced Toxicity

Ethyl methane sulphonate is a direct acting mutagen, which causes toxicity by alkylating DNA. We have tested the protective effect of both the plants extracts against EMS induced toxicity in *Salmonella typhimurium*. The dose of Ethyl methane sulphonate was determined by testing various concentrations and it was found that 1 μl EMS killed about 75% of organisms and thus EMS was used as a 1 μl/plate. Twenty ml of autoclaved nutrient agar was poured in each sterile petriplate and was allowed to solidify. 50 μl of 24-hr old broth culture of *S. typhimurium* was taken in a test tube containing 50 μl of phosphate buffer. To these tubes various concentrations of extracts (0, 2, 4, 6 and 8 mg in separate tubes) and 1 μl of EMS were added aseptically. Tubes were incubated for thirty minutes at 37°C and after incubation, 2ml of molten top agar was added to each tube and poured into the solidified petriplates. Plates were kept in triplicates for each concentration and incubated for 24 hrs at 37±1°C. Number of surviving colonies were counted and percent of survival were calculated by comparing with the control (63).

IV. Inhibition of Serum induced Germ tube formation in *Candida albicans*

Effect of *T. cuneifolia* and *G. glabra* extract on serum induced germ tube induction in *Candida albicans* was studied using a standard strain of *Candida albicans* (MTCC 227) (65). For germ tube induction, *Candida albicans* culture was grown in YPD broth for 24 hrs at 28 ± 1°C in a shaking incubator. 5.0 x 10^6 cells/ml from this culture was used as inoculum for one ml of 25% human serum in a 1.5 ml vials. Different concentrations of *G. glabra* and *T. cuneifolia* extracts were added aseptically in separate vials. The Vials
containing respective quantity of Dimethyl sulfoxide (DMSO) was used as control. All the vials were incubated at 37° C for 90 min. After incubation, a drop of the culture from the vials were taken on a hemocytometer slide and observed under a microscope for germ tubes formation and compared with the control (without drug).

V. Inhibition of Fruit Juice Browning

The inhibition of Fruit Juice Browning could be a good model system for the screening of antioxidant agents. The enzymes like Polyphenol oxidases like catecholase and cresolase mediate oxidation of phenolic compounds present in the fruit juice, which leads to the formation of quinones resulting in the browning of juice (64). In present study we have tested inhibition of browning in apple juice (64).

Hundred grams of apples were purchased from the local market, chilled, peeled and cut into small pieces. These pieces were blended in a mixer using 200-ml ice cold distilled water and filtered through a muslin cloth. To these flasks 1 mg concentration of the different extracts of T. cuneifolia and G. glabra were added. The flasks containing respective quantity of DMSO were used as control. The rate of quinone formation (browning) was determined by reading the OD at 420 nm at five minutes interval.
RESULTS AND DISCUSSION:

I. Anti-tumor activity of *T. cuneifolia* and *G. glabra*:

*Agrobacterium tumefaciens* is a well-known agent inducing crown galls/tumors (a neoplastic disease) in a wide range of dicotyledonous plants (62). Potato Disc assay is one of the most convenient method for rapid screening of natural anti-tumor agents. Incubation of potato discs with *A. tumefaciens* for twelve days induced profuse number of galls (tumors) on potato discs. In the case of discs treated with plant extracts, it was found that the numbers of galls were reduced considerably. In the case of *T. cuneifolia* extract treated discs, average number of gall were found to be eleven while that of *G. glabra* extract treated discs were nine tumors. Both the plant extracts exhibited considerable anti tumor activity i.e. *T. cuneifolia* crude extract showed 50% inhibition of tumor formation which is comparable to that of *G. glabra* extract (59%) at 0.250 mg/ml concentration (Table 1). Extracts of both the plants exhibited no effect on the *in vitro* growth of *A. tumefaciens* up to the 30mg/plate concentration.

Several workers have demonstrated that potato disc assay could be a good model system for the screening of natural antitumor agents (62). They have isolated number of compounds from different plants like Piceatannol from *Euphorbia lagascae*, Phebalosin from *Miermelum minutum* (Forst. f.), Tiliroside from *Eremocarpus setigerus* (Hook). Benth., Plumericin, Isoplumericin and Allamandin from *Allamanda cathartica*, Gonithalenol, Gonithalamin, and Annonacin from *Gonithalamus gigantus* Hook. f., Tubulosine from *Pogopus speciosus* (Jacq.) K. Schum. All these compounds show considerable antitumor activity in animal models as well (62).
G. glabra exhibits variety of compounds like saponins, flavonoids, terpenoids etc. associated with its broad spectrum of bioactivities (1-61). Glycyrrhizin, 18-α glycyrrhetinic acid, isoliquiritigenin, glabridin, licocoumarone etc. are reported to exhibit considerable anticancer activity when tested in different model systems (17,19, 25, 35, 39).
Table 1. Inhibition of *Agrobacterium tumefaciens* induced tumors (Crown Galls) in potato discs by *Taverniera cuneifolia* and *Glycyrrhiza glabra* extracts.

<table>
<thead>
<tr>
<th>Crude Extract (0.5 mg/ml)</th>
<th>Number of tumors/disc</th>
<th>Tumor Induction (%)</th>
<th>Inhibition of Tumor Induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no extract)</td>
<td>22 ± 2.0</td>
<td>100</td>
<td>00</td>
</tr>
<tr>
<td><em>G. glabra</em></td>
<td>09 ± 1.0</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td><em>T. cuneifolia</em></td>
<td>11 ± 1.0</td>
<td>51</td>
<td>49</td>
</tr>
</tbody>
</table>
Figure 1. Effect of *Taverniera cuneifolia* and *Glycyrrhiza glabra* on *Agrobacterium tumefaciens* mediated tumor induction.
II. Protective Effect of Extracts against a Mutagen Induced Toxicity

Ethyl methane sulphonate is a direct acting environmental mutagen, which can react directly with DNA and cause alkylation leading to the inhibition and or stimulation of specific gene expression. Plants harbor compounds, which protect cells from mutagen-induced damage. Mechanism of action of the antimutagenic agents present in plants is not very clear (36-38). Those compounds, which act inside the cell and modify the response of the cell to a mutagen are called as antimutagenic. Those that act on the mutagens outside the cells and inactivate its mutagenicity are called as desmutagenic agents. Protective effects of both the plant extracts were studied using *Salmonella typhimurium* as a model (63). It was found that both the plant extracts exhibit concentration dependent protective effect against EMS induced toxicity. *G. glabra* extract caused about 97% survival of *Salmonella typhimurium* at the concentration of 8 mg/plate (Table 2). 6-mg/plate concentration of *T. cuneifolia* extract supported 75% survival of *Salmonella typhimurium* (Table 2).

Antimutagenic activity of several plants are reported by different workers (35-39). Several compounds of plant origin like Ascorbic acid, ellagic acid and tannins from *E. officinalis* and phenolics and tannins from *C. occidentalis* are known to be associated with antimutagenic activity. Mechanisms of mutagenesis is complex and different in different mutagens and thus antimutagenesis process is also varied. Antioxidant activity of the compounds are hypothesized to be associated with this activities. In addition to this, these compounds are known to possess other bioactivities like, modulation of cytochrome p450 dependent enzyme activity or may interact with reactive electrophiles generated by microsomal proteins or bind with enzymes involved in mutagenesis. *G. glabra* crude extract and components like Glycyrrhizin, 18-α and 18-β glycyrrhetic acid show considerable antimutagenic as well
as desmutagenic activity against ribose-lysine induced mutagenesis (36). Glycyrrhizin, 18-α and 18-β glycyrrhetinic acid do not show any activity against EMS induced toxicity while leaf extract of *G. glabra* show considerable antimutagenic activity against EMS induced toxicity in *S. typhimurium* (36). This activity is postulated to be associated with another compound i.e. glabrene.
Table 2. Protective effect of the ethanol extracts of *Glycyrrhiza glabra* and *Taverniera cuneifolia* against EMS (mutagenic agent) in *Salmonella typhimurium* strain.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Extract (mg/plate)</th>
<th>Number of colonies/plate</th>
<th>Percentage Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (no EMS)</td>
<td>EMS (1 µl/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>288 ± 21.0</td>
<td>64 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>137 ± 11.0</td>
<td>64 ± 9.0</td>
</tr>
<tr>
<td><em>G. glabra</em></td>
<td>4</td>
<td>333 ± 27.0</td>
<td>104 ± 14.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>299 ± 25.0</td>
<td>116 ± 18.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>240 ± 24.0</td>
<td>233 ± 22.0</td>
</tr>
<tr>
<td><em>T. cuneifolia</em></td>
<td>2</td>
<td>242 ± 21.0</td>
<td>125 ± 14.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>272 ± 26.0</td>
<td>163 ± 17.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>300 ± 29.0</td>
<td>224 ± 21.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>312 ± 32.0</td>
<td>159 ± 15.0</td>
</tr>
</tbody>
</table>

± - Indicates standard deviation
Figure 2. Effect of *Taverniera cuneifolia* and *Glycyrrhiza glabra* extracts on EMS induced toxicity in *Salmonella typhimurium*.
Candida albicans is an important pathogen of humans, causing serious health hazards in immunocompromised patient's (65). It is a dimorphic fungus capable of switching to various morphologies (65). Germ tube induction leads to the formation of hyphae, which is one of the important factors, involved in the pathogenicity of Candida albicans (66). It helps in adherence and colonization of tissues. Inhibition of form switching could be a novel strategy in the treatment of invasive candidaisis (65). The drugs available in the market targets and arrests the fungus growth but emergence of drug resistance among the Candida albicans isolates and severe side effects of these drugs has limited their use and necessitated the search for novel and less toxic antifungal agents from natural sources. In the present study, we have tested the efficacy of crude extracts of G.glabra and T.cuneifolia on serum induced germ tube induction in Candida albicans. It was found that T. cuneifolia extract inhibited serum induced germ tube formation in C. albicans considerably i.e. 85 % (Table 3), while G. glabra extracts showed only 3% inhibition (Table 3). Effect of both the plant extracts on the growth of C.albicans tested separately, showed that both of the plant extracts does not affect the C.albicans growth upto 10 mg/ml concentration. Compounds present in the T.cuneifolia extract might be modifying response of C.albicans to the serum and thus affecting the germ tube formation. On the other hand, G.glabra did not inhibit C.albicans growth while very slight inhibition of serum induced germ tube formation was observed (3%) (Table 3).
Table 3. Effect of crude extract of *Glycyrrhiza glabra* and *Taverniera cuneifolia* on germ tube formation by *Candida albicans*

<table>
<thead>
<tr>
<th>Extract (1 ug/ml)</th>
<th>% of GT Induction Germ Tube</th>
<th>% Inhibition of Germ Tube formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No extract</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td><em>G.glabra</em></td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td><em>T.cuneifolia</em></td>
<td>15</td>
<td>87</td>
</tr>
</tbody>
</table>
Figure 3. Effect of *Taverniera cuneifolia* and *Glycyrrhiza glabra* extracts on serum induced germ tube induction in *Candida albicans*.
IV. Inhibition of Fruit Juice Browning

It was found that both the plant extracts inhibited Polyphenol oxidase (PPO) mediated browning of apple juice considerably. Crude extracts of *G. glabra* and *T. cuneifolia* were effective (Figure 4). The chloroform and ethanol fraction of *T. cuneifolia* inhibited browning of apple juice (Figure 4). In addition to this a coumarins, 7-hydroxy coumarin and 7-methoxy coumarin present in *G. glabra* was also found effective (Table 4). Polyphenol oxidase converts mono or dihydroxy phenol into quinines. These quinones are very reactive and further polymerize with other biomolecules like protein or amino acids and form complex compounds. This conversion of hydroxy phenols into quinines thus results in the spoilage of many food products. Control of enzyme mediated browning by using inhibitors of PPO is one of the strategies in food preservation. Many compounds like ascorbic acid and other plant products found effective in this regard.
Table 4. Inhibition of browning in Apple juice by *Glycyrrhiza glabra* and *Taverniera cuneifolia* extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Conc. (mg/ml)</th>
<th>O.D. at 420 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>G.g. (70% alc)</td>
<td>con</td>
<td>1.539</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>1.040</td>
</tr>
<tr>
<td>T.c. (70% alc)</td>
<td>con</td>
<td>1.469</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>1.248</td>
</tr>
<tr>
<td>T.c. (Chloroform)</td>
<td>con</td>
<td>1.536</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>1.262</td>
</tr>
<tr>
<td>T.c. (Ethanol)</td>
<td>con</td>
<td>1.651</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>1.364</td>
</tr>
<tr>
<td>7-Methoxy Coumarin</td>
<td>con</td>
<td>1.542</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.050</td>
</tr>
<tr>
<td>7-Hydroxy Coumarin</td>
<td>con</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.047</td>
</tr>
</tbody>
</table>
A

-♦- control
--•— crude extract of T.c. (1 mg/ml)

B

-♦- control
--•— crude extract of T.c. (1 mg/ml)
Figure 4. Inhibition of Browning in apple juice by extracts (1mg/ml) of G. glabra and T. cuneifolia.

A- Inhibition browning in Apple Juice by 70% ethanol (Crude) extract of G. glabra.
B- Inhibition browning in Apple Juice by 70% ethanol (Crude) extract of T. cuneifolia
C- Inhibition Browning in apple juice by Ethanol fraction of T. cuneifolia extract.
D- Inhibition Browning in apple juice by Chloroform fraction of T. cuneifolia extract.
REFERENCES:


