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Original Article

Antimicrobial, Antiproliferative and Antioxidant Activities of Eethanolic, Ethyl Acetate and Petroleum Ether Extracts of PsidiumGuajava Leaves

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Abstract

Background: To determine the antimicrobial, anti-proliferative and antioxidant activities of ethanolic, ethyl acetate and petroleum ether extracts of Psidiumguajava leaves.

Methods: The antimicrobial activity was assessed using disc diffusion method on Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli and Salmonella typhi. The anti-proliferative activity was determined on HeLa cell lines. The anti-proliferative activity was assessed using MTT assay while antioxidant activity was determined using catalase and superoxide dismutase (SOD) assays.

Results: The MTT results indicate that ethyl acetate and petroleum ether extracts have significantly inhibited the proliferation of HeLa cells as compared to the control. Antioxidant assays showed that catalase activity was insignificant for all extracts however the SOD activity was significant. All the extracts showed inhibitory zones against B. cereus while only ethanolic extract of P. guajava leaves was active against S. aureus.

Conclusion: Psidiumguajava plant leaves extracts can be used as antiproliferative agent against HeLa cell line.

Keywords: Psidiumguajava leaves, antioxidant, antiproliferative

Introduction

Herbal medicine usage is the most established manifestation of health awareness known to human beings. The medicinal use of plants is followed in different countries as they are the source of efficient drugs. More than half of all current clinical medications contain natural products derived from plants. These products are of utmost importance in medication advancement in the pharmaceutical industries.[1]According to World Health Organization (WHO) primary health care of more than 80% of the world population is fulfilled by the traditional medicine.[2]

Psidiumguajava commonly known as guava belongs to family Myrtaceae. It is native of Central America but due to its adaptability to a diversity of soils, it is now grown all over the world. This plant is widely used in treating different conditions and is of utmost importance for many indigenous systems of medicine.[3]

The plant parts are rich in tannins, phenol, triterpenoids, minerals, vitamins, alkaloids and flavonoids.[4-6] Different plant parts have been used to treat different conditions like malaria, food poisoning, emesis, dysentery, wounds, ulcers and a number of other conditions.[7-9] Many researchers are interested in finding anticancer drugs. Different studies have been conducted to find anticancer medications from plants.[10] Guava extracts have also been reported for antioxidant activities.[11,12]

Materials and Methods

The study was done at Microbiology laboratory and tissue culture laboratory of Centre for Research in Molecular Medicine (CRiMM) at The University of Lahore.For the preparation of plant material leaves were separated and washed with tap water. They were cut into smaller pieces and air dried for 4 weeks. They were crushed using electric blender and reduced to fine powder. They were stored in zipper plastic bags.[13]The solvents used for study were ethanol, ethyl acetate and petroleum ether.

Extracts were prepared using maceration method.[9]The quantity 100 gm of leaves powder was mixed in 200 ml of each solvent. They were kept in air tight bottles for 1 week. The bottles were routinely shaken during this time. The extract solutions were than filtered with filter paper Whatman no. 1 and filtrate was collected. The filtrate was concentrated under rotary evaporator

until powdery extracts were obtained. The extracts were weighed and kept at 4°C until further use.[14]

For the preparation of standard concentration of leaves extracts 100mg of each extract was weighed and dissolved in 5ml of Di-Methyl Sulfoxide (DMSO). Thus 20mg/ml of stock was obtained as a standard concentration of ethanolic, ethyl acetate and petroleum ether extracts. The samples were labeled and stored in refrigerator.[15]

Microorganisms and preparation of culture media included ATCC standard bacterial strains tested include Staphylococcus aureus (29213), E. coli (25922), Salmonella typhi (14028), Bacillus cereus (11778), Pseudomonas aeruginosa (27853) and Klebsiella pneumoniae (10031). The medium used for the detection of sensitivity was Mueller Hinton agar (MHA). The sterility of agar plates was verified after overnight incubation at 37°C.[14] The Kirby Bauer disc diffusion method was employed to determine the antibacterial activity of P. guajava leaves extracts. Petri dishes of Mueller Hinton Agar were inoculated with test microorganisms matched with 0.5 McFarland standard solution. The filter discs were prepared using punch machine. The filter paper Whatman no 1 was used which was punched and holes were made 6 mm in diameter. The filter paper discs were placed in Petri dish and were sterilized using Hot air oven.[16] Subsequently, sterile filter discs impregnated with 100 µg of each extract were placed and plates were incubated at 37°C for 24 h. DMSO was used as negative control. Amikacin, cefotaxime, ampicillin, vancomycin, gentamicin, nitrofurantoin and erythromycin were used as the positive control. Inhibition of bacterial growth was determined by the presence and size of clear zone around the discs. These inhibition zones were interpreted in millimeter according to the standard of the antibiotic sensitivity test manual.[17]The growth inhibition diameter was an average of three measurements.

For the preparation of working solution the stock solutions were further diluted to a concentration of 1mg/ml in Phosphate buffered saline (PBS) solution. These solutions were filtrated via syringe filter of 0.22 µm size and stored in sterile falcon tubes. Paraffin tape was applied at the caps. They were stored in refrigerator until further required. These solutions were labeled as pre-treatment solutions.

For culture of cell line the ATCC standard HeLa cell line CCL-2 maintained in DMEM solution supplemented with fetal bovine serum, streptomycin (100 mg/ml) and penicillin (100 IU/ml) was incubated in CO2 incubator at 37° C. Cells were seeded at $1x10^5$ cells/ml adjusting cell concentration daily by addition of fresh medium.[18]The treatment of cell line was processed under laminar flow. The HeLa cancer cells ($2x10^4$ cells/well) were seeded into 96 well micro titer plate. The plate was incubated in CO_2 incubator. After 24 hours of incubation the cells were observed for adherence. Medium was decanted and 200 µl of pre-treatment plant extracts were added. The culture plate was incubated in CO_2 incubator for 24 hours. Each plant extract was then harvested in eppendorf tubes. This was labeled as post-treatment solutions. Both pre-treatment and post-treatment solutions were used to determine the antioxidant activity through analysis of SOD and catalase. The treatment of cell line was performed in triplicate.[18]

Determination of cell survival was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.Cell viability was checked using MTT assay. The MTT is converted into formazan which is a colored product and serves as useful indicator for checking cell viability.25 µl of MTT reagent was added to each well containing treated cell line. The plate was incubated at 37°C in CO_2 incubator for 2 hours. After 2 hours plate was taken out of incubator and 100µl of 10 % Sodium dodecyl sulphate (SDS) was added to respective wells. The plate was again incubated in CO_2 incubator at 37°C for 2 hours. After 2 hours of incubation period, absorbance of 96 wells plate was read at 570 nm. The number of viable cells in each well was found proportional to intensity of the absorbance of light.[19]

For antioxidant assays the 96 well plate was used to test the catalase and superoxide dismutase activity. The catalase activity was determined by the method of Beers and Sizer. 12.5 µl of 1M phosphate buffer solution was added in each well of 5^{th} to 8^{th} row of 96 well plate. 31.25 µl of hydrogen peroxide (100mM) was now poured in respective wells. From these four rows two were chosen to test pre-treatment solutions activity and other two were chosen for post-treatment activity. In pre-treatment rows 206.25 µl of pre-treatment plant extracts were added while the same quantity of post-treatment plant extracts were added in post-treatment rows. After one minute the absorbance was determined at 240 nm. One unit of CAT was defined, as the amount of enzyme required for decomposing 1µmol of peroxide per min, at 25° C and pH 7.0.[20] Superoxide dismutase (SOD) assay was performed according to the method described by Winterbourn. The first four rows from row 1 till row 4 were chosen to test SOD activity. The rows were labeled as pre-treatment and post-treatment for both

plants. First of all 25 µl of 1 M potassium phosphate buffer (KH_2PO_4) was added in each well of first four rows. Now 56.5 µl of 10 mMNitroblue tetrazolium (NBT) was added in respective wells. After the addition of NBT 5 µl of 5 mM Ethylene diamine tetraacetic acid (EDTA) was added in respective wells. 32.5 µl of 100 mM methionine was now added in respective wells. After this 10 µl of 2 mM riboflavin was poured in respective wells. 121 µl of pre-treatment plant extracts were added in pre-treatment rows while the same quantity of post-treatment plant extracts were added in post-treatment rows.Absorbance was recorded at 560 nm after 10 minutes. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.[21]

Results

Antibacterial activity study revealed that the maximum inhibitory zone was seen against B. cereus by the leaves extracts. No inhibition was found against Gram negative bacteria (Table 1). In antiproliferative activity the ethyl acetate and petroleum ether extracts showed significant results as compared to the control. The ethanolic extract was insignificant (Table 2).

Table 1 Antibacterial activity of Psidiumguajava leaves extracts and controls

Parts	Solvents	Zone of inhibition in mm (Mean±Standard deviation)					
		S. aureus	E. coli	P. aeruginosa	B. cereus	K. pneumoniae	S. typhi
Leaves	E	7±1	0	0	13±1	0	0
	E.A	0	0	0	13±1	0	0
	P.E	0	0	0	12±1	0	0
Controls	AK30	13±1	20±1	17±1	24±1	17±1	20±1
	CTX30	30±1	22±1	16±1	9±1	24±1	20±1
	VA30	13±1			14±1		
	AMP10	11±1	10±1	0	0	0	18±1
	CN10	15±1	20±1	13±1	21±1	14±1	16±1
	F300	15±1	0		15±1	0	14±1
	E15	10±1	13±1	0	24±1	16±1	0
	DMSO	0	0	0	0	0	0

E- Ethanol; E.A- Ethyl acetate; P.E Petroleum ether ;Positive controls: AK30-Amikacin; CTX30-Cefotaxime; VA30- Vancomycin; AMP10- Ampicillin, CN10-Gentamicin; F300-Nitrofurantoin; E15-Erythromycin ;Negative control: DMSO-Dimethyl sulfoxide

Table 2: Anti-proliferative activity of Psidiumguajava leaves on HeLa cell lines

Extracts	Absorbance in nm (Mean±Standard deviation)
P. guajava leaves ethanol	2.26
P. guajava leaves ethyl acetate	1.33
P. guajava leaves petroleum ether	1.41
Control (HeLa cells)	2.35

The catalase assay of Psidiumguajava leaves extracts showed insignificant catalase activity. However the post-treatment ethanolic extract has better result as compared to its pre-treatment extract. The post-treatment petroleum ether extract have same result as that of its pre-treatment extract (table 3). Superoxide dismutase (SOD) assay showed that post-treatment ethanolic and ethyl acetate extracts of leaves have significant SOD activity. The post-treatment petroleum ether extract of leaves was significant as compared to the control however the result was insignificant comparative to its pre-treatment extract (Table 4).

Table 3: Catalase activity of Psidiumguajava leaves extracts on HeLa cell line

Extracts	Plant codes	Absorbance in nm (Mean±SD)
Pre-treatment extracts	PGLE	0.08
	PGLEA	0.09
	PGLPE	0.07
Post-treatment extracts	PGLE	0.13
	PGLEA	0.05
	PGLPE	0.07
Control	HeLa cells	0.17

PGLE- Psidiumguajava leaves ethanol; PGLEA- Psidiumguajava leaves ethyl acetate; PGLPE-Psidiumguajava leaves petroleum ether;Pre-treatment extracts: Extracts before application on HeLa cell line;Post-treatment extracts: Extracts after application on HeLa cell line

Table 4: SOD activity of Psidiumguajava leaves extracts on HeLa cell line

Extracts	Plant codes	Absorbance in nm (Mean±SD)
Pre-treatment extracts	PGLE	0.24
	PGLEA	0.30
	PGLPE	0.31
Post-treatment extracts	PGLE	0.30
	PGLEA	0.48
	PGLPE	0.28
Control	HeLa cells	0.23

PGLE- Psidiumguajava leaves ethanol; PGLEA- Psidiumguajava leaves ethyl acetate; PGLPE-Psidiumguajava leaves petroleum ether;Pre-treatment extracts: Extracts before application on HeLa cell line;Post-treatment extracts: Extracts after application on HeLa cell line

Discussion

The leaf of P. guajava showed inhibitory zones against S. aureus complementing the findings of Vieira et al.

who also reported the antibacterial effect of guava leaves against S. aureus.[22] Another complementary report by Gnan and Demello found good antimicrobial activity of guava leaf against different strains of Staphylococcus aureus.[23] The alcoholic extract of leaves was found more active against S. aureus than aqueous extract.[24] The P. guajava leaves extract was found to inhibit S. aureus.[25] The ethanolic, petroleum ether and aqueous extract of P. guajava leaves were found to have strong antibacterial activity against S. aureus which is contradictory in case of petroleum ether but complementary in ethanolic extract.[26] All the extracts of Psidiumguajava leaf were found to have inhibitory zones against S. aureus.[27] The study done by Meigy et al. showed that the author tested different concentrations of Psidiumguajava extracts. The report is complementary in having inhibitory zones against S. aureus but there was no significant difference between the low concentrations however the increased concentrations showed greater inhibitory zones.[28]

In this study no antimicrobial activity of leaves extracts of P. guajava was found against K. pneumoniae which is contrary to the findings of Yushau and Geidam who found that the ethanolic and aqueous extract of leaves respectively, were active against it.[4,29] Complementary report where no effect was observed on K. pneumoniae was done by Khaled.[30]In present study all extracts of P. guajava leaves showed significant antibacterial activity against B. cereus complementing a study where the methanolic and ethanolic extracts were found to have antibacterial activity against B. cereus. The methanolic extract has stronger inhibition than ethanolic extract.[31] Another complementary study reported that flavonoids present in guava leaves inhibit B. cereus.[32]All the extracts of Psidiumguajava leaf were found to have inhibitory zones against B. cereus complementing this study.[27] The report of Deepa et al. is also supporting this study.[33] No contradictory evidence was found in these reports.

Psidiumguajava leaves extracts did not show any activity against E. coli, Salmonella typhi and Pseudomonas aeruginosa. The results were comparable to a study where ethanolic extract of Psidiumguajava leaves did not show any antimicrobial activity against E. coli.[29] Contradictory findings were noted with alcoholic extract of guava leaves which was found to inhibit the growth of E. coli and extract was found to be bacteriostatic.[25] The extract of P. guajava possessed antibacterial activity against S. typhi contradicting this study but did not show any activity against E. coli complementary to this study.[4] Another complementary finding reported that the alcoholic extract of leaves of P. guajava was found inactive against E. coli and Pseudomonas aeruginosa.[24] Contradictory report of P. guajava alcoholic extract of leaves was found to inhibit growth of E. coli and P. aeruginosa.[25] The ethanolic and petroleum ether extract of P. guajava leaves was found to have strong antibacterial activity against E. coli which is contradictory to this study. P. aeruginosa was found to be resistant to the action of petroleum ether extract of P. guajava leaves but not for ethanolic extract for which they were sensitive.[26]

Contrary to the findings of this study alcoholic extract of guava leaf was found to have antiproliferative activity on HeLa cell lines.[13] Psidiumguajavalevaes showed moderate cytotoxic activity on HeLa cell lines which is contradicting to this study.[34] Petroleum ether extract of Psidiumguajava leaf did not show any activity on HeLa cell lines which is contradictory to this study.[12] In another study ethanolic extract of Psidiumguajava leaf showed significant antiproliferative activity on HeLa cell lines contradicting this study.[35] Another study complementary to this study showed that alcoholic extract of P. guajava leaves did not have any antiproliferative activity on HeLa cell lines.[12] No reports have been found on catalase and SOD activity of Psidiumguajava plant extracts on HeLa cell line.

Conclusion

1. Psidiumguajava plant leaves extracts can be used as anti-proliferative agent against HeLa cell line.
2. At the tested concentration only B. cereus was inhibited by all the extracts therefore further studies can be conducted on the basis of findings of this study to discover new compounds.

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