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Research Article

ANTIMICROBIAL EFFECTS OF MEDICINAL PLANTS AND THEIR COMPARATIVE CYTOTOXIC EFFECTS ON HEPG2 CELL LINE

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ABSTRACT

Objective: To evaluate the antimicrobial property of few medicinally important plants and vegetables and also to evaluate their cytotoxicity on HepG2 cell line and normal lymphocytes.

Methods: Crude extracts were prepared from selected plant parts using water as the solvent following standard protocols. Antimicrobial activity was assessed using agar well diffusion method and MIC was determined using the broth dilution assay. Cytotoxicity of the same extracts on HepG2 cell line and human lymphocytes were assessed using MTT assay and trypan blue dye exclusion methods.

Results: Liquorice, babul, sarpagandha, amla, periwinkle, green tea have exhibited antimicrobial as well as cytotoxic properties. Beetroot and aloe vera have shown antimicrobial property but not cytotocity. For many plants antimicrobial property did not correlate with cytotoxic property. The plants showing cytotoxicity to HepG2 were least toxic to normal lymphocytes.

Conclusion: This study shows that the active principle of plants against microbes are different from the active principles which are cytotoxic to cancer cells.

Keywords: Antimicrobial, Cytotoxic, Amla, Babul, Liquorice, Beet root, HepG2 cell line, Lymphocytes.

INTRODUCTION

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. Genes for resistance to antibiotics, like the antibiotics themselves, are ancient [1]. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in India. According to World Health Organization [2], medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency [3]. Current trends in drug development process are focused on natural sources, especially sources of plant origin due to some proven correlation between the folkloric medicinal uses of some of these plants to biological activity. Hence the use of plant materials to prevent and treat infectious diseases successfully over the years has continued to attract the attention of scientists worldwide [4-7]**.** The aim of the present study was to evaluate the antimicrobial effects of few medicinally important plants and some common vegetables against four strains of bacteria and three fungal species and also to evaluate their cytotoxicity on the liver cancer cell line $HepG_2$ and on normal human lymphocytes.

MATERIALS AND METHODS

Microorganisms tested

The following microorganisms were used in the present study were procured from Microbial Type Culture Collections, Chandigarh, India.

Bacteria

Pseudomonas aeruginosa (MTCC 4727), *Bacillus subtilis* (MTCC 7419), *Staphylococcus aureus* (MTCC 3381) and *Enterobacter aerogenes* (MTCC 7325), *Escherichia coli* (isolated in the laboratory). The bacteria were cultured on Nutrient agar plates at 37ºC.

Fungi

Candida albicans (MTCC 3071), *Aspergillus niger* and *Penicillium* sp. (isolated in the laboratory) and these fungi were grown on Potato Dextrose Agar at room temperature.

Cell lines

HepG2 cell line was procured from National Center for Cell Sciences(NCCS), Pune, India and were grown in complete DMEM medium (2mM L-glutamine, 100 g ml^{-1} of streptomycin, and 100 U ml^{-1} of penicillin) supplemented with 10% fetal bovine serum (HIMEDIA) and maintained in a 5% CO_2 humidified incubator at 37°C. Cells were seeded at a density of 1×10^5 cells/ml, except where otherwise indicated.

Isolation of lymphocytes

Lymphocytes were obtained from the blood of five healthy male and female individuals, about 20 years of age, apparently free from infection by pathogenic agents and had not been under any treatment for the last six months. HiSep medium (HIMEDIA, India) was used for the isolation. Cells were suspended in complete RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (HIMEDIA, India), 5 g ml^{-1} phytohemagglutinin (PHA) and maintained at 37°C in a 5% CO_2 humidified incubator[8]. Lymphocytes were used as control cells to assess the cytotoxicity of plant extracts.

Plant Material

Ginger (*Zingiber officinale),* Amla/Indian gooseberry (*Phyllanthus emblica)*, Beetroot (*Beta vulgaris***)**, Carrot *(Daucus carota),* Periwinkle (*Vinca rosea*), Aloevera *(Aloevera),* Shanger/liquorice (*Glycyrrhiza glabra),* Babul (*Acacia arabica),*Sarpgandha *(Rauvolfia serpentine),* and Henna (*Lawsonia inermis)* were chosen for the study. The air-dried leaves and plant parts were collected and ground into powder using a sterile pestle and mortar. The soluble ingredients in the grounded plant parts were extracted by

solubilization using water as the solvent. 5g of each of the dried plant material were extracted by successive soaking for 2-4 hrs using 100ml of distilled water in a 250ml sterile conical flask. The extracts were filtered using Whatmann filter paper No 1. They were filter sterilized with the help of Millipore Syringe filters of 0.2µm pore size before use. The extracts were stored at 4°C for further use.

Antimicrobial activity

The modified agar well diffusion method of Perez *et al*. [9] was employed. Nutrient agar and Potato dextrose agar were used for bacteria and fungi respectively. Once the agar was solidified, 50µl of the different bacterial and fungal cultures were spread onto the plates using a sterile glass spreader. The plates were punched with six millimeter diameter wells and filled with 25µl of the plants extracts and blanks (distilled water which served as the negative control). Simultaneously, streptomycin (100µg/ml) and nystatin (100µg/ml) were used as positive controls for bacteria and fungi respectively. The tests were carried out in triplicates. The bacterial plates were incubated at 37°C for 24 hrs, and fungal plates at room temperature. The diameter of the zone of inhibition was measured in millimeters at 24 hrs and 120 hrs for bacteria and fungi respectively.

The minimum inhibitory concentration (MIC)

A loop full of bacterial and fungal cultures from the slant was inoculated into nutrient broth and potato dextrose broth respectively and incubated at 37°C for 24hrs for bacteria and at room temperature for 4-5 days for fungi. The fresh broth (20ml) was seeded with 0.25ml of 24hr bacterial broth culture or 4-5 day's fungal broth culture. Then 0.2ml of the extract was added to 1.8ml of seeded broth which was the 1st dilution. 1ml of the solution was diluted further with 1ml of the seeded broth to produce 2nd dilution and the procedure was repeated until six dilutions were obtained. A set of tubes containing only seeded broth were kept as control. After incubation for 24hrs at 37°C for bacteria and after 4-5 days at room temperature for fungi, the last tube with no visible growth of bacteria or fungi was taken to represent MIC of test samples which was expressed in mg/ml. The broth dilution assay was also carried out with streptomycin for bacteria and nystatin for fungi in the same way as the extracts and MIC values of streptomycin and nystatin were determined [10].

Determination of cell concentration and viability by Trypan blue dye exclusion

Lymphocytes treated with different concentrations of plant extracts for 2 days (48 hrs). At the end of treatment period, the cells were counted with the aid of a hemocytometer and cell viability was determined by trypan blue dye exclusion method [11]. Trypan blue was prepared at a concentration of 0.4% in phosphate buffered saline (PBS)

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay was performed to assess the cytotoxicity of the plant extracts. (MTT is a yellow dye, which is reduced into purple formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells). HepG2 cells and lymphocytes were cultured in 96-well microtiter plates and were treated with varying concentrations of different plant extracts for 48 hrs. At the end of treatment period, to each well, 20 µl of MTT was added. After addition of MTT, the plates were incubated for 3 h in a dark chamber. Then, 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 540 nm using ELISA reader [12].

Statistical Methods

All experiments were carried out in triplicates. The results were calculated as mean ± standard error (SE) values. The significance was calculated using one-way analysis of variance (ANOVA) and Student's t-test. A value of $P<0.05$ was taken as statistically significant.

RESULTS

Antimicrobial activity

Among the plants, ginger (*Gingiber officinale*) extract was highly effective and it could inhibit the growth of all three tested fungi and the highest zone of inhibition was against *C. albicans* (6.08mm). But ginger had not inhibited the growth of any of the tested bacteria (Figure 1). Babool has shown highest zone of inhibition against *Penicillium* sp. (11.2 mm). Green tea could inhibit only *A. niger* (2.87mm). Henna (*Lawsonia inermis*) was effective against only *C. albicans* with a zone of inhibition 10.08mm. Liquorice (*Glycyrrhiza glabra)* extract was ineffective against all the tested fungi, but at the same time it was highly active against the growth of all bacteria with highest zone of inhibition against *E. coli* (18.33 mm), and least against *E. aerogenes* with a zone of inhibition of 12.66 mm (Figure 2). Babool was next to liquorice with highest zone of inhibition against *B. subtilis* (17.33mm), followed by *P. aeruginosa* (12.56 mm) and *S. aureus* (12.08mm). Beetroot was able to inhibit all the tested bacteria except *S. aureus*, with highest zone of inhibition against *E. aerogenes* (9.25mm). Amla extract was able to inhibit the growth of *S. aureus* and *P. aeruginosa* with the zones of inhibition, 5.91mm and 7.25 mm respectively. Green tea was inhibiting the growth of only *S. aureus* and *P. aeruginosa.*

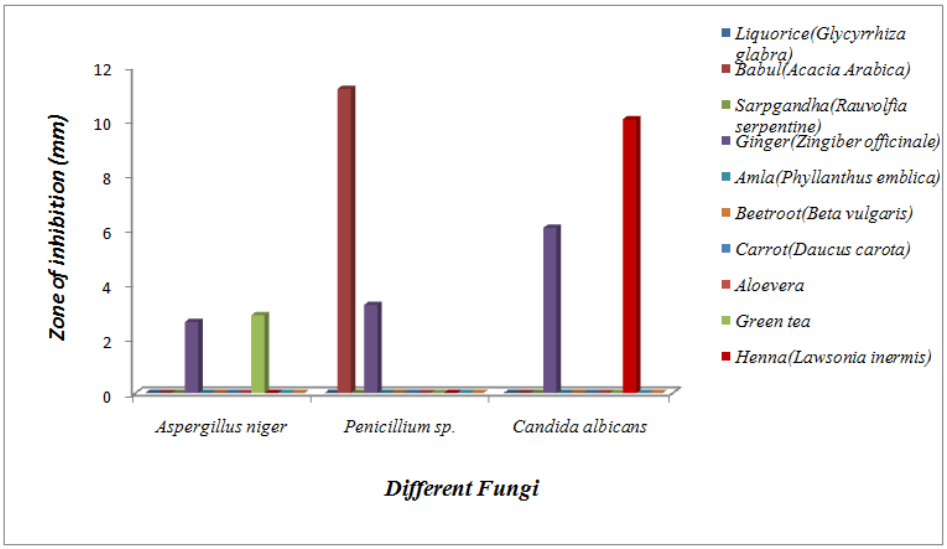

Fig. 1: Antifungal activity of different plant extracts

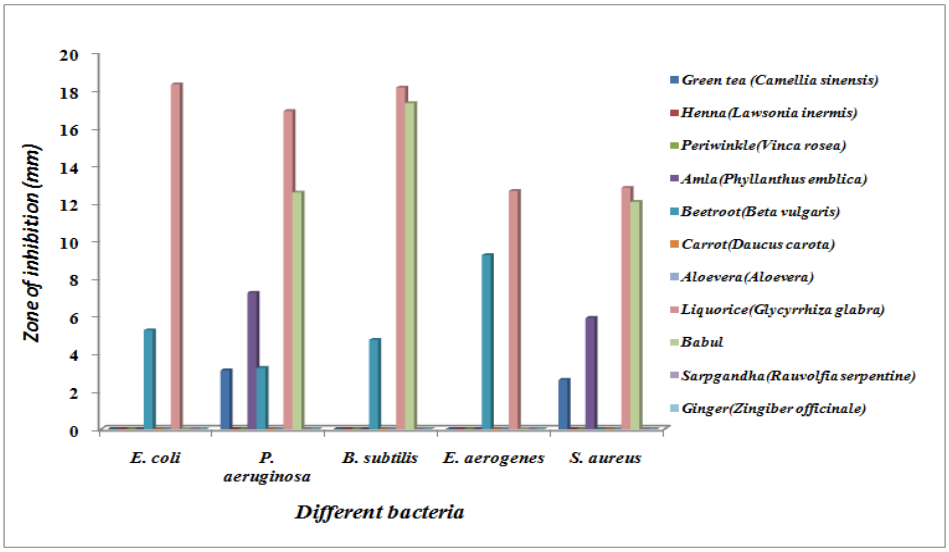

Fig. 2: Antibacterial activity of different plant extracts

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of liquorice was 0.5mg/ml against *E. coli*, *B. subtilis*, *P. aeruginosa* and 5.0 mg/ml against *S. aureus* and *E. aerogenes.* The MIC of babul against *B. subtilis*, *S. aureus* and *P. aeruginosa* was 0.5mg/ml (Table 1). The MIC of beetroot was 5mg/ml against *B. subtilis*, *P. aeruginosa* and *E. coli* and 0.5mg/ml against *E. aerogenes.* The MIC of green tea and amla against *S. aureus* and *P. aeruginosa* were 50 mg/ml and 5mg/ml respectively.

The minimum inhibitory concentration of ginger was 5mg/ml against all the fungi, the MIC of babul (*Acacia nilotica*) was 0.5mg/ml against *Penicillium* and that of green tea was 50mg/ml against *A. niger*. The MIC of henna against *C. albicans* was 0.5mg/ml (Table 2).

Table 1: Minimum Inhibitory Concentration of different plant extracts on bacteria

	Different medicinal Plants					
Organisms	**Liquorice**	**Babul**	**Beetroot**	**Amla**	**Green tea**	**Streptomycin**
E. coli	0.5 mg/ml	-	5.0 mg/ml	-	-	100 µg/ml
B. subtilis	0.5 mg/ml	0.5 mg/ml	5.0 mg/ml	-	-	100µg/ml
E. aerogenes	5.0 mg/ml	-	0.5 mg/ml	-	-	100µg/ml
S .aureus	5.0 mg/ml	0.5 mg/ml	-	5.0 mg/ml	50 mg/ml	100µg/ml
P. aeruginosa	0.5 mg/ml	0.5 mg/ml	5.0 mg/ml	5.0 mg/ml	50 mg/ml	100µg/ml

Table 2: Minimum Inhibitory Concentration of different plant extracts on fungi

	Different medicinal Plants				
Organisms	**Babul**	**Ginger**	**Henna**	**Green tea**	**Nystatin**
C. albicans	-	5.0 mg/ml	0.5 mg/ml	-	100 µg/ml
Penicillium sp.	0.5 mg/ml	5.0 mg/ml	-	-	100µg/ml
A. niger	-	5.0 mg/ml	-	50 mg/ml	100µg/ml

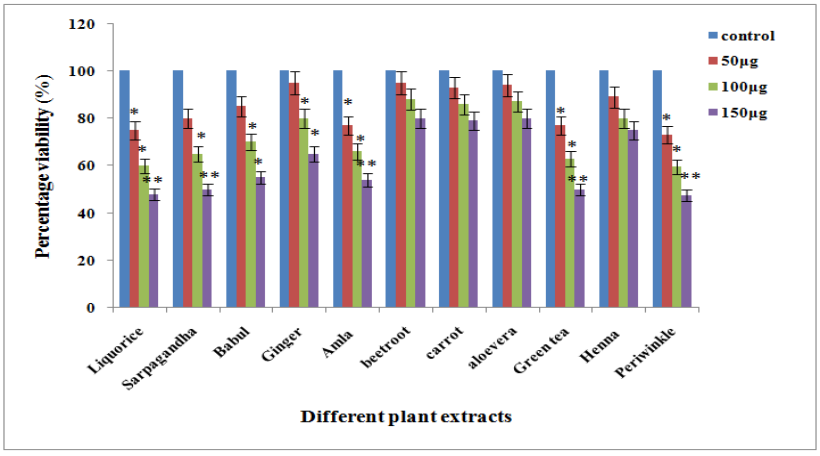

Fig. 3: Effect of the different plant extracts on HepG2 cell line. *p<0.01 compared with control. Two stars indicate high significance, with p<0.0001

MTT assay for cytotoxicity

When the different plant extracts were added at different concentrations (50, 100 and 150 µg/ml) to cultured HepG2 cells, and incubated for 48 hrs, it was observed that, liquorice, sarpagandha, amla, green tea and periwinkle were cytotoxic to the liver cancer cell line at all the tested concentrations (Figure 3). The IC_{50} value for these were found to be <150µg/ml. The cytotoxicity of liquorice was equal to that of periwinkle (*Vinca rosea*), which produces vincristine, the well known anticancer agent. Cell count and percentage viability of HepG2 cells treated with *G. glabra, R. sarpentina*, *P. emblica, C. sinensis* and *V. rosea*, clearly indicate that these plant extracts inhibit the proliferation of HepG2 cells at 50, 100 and 150 µg/ml concentrations (percentage viability decreased as the concentration of extracts increased) when compared to that of the controls.

When the same plant extracts were added to normal lymphocytes and checked after 48hrs for cell viability by MTT assay, it was observed that the percentage viability was nearing to 100 in almost all the treatments (Figure 4) and the differences were not statistically significant ($p>0.05$). Periwinkle and liquorice treatments have reduced the percentage viability of lymphocytes to 90% at 50 and 100µg/ml concentrations.

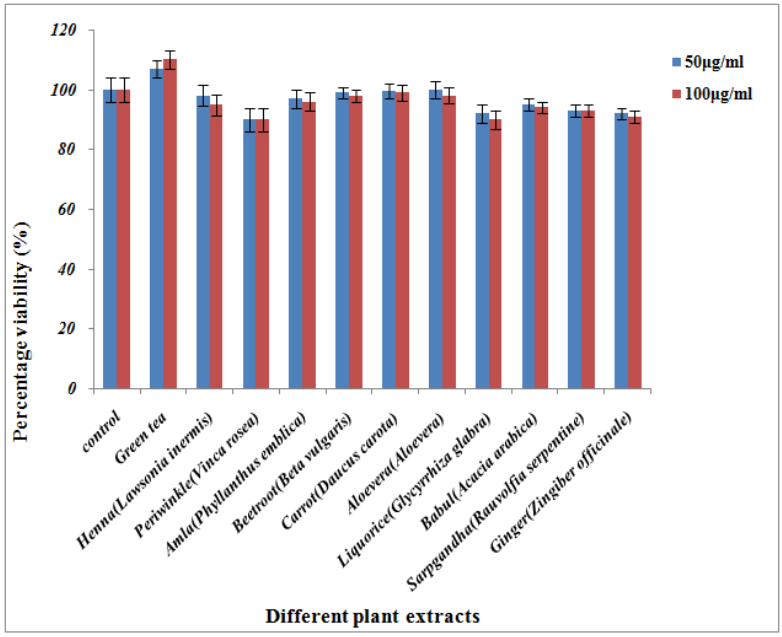

Fig. 4: Effect of the different plant extracts on normal lymphocytes.

These results indicate that the plant extracts which were inhibitory to the proliferation of HepG2 cell line were not cytotoxic to lymphocytes and appear to be quite safe for humans. Further animal studies are required to prove this, which will be taken up in the next phase of our research. When cell concentration was tested by trypan blue dye exclusion method, the same results as shown by MTT assay were observed for both HepG2 cells and lymphocytes (results not shown).

DISCUSSION

Among the tested medicinal plants *G. glabra* has shown potent antibacterial effects against all the tested bacteria with an MIC of 0.5-5.0 mg/ml. Earlier results have also shown that the methanol extract of aerial parts of *G. glabra* has antibacterial activity against several kinds of bacteria [13]. Several flavonoids with C5 aliphatic residues were isolated as the effective constituents of licorice against methicillin-resistant *Staphylococcus aureus* (MRSA) and restored the effects of oxacillin and β- lactam antibiotic against MRSA [14,15]. Chopra *et al*. [16] reported that the methanolic extract of *G. glabra* possess compounds with antimicrobial potentials that can be further explored for antimicrobial activity. In our study, it was found that licorice is inhibiting the growth of only the cancer cells and not the lymphocytes, indicating that it is quite safe for human consumption. In agreement with our results earlier studies also indicated that the aqueous extract of *G. glabra* inhibits the *in vivo* and *in vitro* proliferation of Ehrlich ascites tumor cells and inhibits angiogenesis in *in-vivo* assay, peritoneal and chorioallantoic membrane assays [17]. Also, the ethanol extract of *G. uralensis* root induced apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells [18]. Licorice root has been identified by the National Cancer Institute as possessing cancer-preventive properties [19,20]. It can be concluded that liquorice can be effectively used for the treatment of hepatocellular cancers without much side-effects to the normal body cells.

In our study, ginger had exhibited antifungal activity but not anti bacterial activity. Earlier, Samy [21] reported that when they used methanolic extract of ginger, it did not inhibit *S. aureus* or *E. coli*. However, Indu *et al.,* [22] using a different extract preparation of ginger, reported that it inhibited the growth of *E. coli*. It appears that the antimicrobial activity and cytotoxic properties of plant extracts vary depending upon the solvent of extraction as well as the methods of extraction.

Babul was next only to *G. glabra* to have antibacterial effects against *B. subtilis*, *P. aeruginosa* and *S. aureus*. Both *G. glabra* and *A. arabica* have shown cytotoxic effects on HepG2 cells but not to lymphocytes. Their antibacterial effects correlated with their cytotoxic effects. Beetroot has exhibited antibacterial effects but no cytotoxicity to HepG2 cells. So there was no correlation between antimicrobial and cytotoxic effects. In the case of *Vinca rosea* we could not observe any antimicrobial effects against the tested microbes but it had potent cytototoxicity to HepG2 cells. Again, no correlation existed between these two activities. Amla has antimicrobial effects on certain bacteria and also had cytotoxic effects on HepG2 cells. In case of *Aloe vera*, henna and carrot no antimicrobial effects were observed and cytotoxic effects to HepG2 cells were also not observed. So there was strong correlation between their antimicrobial and cytotoxic effects. Here we can say that the active principles of the plants which show antimicrobial effects could be different from the active components of the same plants which exhibit cytotoxic effects against the hepatocellular cancer cell line, HepG2. We can conclude that the medicinal plants which have potent anti microbial effects are not

always exhibiting anticancer properties. The plants which show both antimicrobial and cytotoxic properties may have different active components which are responsible for these two different activities.

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