ANTIBIOFILM ACTIVITY AND COMPOSITION OF THE PETROLEUM ETHER SOLUBLE FRACTION OF *PSIDIUM GUAJAVA LINN*.

SARA[1], NAZISH DILDAR[1], SYED NAWAZISH ALI[*1], SABIRA BEGUM[2], SAIQA ANDLEEB[3] AND BINA S. SIDDIQUI[2]

[1]Department of Chemistry, University of Karachi, Karachi-75270, Pakistan
[2]HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
[3]Microbial Biotechnology Laboratory, Department of Zoology, University of Azad Jammu and Kashmir, Muzaffarabad, 13100, Pakistan
Corresponding author's e-mail: syed.nawazish@gmail.com

خلاصہ

دنیا بھر میں ادویاتی خصوصیات رکھنے والے نباتات کو محفوظ، منفرد اور موثر ضد خُردحیاتیاتی عامل (anti-microbial agent) تسلیم کیا جاتا ہے۔ حالیہ تحقیق کا بنیادی مقصد *Psidium guajava* (امرود، خاندان *Myrtaceae*) کے پتوں کے Extract سے حاصل ہونے والی پیٹرولیم ایتھر حل پزیر کسر (PG-PES) کی ضد خُرد حیاتیاتی فلمی (anti-biofilm) صلاحیت اور اس کسر میں موجود کیمیائی اجزائے ترکیب کا جائزہ لینا ہے۔ یہاں یہ بات بھی اہم ہے کہ یہ پودا Tropical اور Sub-Tropical خطے میں مختلف انواع کی بیماریوں میں اکسیر کا درجہ رکھتا ہے۔ ہماری بہترین معلومات کے مطابق اس پودے کی غیر قطبی کسر کی Anti-Biofilm سرگرمی کو اس کے کیمیائی اجزائے ترکیب کے ساتھ سائنسی معلومات میں پہلی مرتبہ پیش کیا گیا ہے۔ حالیہ تحقیق میں اس کسر کی Anti-Biofilm سرگرمی کو انسانی کلینیکل جرثوموں کی سات مختلف اقسام کے خلاف جانچہ گیا ہے۔ اور معیاری دوا Ciprofloxacin سے بھی نتائج کا موازنہ کیا گیا۔ *Staphylococcus aureus* *Pseudomonas aeruginosa* (82.0%) , *Klebsiella pneumoniae* (74.8%), *Escherichia coli* (84.5%) اور (84.0%), *Serratia marcescens* (68.8%) بیکٹیریا کے خلاف اس کسر کا موثر anti-biofilm اثر پایا گیا جبکہ *Streptococcus pyogenes* (36.0 %) بیکٹیریا کے خلاف نسبتاً کم اور Staphylococcus *epidermidis* (7.9%) بیکٹیریا کے خلاف انتہائی معمولی anti-biofilm سرگرمی کا مشاہدہ کیا گیا اس کسر کی GC/GCMS تجزیہ کاری کے نتیجے میں سترہ کیمیائی مرکبات کی تشخیص ممکن ہو سکی۔ ان کیمیائی اجزاء میں D-friedoolean-14-en-3-one(13.75%) ، tert-butyl 1-[4-(2,6-ditert-butyl-4-methoxyphenoxy)-3-nitro-4-oxobutyl] pyrrolidine-2-carboxylate (13.15%), globulol (12.23%), 4,4,8-trimethyltricyclo [6.3.1.0(1,5)] dodecane-2,9-diol (11.22%) اور isoaromadendrene epoxide (7.79%) شامل ہیں۔

موجودہ تحقیقات نہ صرف PG-PES کسر میں مختلف حیاتیاتی سرکردہ (bioactive) کیمیائی مرکبات (اجزاء) کی موجودگی کی تصدیق کرتی ہے بلکہ جانچے گئے بیکٹیریا کے خلاف اس کسر کے زود اثر اور قدرتی anti-biofilm عامل ہونے کی عکاسی بھی کرتی ہے۔

Abstract

Medicinal plants are considered as safe, significant and effective antimicrobial agents worldwide. The objective of present study is to determine antibiofilm potential and chemical composition of petroleum ether soluble fraction (PG-PES) of leaves extract of *Psidium guajava* Linn. (Guava; family *Myrtaceae*). *Psidium guajava* is a well known plant of tropical and subtropical region which is commonly used in various folkloric remedies. To the best of our knowledge, the scientific information on antibiofilm activity of non-polar fraction of this plant along with its chemical composition is first time reported. The antibiofilm activity was tested against seven human clinical pathogens with the comparison of standard drug ciprofloxacin. The strong antibiofilm effect was observed against *Escherichia coli* (84.5%), *Staphylococcus aureus* (84.0%), *Pseudomonas aeruginosa* (82.0%), *Klebsiella pneumoniae* (74.8%), *Serratia marcescens* (68.8%), while it showed moderate result against *Streptococcus pyogenes* (36.0%) and little effect against *Staphylococcus epidermidis* (7.9%).The chemical composition of this fraction identified the presence of seventeen compounds by GC/GC-MS analysis. The main constituents were D-friedoolean-14-en-3-one (13.75%), tert-butyl 1-[4-(2,6-ditert-butyl-4-methoxyphenoxy)-3-nitro-4-oxobutyl]pyrrolidine-2-carboxylate(13.15%),globulol (12.23%), 4,4,8-trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol (11.22%) and isoaromadendrene epoxide (7.79 %).These investigations endorsed the incidence of various bioactive phyto constituents and also explored the use of PG-PES as an efficient, natural anti-biofilm agent against tested bacteria.

Key words: *Psidium guajava,* petroleum ether soluble fraction, antibiofilm activity, GC/GC-MS analysis, phytochemical constituents

Introduction

Psidium guajava Linn. (Family: *Myrtaceae*) commonly known as Guava tree native to tropical Central America from Southern Mexico to Northern South America, is used as a nutritional medicinal plant globally. Indigenous system of medicine have seen the use of different parts of this plant to heal from diarrhea, vomiting, pulmonary disorders, ulcers, bowels infections and cough (Fazlin, Ahmad, & Lim, 2002; Lin, & Lin, 2020; Parvez, Shakib, Khokon, & Sanzia, 2018). Several pharmacological investigations have also confirmed antimicrobial, antipyretic, anti-inflammatory, antioxidant, antidiabetic, anticancer, antihypertensive, antimalarial, antispasmolytic, cardio, neuro and hepato-protective properties of this plant (Anand *et al*., 2016; Diaz de Cerio, Verardo, Gómez-Caravaca, Fernández-Gutiérrez, & Segura-Carretero, 2017; Sultana *et al*., 2020). Literature of the plant also revealed the presence of medicinally important chemical constituents including tannins, lectins, vitamins, flavonoids, terpenoids and carotenoids responsible for antibacterial activity (Bhagavathy, Mahendiran, & Kanchana, 2019; Lin & Lin, 2020; Ugboko, Nwinyi, Oranusi, Fatoki, & Omonhinmin, 2020).

The world population is continuously overwhelmed with the increasing threat of multidrug-resistant bacteria which have become one of the crucial concerns for public health. The microbial communities known as biofilms, endure in the antagonistic atmosphere are extremely resistant against antimicrobial agents to produce harmful infections. The bacteria have shown adhesion ability on to a biotic surfaces which play significant role in food industries through biofilm formation to contaminate food processing environment (Costa *et al*., 2018).It is reported that more than 90% food borne human illness is caused by consumption of contaminated pathogenic microbial food which is a great problem for public health (Friedman, Henika, & Mandrell, 2002; WHO, 2015, 2020).

Since biofilm is considered as a crucial virulence factor therefore new strategies to control microbial infections by biofilm inhibition is of great interest (Parrino *et al*., 2019). Naturally occurring active compounds in plants strive as anti-microbial agents and plays a vital role to inhibit biofilm formation in the wide range of infections (Khameneh, Iranshahy, Soheili, & Fazly Bazzaz, 2019; Slobodníková, Fialová, Rendeková, Kováč, & Mučaji, 2016; Trentin *et al*., 2015). To the best of our knowledge, there has been no scientific information on antibiofilm activity of non polar fraction of *P. guajava* leaves extract against mentioned bacteria using crystal violet (CV) reduction assay with its chemical composition by GC-FID and GC-MS techniques.

Materials and Methods

Sample collection: The collection of plant was achieved from Karachi, Pakistan in summer (March 2018). A herbarium voucher specimen number (KUH-GH N0.53976) is deposited in University of Karachi at the Department of Botany after identification at the same department. Petroleum ether soluble fraction (PG-PES) was obtained by systematic fractionation of alcoholic extract of the leaves(Begum *et al*., 2014).

Anti-biofilm Assay: Anti-biofilm activity of petroleum ether fraction was analyzed by crystal violet method with little modification (O'Toole, 2011). Bacteria were cultured in special borosilicate glass tubes (Minitek, USA) in a nutrient broth medium (2 ml) and PG-PES (30 mg/ml) at 37°C for overnight. Ciproflaxcin was used in this experiment as positive control and nutrient broth was used as negative control. The attached cells were stained by crystal violet (0.1%, 125 µl) after incubation and removal of broth medium. Incubation of Borosilicate tubes were carried out at room temperature for 15 minutes. The excess of unattached cells and dye was removed by rinsing with water. After completing the staining of film, acetic acid (30%) was gradually added to dissolve crystal violet. The above mentioned conditions were adopted for incubation of this solution. Quantification of solubilized crystal violet was done at 550nm by using a SHIMADZU UV-1601 spectrophotometer (30% acetic acid in water was used as blank).

Gas Chromatography (GC): The analysis of PG-PES was accomplished by gas chromatography on Shimadzu 17-A equipped with Zebron™ ZB-5 capillary column. The required conditions for Gas chromatography (GC) were as follows: flame ionization detector temperature (260^{o}C), sample injector temperature (240^{o}C), column temperature (75^{o}C for 3mint, increased to 200^{o}C at a rate of 7^{o}C/mint, and then held at 240^{o}C for 30 mint), carrier gas (Nitrogen with flow rate 10.939 mL/mint, 89 kPa).

Gas Chromatography-Mass Spectrometry (GC-MS): GC-MS–experiments were performed on Agilent Technologies 7000 GC/MS Triple Quad gas chromatograph, equipped with ZB-5MS combined with a Jeol (JMS-600H) mass spectrometer functioning in EI mode with ion source at 250^{o}C with 70 eV. Depending upon the response of the detector, the volume of carrier gas was adjusted from 1.0-5.0 mL/min.

Identification of the Constituents: The petroleum ether fraction (*loc. cit*) was analyzed by gas chromatography (GC-FIDand GC-EIMS) (Masada, 1976).The recognition of components were done by comparison of reported mass spectral data in NIST retention index data base library (NIST, 1998).

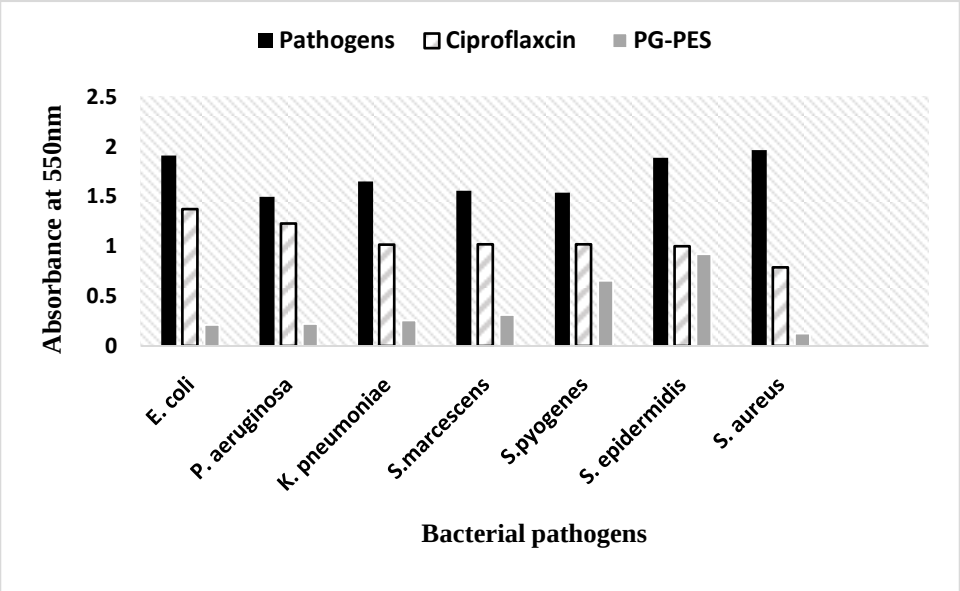

Fig 1.Biofilm Inhibitory Potential of PG-PES

Table 1. Composition of the Petroleum Ether Soluble Fraction (PG-PES) of *Psidium guajava* Linn. Leaves

Compounds[a]	RI[b]	%	Compounds[a]	RI[b]	%
Caryophyllene	1494	1.34	(3E,12Z)-nonadeca-1,3,12-triene-5,14-diol	2241	4.25
α-Cubebene	1344	4.39	Hexahydrofarnesyl acetone	1754	1.99
(+)-Epi-bicyclosesquiphellandrene	1435	2.73	4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	1840	11.22
Acetic acid, 7-hydroxy-1,3,4,5,6,7-hexahydro-2H-naphthalen-4α-yl-methyl ester	1740	1.38	Ethyl palmitate	1978	3.39
Globulol	1537	12.23	2-(2-ethylhexoxycarbonyl)benzoic acid	2162	3.87
2-Methylene-6,8,8-trimethyl-tricyclo[5.2.2.0(1,6]undecan-3-ol	1599	2.75	Tert-butyl 1-[4-(2,6-ditert-butyl-4-methoxyphenoxy)-3-nitro-4-oxobutyl]pyrrolidine-2-carboxylate	3553	13.15
β-Caryophyllene oxide	1507	3.2	D-Friedoolean-14-en-3-one	2869	13.75
2-Naphthalenol, 2,3,4,4a,5,6,7-octahydro-1,4a-dimethyl-7-(2-hydroxy-1-methylethyl)	1901	3.8	Stigmast-4-en-3-one	2714	3.55
Isoaromadendrene epoxide	1281	7.79	Total identified		94.78

[a] List of compounds are based on their elution order from a Zebron™ ZB-5 capillary column;
[b] RI (retention index): RI-non isothermal Kovatsretention indices on an ZB-5MS column relative to C10–C30 n-alkanes.

Results and Discussion

The antibiofilm activity of the fraction was evaluated against seven human clinical pathogens including four Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*) and three Gram-positive(*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*,) bacteria causing food poisoning, pneumonia, skin, urinary and respiratory infections (Prestinaci, Pezzotti, & Pantosti, 2015; Sizar & Unakal, 2019). The PG-PES fraction has shown remarkable inhibition of biofilm against tested bacterial pathogens when compared with standard drug (ciprofloxacin) (Fig.1). The strong antibiofilm effect of this non-polar fraction was observed against *E. coli* (84.5 %), *S. aureus* (84.0%), *P. aeruginosa* (82.0%), *K.*

pneumoniae (74.8%), *S. marcescens* (68.8%), while it showed moderate effect against *S. pyogenes* (36.0%). The difference in the antibiofilm activity is probably due to surface chemical affinity between the plant constituents and the biofilm matrix.

The results obtained by chemical analysis of PG-PES are presented in Table 1. Seventeen constituents from different classes of natural products have been identified by GC and GC-MS were found to be 94.78 % of the fraction. The main constituents of the fraction were D-friedoolean-14-en-3-one (13.75%), tert-butyl 1-[4-(2,6-ditert-butyl-4-methoxyphenoxy)-3-nitro-4-oxobutyl]pyrrolidine-2-carboxylate (13.15%), globulol (12.23%), 4,4,8-trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol (11.22 %) and isoaromadendrene epoxide (7.79 %).

Conclusion

The current phytochemical analysis of non polar fraction of *P. guajava* leaves confirms the presence of different bioactive components and strongly suggests the use of PG-PES as an effective natural biofilm disrupting agent against tested bacteria.

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