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Evaluation of antimicrobial and cytotoxic activities of the extracts of *Capsicum annuum* and *Zinziber officinale*

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Abstract: *The antibacterial and cytotoxic activities of methanol extract and Ethyl acetate extract of Capsicum annuum and Zinziber officinale was investigated. Antibacterial activity was assayed against Gram positive and Gram negative bacteria using the disc diffusion method and Minimum Inhibitory Concentration (MIC) were also evaluated. Cytotoxicity was measured against , HeLa, a human cervical carcinoma cell line and to compare to the normal Vero a kidney epithelial cells extracted from an African green monkey cell line using the Cell Counting Kit-8 (CCK-8), a Non-Radioactive colorimetric Cell Proliferation and Cytotoxic Assay. The strongest cytotoxic activity against the Hela cell line was detected for methanolic extracts of Z. officinale at IC50 = 85 µg/ml and no cytotoxicity was observed on Vero cell line. The best incubation time for this extract was 48 h. Most of the tested extracts were active against the tested bacterial strains. The methanol extract of Z. officinale was found to be the most active against all the tested bacteria. The MIC of the extract of Z. officinale of the tested bacteria ranged between 31.2 to 125 µg/ml. The preliminary screening of antibacterial and cytotoxic efficacy study of C. annuum and Z. officinale indicates the medicinal importance of these plants in control of pathogenic bacteria. This study has also shown that the methanol extract of Z. officinale have potent anticancer activities in vitro and consequently, could potentially be a source for a pharmacologically active products suitable for development of novel anticancer agents.*

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I. Introduction

Medicinal plants have been being used from ancient times and their utility has been increasing day by day. For a long time, plants have been a vital source of natural products for human wellbeing. Treatment of infectious diseases is becoming difficult due to the development of multiple drug resistance bacteria[1]. Thus, it is the high time to search for newer antibiotic sources be a continuous process. Plants are one of the most important sources in the search for novel and alternative antimicrobials[2,3]. Plants have evolved a various kind of chemical strategies to defense against pathogens as a result phytochemicals are good source of antimicrobial compounds. These phytochemicals are called secondary metabolites, e.g. tannins, alkaloids and flavonoids, these are found to have antimicrobial properties *in vitro*. Different types of phytochemicals may act as antioxidants, antimicrobial, anti-inflammatory and anti-cancer agents, which making the use of plants more beneficial[4,5].

Research on different herbal plants have shown the anti-proliferative and cytotoxic activities of various phytochemicals against different cancer cells. Cancer has always been a major threat to mankind and has been characterized by proliferation of abnormal cells. Various treatments of cancer such as chemotherapy and surgery have limited effectiveness, could create adverse side effects and resistant to tumor cells[6]. Chemotherapy treatment is designed to target rapidly dividing cells. Unfortunately, it may not distinguish between cancer cells and other rapidly dividing cells. The body's immune system cells are equally rapidly dividing and may become the target of chemotherapy. Investigations for finding new plant based antimicrobial and anticancer compounds are essential[7].

Naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the crisis of drug resistance found in synthetic drugs is also reduced[8]. Spices and herbs have been used for thousands of years by many cultures to improve the taste and flavor of food. Microbial inhibitory activities of spices and their derivatives have been investigated by many researchers[9,10]. Studies have shown that both Gram positive and negative food borne and water borne bacteria are inhibited by garlic, onion, cinnamon, cloves, thyme and sage[11].

Capsicum annuum (chili pepper) and *Zinziber officinale* (ginger) are widely used as spice. *C. annuum* is a member of Solanaceae family and the family of *Z. officinale* is Zingiberaceae[12,13]. The underground rhizome of the gingeris used for cooking as savory ingredients. On the other hand fruits and seeds of chili peppers are widely used in many cuisine for spicy taste. Research has shown that these plants has activity against food borne pathogenic bacteria[14,15,16].

The aims of the research work was to evaluate the antimicrobial activity of *C. annuum* and *Z. officinale* extracts against Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* and *Salmonella typhi*) and to determine the cytotoxicity of *C. annuum* and *Z. officinale* extracts on HeLa, a human cervical carcinoma cell line and Vero cell line, a kidney epithelial cells extracted from an African green monkey.

II. Material And Methods

Plant Materials

Fruits of *C.annuum* and *Z. officinale* rhizomes were purchased from Dhaka New Market. The collected plant material was healthy and free from any deformities. The plant materials were thoroughly washed under tap water, dried in the shed and then ground into coarse powder with the help of mortar and pestle. These powders were stored in airtight bottles at room temperature until needed for future use.

Preparation of plant extracts:

10 g of each of the samples of dried plant material was extracted extensively in 50 ml methanol (Ajax, AR grade) and ethyl acetate for 72 hours at room temperature with gentle shaking. The extract was filtered through filter paper (Whatman No. 1) and concentrated at reduced pressure using a rotary evaporator at 40 °C. The resultant pellet was then stored at 4°C in air tight screw-cap tube.

Extract dissolution

For the dissolution of extract pellets were dissolved in 2.5% dimethyl sulfoxide (DMSO) and filter-sterilized through a 0.45-µm Millipore filter. They were then diluted to the desired concentrations with culture medium for the cytotoxicity tests and with distilled water for the antibacterial assays.

Microorganisms used and growth conditions:

Bacterial strains, including both Gram-positive and Gram-negative obtained from Food Analysis and Research Laboratory, Centre for Advanced Research in Sciences, University of Dhaka. The bacterial strains include *Enterococcus faecalis, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* have been selected for the present study. The pure cultures of bacteria was maintained on Tryptone Soy Agar (TSA) and stored at 4ºC until required for study. The TSB (Tryptone Soy broth) and the Mueller-Hinton agar were used, respectively, for growing and diluting the microorganism suspensions and for the antibacterial assays.

Antibacterial assay

The antibacterial sensitivity was carried out by the disc diffusion method[17]. The microtiter-plate was used to determine the minimum inhibitory concentration (MIC) according to the guidelines recommended by the NCCLS and CLSI with modification[18,19]. For the antibacterial activity test, the bacteria were aerobically cultured in TSB at 37 °C for 24 h, and then suspended in sterile saline at a density equivalent to that of the 0.5 McFarland standard. Bacterial suspensions with a concentration of 10^5cfu/ml were used for in vitro antibacterial activity test.

Disc diffusion

The antimicrobial activities of each extract were investigated by disc diffusion method, Sterile paper discs (6 mm in diameter) were soaked separately with 30 µl of plant extracts at a concentration of 200 mg/ml and then dried (6 mg dried extract/disc). These discs were placed on Mueller-Hinton agar plates, previously swabbed with the test organisms at a concentration of 10^5 CFU/ml. Filter paper discs containing DMSO without any test compounds served as a control and no inhibition was observed. The plates were then incubated at 37°C for 24 hours. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was measured and recorded. Each assay was performed in triplicate and repeated three times. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the discs. The results were evaluated by measuring the areas with no bacterial growth.

Microdilution test

MIC

Minimum inhibitory concentrations (MIC) of each extracts were determined by a broth microdilution assay using sterile 96-well micro titer plates filled with a 150 µl aliquot of Mueller-Hinton broth. To the first column of wells 150 µl of the original extracts were added and mixed with the broth in the wells using a pipette tip. After mixing, 150 µl of the mixture was transferred to the next well in each row and this process was continued making 2 fold dilutions until column #8. The final concentrations of the plant extracts ranged from 0.4 µg/ml to 250 µg/ml. Each well was inoculated with 10 µl of inoculum (10^5 CFU/ml) and incubated at 37°C

for 18h. Only medium was served as negative control and bacterial growth without any extract was considered as positive control.

Absorbance reading at 650 nm wavelength for each plate was measured after incubation at 37°C for 18 hours. Bacterial cell viability and minimum inhibitory concentration (MIC) values were determined by observing the turbidity and the absorbance reading of the suspension post-incubation. The lowest concentrations of plant extracts with clear suspensions were considered as the MIC values.

MBC

The minimum bactericidal concentration (MBC) of each extract was determined by withdrawing 20 µl of the bacterial broth suspension and then spreading it on TSA plates, which were then incubated at 37°C for 24-48 h. The lowest concentration of the extract at which no bacterial growth was observed, was considered as the MBC.

Cytotoxicity assay

Animal Cell line and culture medium:

The HeLa and Vero cell lines were maintained in DMEM (Dulbecco's Modified Eagles' medium) containing 1% penicillin- streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine Serum (FBS). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were subcultured after a monolayer formed on the flask. The cells were detached by using trypsin and then by adding complete medium to inhibit the reaction.

Cell treatment and cytotoxic assay:

Cytotoxicity was examined by using Cell Counting Kit-8 (CCK-8), a Non-Radioactive colorimetric Cell Proliferation and Cytotoxic Assay kit (Sigma-Aldrich, USA). Cell Counting Kit-8 (CCK-8) allows very convenient assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron mediator. CCK-8, being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells[20]. Manufacturers instruction was followed to perform the assay. HeLa Cells (2×10^4/100 µl) and Vero cells (1.5×10^4/100 µl) were seeded onto 96-well plate and incubated at 37°C+5% $CO_2$for 24 h. The cells were then treated with various concentrations (25, 50, 100 and 200 µg/ml) of methanol extracts of *C. annuum* and *Z. officinale*. 2.5% DMSO was served as control. Cytotoxicity was examined under an inverted light microscope after 48h of incubation. Then, CCK-8 reagent (10 µl) was added to each well and incubated for three hours. After the incubation the absorbance was read at 450 nm using an ELISA microplate reader (EPOCH, BioTek, USA). The results were expressed as the percent optical density of treated cells to that of the control cells. The assay was performed in duplicate to calculate an IC_{50} of the cell population for each extracts. According to the following equation IC_{50} value were calculated.

Viability (%) = (optical density of sample/optical density of control) x 100

III. Result

Antibacterial assay

The in vitro antibacterial potential of the three selected medicinal plants was evaluated against both Gram positive and negative bacteria using disc diffusion method to find out the zone of inhibition and microdilution method to determine their MIC and MBC values.

Disc diffusion method

The antibacterial activities of extracts were evaluated by the diameter in mm of the inhibition zone around the disk; these diameters are reported in Table no 1. For the interpretation of antibacterial assay results, the following scale of measurement were adopted: zone of inhibition of >15 mm as strongly inhibitory, 10-15 mm as moderately inhibitory, and <10 mm as not inhibitory, according to Carović-Stanko et al. (2010)[21].Thus many of tested extracts were found to be active against different bacteria.

The disc diffusion method was initially performed to determine the antibacterial activities of the two medicinal plants against Gram positive and negative bacteria. The methanol extract of *C. annuum* showed inhibition zone of 15.5±0.40 mm, 10.5±0.40 mm and 12.1±0.47 mm and ethyl acetate extract of the same plants showed inhibition zone of 14.16±0.23 mm, 11.83±0.62 mm and 10.0±0.40mm against *S. aureus*,*E. coli* and *S. typhi* respectively. The extracts of *Z. officinale* rhizome showed inhibitory effect against all the tested bacteria.

The methanol extract of *Z. officinale* exhibited maximum inhibition zone on *S. typhi* followed by *Enterococcus faecalis, S. aureus* and *E. coli.* The DMSO and sterile water used as negative controls showed no inhibitory effect.

Z. officinale were seen most effective, with at least causing 15 to 28 mm in diameter sizes of zone of inhibition against any bacterium. The extract of *Z. officinale* showed the highest value of inhibition zone against *S. typhi* (28 mm). Methanolic extracts showed strongest inhibition against both Gram positive and negative bacteria than ethyl acetate extract of plants.

Table 1: Antibacterial activity of selected plant extracts determined by disc diffusion method.

Plants	Solvent	Inhibition zone (mm)			
		Gram +ve bacteria		Gram -ve bacteria	
		Enterococcus faecalis	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhi*
Capsicum annuum	Methanol	-	15.5±0.40	10.5±0.40	12.1±0.47
	Ethyl acetate	-	14.16±0.23	11.83±0.62	10.0±0.40
Zinziber officinale	Methanol	27.5±0.40	22.5±0.40	15±0	28.16±0.23
	Ethyl acetate	25.5±0.40	15.16±0.23	10.83±0.62	22.66±0.84

"-" indicates no zone of inhibition.

MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) activity of methanol extracts of *C. annuum* and *Z. officinale* are presented in Table no 2. The MIC values of the extracts ranged from 31.2 µg/ml to 250 µg/ml. The extract of *Z. officinale* revealed a lowest MIC value 31.2 µg/ml and MBC value 62.5 µg/ml against *S. aureus*. The MIC value of the same extracts was 62.5 µg/ml against both *E. faecalis* and *S. typhi* and MBC value were 125 µg/ml and 250 µg/ml respectively. In case of *E. coli* the MIC value was 125 µg/ml for the extract of *Z. officinale* and 250 µg/ml for the extract of *C. annuum.* Against *E. coli* the MIC was not detected up to 250 µg/ml. The MBC value of *C. annuum* was not detected at up to 250 µg/ml concentrations.

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the tested plant extracts determined by microdilution method.

Plants	Gram +ve bacteria				Gram -ve bacteria			
	Enterococcus faecalis		*Staphylococcus aureus*		*Escherichia coli*		*Salmonella typhi*	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Capsicum annuum	-	-	250	-	250	-	-	-
Zinziber officinale	62.5	125	31.2	62.5	125	250	62.5	250

"-" indicates MIC and MBC not detected at up to 250 (µg/ml)

Cytotoxic assay

In this study methanol extract of *C. annuum* and *Z. officinale* were studied. Results of the cytotoxicity of extracts on HeLa and Vero cell lines are demonstrated with both CCK-8 assay and morphological appearances of the cultures under microscope after 48 h of post treatment of the extract (Fig. 1). *Z. officinale* only showed cytotoxic activity against HeLa cell line and no cytotoxicity was observed against Vero cell line. *C. annuum*, showed cytotoxic activity against both cell lines. The IC_{50} values for HeLa cell line were 146 µg/ml and 85 µg/ml of the extract of *C. annuum and Z. officinale* respectively. For Vero cell line IC_{50} values were more than 200 µg/ml for *C. annuum.*

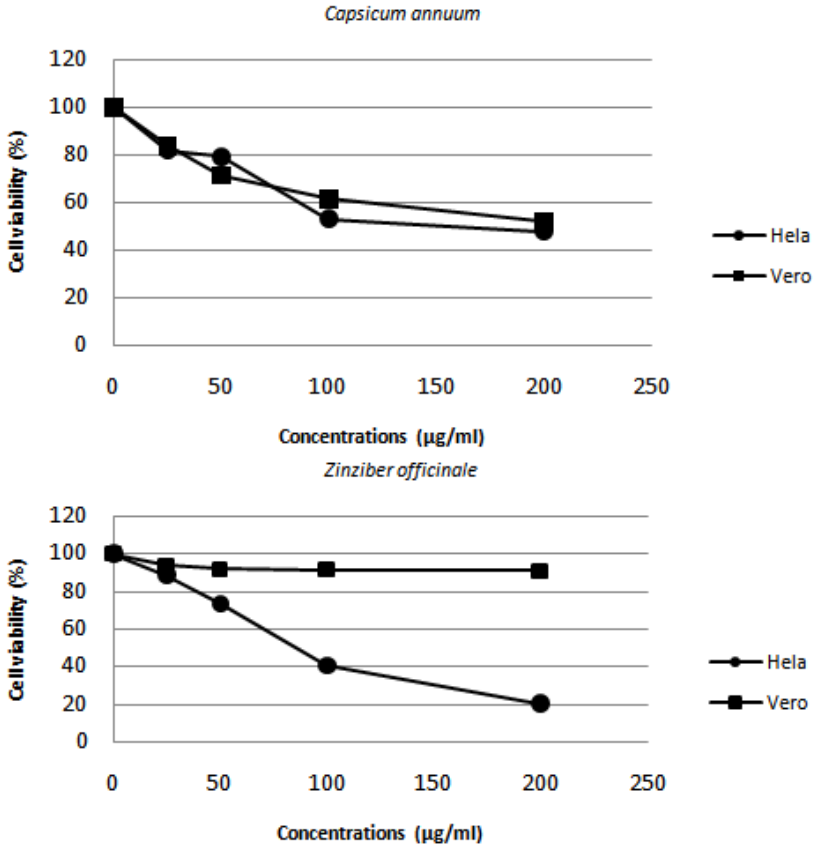

Fig. 1 Cytotoxic activity of methanol extract of *C. annuum* and *Z. officinale* extracts on HeLa and Vero cell line determined by CCK-8 cell proliferation and cytotoxicity assay.

IV. Discussion

Natural products which are obtained from medicinal plants are chemically diversified, for this reason, these have been used as bases for discovering new drugs. The search for therapeutic drugs obtained from herbal plants, particularly edible plants, has grown throughout the world[22,23]. In this study, the antibacterial and cytotoxicity properties of two important medicinal plants were investigated.

The methanol extracts were the most active as compared to the ethyl acetate extracts. These findings are similar to those of other authors[24] who reported that the methanol extract of *Z. officinale* has moderate antibacterial activity against *S. aureus* and *E. coli* while the ethyl acetate extract was less effective against these bacteria.

The methanol extract of *Z. officinale* showed the broadest antibacterial activity by inhibiting growth of both Gram positive and negative bacteria tested (the diameter of inhibition zone,10-28 mm). These results agree with observations of several workers[25,26,27,28]. They also showed that crude extract of *Z. officinale* inhibit the growth of certain Gram positive and negative bacteria. These results are contradictory to the observations of Indue et al.[29], who had reported that the *Z. officinale* extracts did not show any antibacterial activity *E.coli* and *Salmonella* sp.

In case of the metanol and ethyl acetate extract of *C. annuum*, all the tested bacteria were inhibited except *E. faecalis.* Previous studies revealed that *C. annuum* var. *glabriusculum* have strong antimicrobial activity against *Vibrio cholera*[30]. It is also reported that the aqueous extract from fresh *Capsicum* species showed varying degree of inhibition against *Bacillus, Salmonella, Streptococcus* and *Clostridium* spp[31]. Keskin et al.[32] reported that *C. annuum* methanol extracts weren't susceptible to other bacteria except *P. aeruginosa*. According to Molina -Torres et al.[33] MIC for *E. coli* was 300 µg/ml, in this study 250 µg/ml MIC values were required. Rose Koffi-Nevry, et al.[12] reported that flavanoids and other active components like capsaicin have potential antibacterial activity as it is major compound from *Capsicum* species. Based on previous studies it can be assumed that in the present study, the flavanoids or capsaicin might be act as antibacterial compound of capsicum extract against *S. aureus, E. coli* and *S. typhi*.

Cervical cancer is the fourth most common cancer in women worldwide[34]. Bray et al (2018)[35] reported that approximately 90% of deaths from cervical cancer occurred in low and middle income countries. The chemotherapeutic drugs affected the cancer cells as well as normal cells also affected. For this reason, use of natural compounds extracted from herbs and medicinal plants has become a global trend.

In this research, methanol extract of all plant materials were tested for their cytotoxic activity using the CCK-8 colorimetric assay. The plant extracts were prepared and examined for their *in vitro* cytotoxic effect against HeLa and Vero cells. In screening result, *Z. officinale* has shown lowest IC_{50} (85 µg/ml) on HeLa cell line than *C. annuum* extracts.

Previous studies showed that *Z. officinale* and its active ingredients inhibit growth and induce apoptosis in a variety of cancer types, including skin, ovary, colon, breast, cervix, mouth, kidney, prostate, stomach, Pancreas, liver and brain cancer[36,37]. Based on the results obtained from the CCK-8 assay, the prepared extract showed cytotoxic activity against cervix cancer cells. But does not show any activity against normal Vero cell line.

Nalbantosy et al.[28] [22] reported that the ethanol extract of *Z. officinale* exhibited cytotoxic effect on HeLa cancer cells with IC50 values of 33.78 µg/ml for 48 h treatment.

V. Conclusion

The preliminary screening of antibacterial and cytotoxic efficacy study of *C. annuum* and *Z. officinale* indicates the medicinal importance of these plants in control of pathogenic bacteria. This study has also shown that *Z. officinale* have potent anticancer activities *in vitro* and consequently, could potentially be a source for a pharmacologically active products suitable for development of novel anticancer agents. Further phytochemical studies are necessary to provide relevant information for development of these plants as potential effective treatments against bacterial infection and diseases. These plant extracts should be searched in detail to find the compounds responsible for the anticancer activity by isolation and purification studies in the future.

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