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The Phytochemical and Comparative Anticancer Study of Methanolic and Chloroform Extracts of *Psidium guajava* L. Leaves of Pakistani Origin

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ABSTRACT

The chief focus of our study is to evaluate the phytochemical and anti-cancer activity of methanol (PGM) and chloroform extracts (PGC) of the leaves of *Psidium guajava* (guava) collected from local area of district Sialkot, Pakistan. Shade dried milled leaves was subjected to extraction (maceration) with methanol and chloroform. Quantitative and qualitative screenings by GC-MS and phytochemical techniques were performed. Then different secondary metabolites and phytochemical compounds were identified which are typically associated with the existence of therapeutic characteristics. *Psidium guajava* has been extensively used as herbal remedies like, anti-diarrheal, antihypertensive, antibacterial, antifungal as well as to control obesity, ulcer, diabetes. In this study, both extracts of *P. guajava* were evaluated for their anticancer activities against HeLa cell-lines (cancerous cells). The healthiest anticancer response in the form of cell-line suppression was perceived with 200µg/mL of both extracts, PGM showed 81% and PGC exhibited 91% while the standard drug doxorubicin presented around 76% inhibition. The comparative better result was seen with chloroform extract than methanolic abstract. In conclusion, the chloroform and methanol extracts of our nominated plant from Pakistan origin has a good source of phytochemicals that revealed an outstanding anti-cancer potential.

Keywords: *Psidium guajava*, anticancer, phytochemicals, methanol extracts, secondary metabolites.

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INTRODUCTION

Natural compounds obtained from animal and plant sources have been used to treat various diseases of human being earlier around 6000 BC. It is strongly believe that natural plants still have a strong therapeutic benefits and providing a foundation for the isolation and synthesis of novel medicinal compounds. According to the assessment of WHO, more than 80% of the world population trusted on herbal medication on primary health care level. Regarding the management of chronic and acute illness in China, Pakistan and India, the tradition herbs also have a significant role[1]. Leaves of *Psidium guajava* have incredible significance to manage the various diseases worldwide with better patient compliance when compared to the allopathic system of treatment. *Psidium guajava* belongs to Myrtaceae family and its plant around 20-feet long. Leaves are 05 to 15 cm long having bulging pinnate veins and oval blade like shape. It is preferably grown in dry weather and extensively cultivated in tropical/subtropical parts of the world including; Asia, Europe, Africa, America and Mexican region. *P. guajava* tagged by various nations such as; amrood in Pakistan, banjiro in Japan, goiabeiro in Portugal, goyave in France, guava in English and guayave in German[2]. Traditionally, different parts of guava have been recommended for the treatment of wounds, lesions, ulcers, diarrhea, cholera, hypertension, obesity and control of Diabetes mellitus[3]. Antibacterial, antioxidant, leishmanicidal, and hepatoprotective properties have seen in leaves of this plant[4]. Through GC-MS analysis and phytochemical techniques, various compounds were identified from the methanol and chloroform extracts of *P. guajava*.

The mortality rate with cancer is high in both developed and under-developed countries worldwide, because of population growth and age seniority besides embracing cancer triggering activities[5]. Treatment of cancers particularly the solid cancers have been treated conventionally by chemotherapy, surgically removing and radiation therapy, with a bit less success rate[6]. In addition

conventional chemotherapeutic compounds were found to be not only toxic and inhibit the growth of cancerous cells but also have a drastic effect on the progress of normal cells in the body[7]. Products from natural sources on the other hand, comparatively harmless and biologically compatible to the living tissues[8]. Hence, the natural plants have become a prime goal for the exploration and recognition of novel anticancer medicine[9]. Though the anticancer activities of essential oils, polysaccharides and other extracts in various parts of *Psidium guajava* was studied, but limited studies and assessment were performed on the real potential of guava leaves from indigenous area of Pakistan origin. Since, our study was mainly focused on the evaluation of anticancer activity of methanol and chloroform extracts of *Psidium guajava* leaves because of easy access of everybody in our region. In addition, comparative anticancer potential of methanol and chloroform extracts was also done and superior antitumor response was seen with our extracts.

MATERIALS AND METHODS

Plant Collection

Fresh leaves of *Psidium guajava* were collected from the main Orchard besides Sialkot International Airport, Sialkot, Pakistan. Plant sample was deposited and obtained Voucher Specimen No. GC-Herb-Bot-2408 after identification from the Dr. Sultan Ahmed Herbarium, Department of Botany, Government College University, Lahore, Pakistan.

Drying and Extraction

After collection of leaves of *P. guajava* were shad dried for the period of fortnight and made course powder with grinder. Extraction of plant material was done through maceration with methanol and chloroform. About 05-liter analytical grade methanol (Sigma made in Australia) was used for the maceration of 01-Kg powder of plant in glass pot with vigorous mechanical stirring twice daily for 01 week at room temperature. Then, muslin cloth was used to filter the mixture and filtrate was passed through Whatman filter paper grade 01 (Sigma-Aldrich). The Rotary Evaporator (IKA HB10 Basic, Made in Germany) was used to achieve the concentrated dry masses from the filtrate at 35±5˚C under reduced pressure. Resulting % age yield of methanolic extract of *P. guajava* (PGM) was found 8.4% and stored at 04˚C in refrigerator for the further investigations[10].

Residual material after extraction with methanol was dried, weighed (991 g) and macerated for 01-Wk in glass bottle with 05-L chloroform (MERCK) analytical grade. Mixture was filtered and dried as done with methanol method. The resulting semisolid material (3.2% Yield) was obtained, assigned code PGC and stored in well closed flask (04˚C).

Phytochemical studies

The phytochemical investigation of crude powder of plant was performed and recognized the existence of phytochemical constituents of various classes such as; alkaloids, anthraqinones, catechins, flavonoids, phenolic compounds, saponins, steroids and tannins[11].

GC-MS Analysis

Eight different compounds were identified from methanolic extracts of *P. guajava* using GC-MS Agilent Technologies (GC Model: 7890A, MS Model: 5975C). The ˚GC-MS protocol˚ was employed with slight temperature modification[12]. At the start, temperature was adjusted at 110˚C for 02 minutes and continually raised at the rate of 10˚C per min up-to 280˚C.

Anticancer Activity

Method of analysis

Anticancer activity of methanolic (PGM) and chloroform (PGC) extracts of *Psidium guajava* was evaluated in our study. Bioassay protocol was employed as; firstly Dulbecco's Eagle medium was modified with 10% foetal bovine serum in 75ml flask. Then medium was inoculated with HeLa cell-line (cervical carcinoma) by incubating at 37˚C in the presence of 5% CO_2. The confluence after collection was planted in 96-well plates pre-treated with tissue culture. After around 24-hour incubation of well plates, methanol and chloroform crude extracts of *P. guajava* were added in doses of 50µg, 100µg and 200µg/mL each in triplicate and made incubation for the period of 48-hour. The positive standard drug doxorubicin was used in MTT assay along 200µl MTT at the dose of 50µg/mL. Then added into wells of test chamber and incubated around 03 hours at 37˚C. The tetrazolium salt was reduced to form formazan crystals after specified time of incubation period. Then solution of crystals was made by dissolving them in 100µL of DMSO. The absorbance was measured at 570-nm with the help of micro-plate reader[13]. The percentage inhibition or suppression of viable cells was determined by using following formula.

$$\text{Percentage Inhibtion} = 100 - \frac{(\text{average of OD. of test sample - average of OD. of –ve control})}{(\text{average of OD. of +ve control - average of OD. of -ve control})} \times 100$$

Study of Acute Toxicity on Healthy Animal

Three groups of healthy Sprague Dawley rats were made after 12-hr fasting and each group have had 05-rats. First group was given orally 750 mg/kg of methanol extract (PGM) and 2nd group was administered 750 mg/kg extract of chloroform (PGC). Third one was nominated as control who received only 10% DMSO. After 4-hr, 24-hr and 168-hr administration of extracts, signs and symptoms were observed. There was no any toxic sign like mortality and morbidity was noticed in any animal.

Statistical Analysis

Data was presented using Mean ± SEM and analyzed through Graph-Pad PRISM (5.02). The quantitative variable comparison was intended with One-Way ANOVA followed by Tukey's multiple comparison ˚Student's t-test˚. The P value ≤ 0.05 was considered as statistically significant.

The comparative anticancer potential of two extracts

The comparative anticancer activity of both extracts PGM and PGC with different concentration was evaluated and found chloroform extract had comparatively better control of cancer cells suppression than methanol extract of *P. guajava*.

RESULTS

Phytochemical Screening

The qualitative phytochemical screening of the crude powder of both plants was done in Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Bahauddin Zakariy University, Multan, Punjab, Pakistan. The final outcomes were acknowledged in the **Table 1**[11,14].

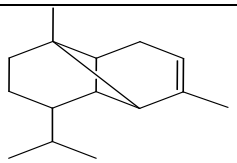
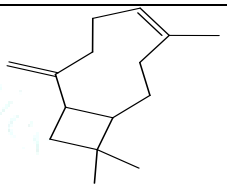
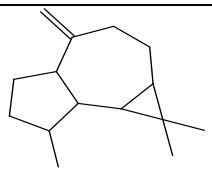
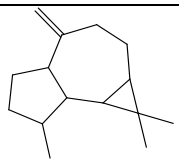
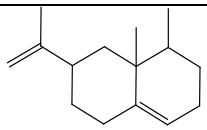
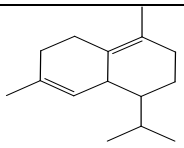
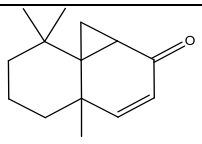
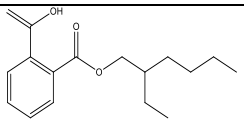
Table 1: **Phytochemical screening (Qualitative) of Crude Powder of *P. guajava***

S#	Phytochemicals	Test	Result
1.	Alkaloids	Dragendorff's	+
2.	Anthraquinones	Magnesium acetate	+
3.	Cardiac glycosides	Ferric chloride	+
4.	Flavonoids	Sodium hydroxide	+
5.	Saponins	Froth	+
6.	Steroids	Acetic anhydride	+
7.	Tannins	Ferric chloride	+

GC-MS studies

Through the use of ˚GC-MS Agilent˚ Technologies (GC Model: 7890A, MS Model: 5975C), 08-compound were identified from methanol extract of *P. guajava* (PGM) and enlisted with retention time, %age content, chemical name, molecular formula, molecular weight and structural formula (**Table 2**).

Table 2: List of constituents in methanol extract of *P. guajava* identified by GC-MS

Sr.#	R_t (min)	Name of identified component	% of content	Mol.wt g/mol	Mol. formula	Structure
1	11.100	Copaene	11.725	204	$C_{15}H_{24}$	
2	11.799	Caryophyllene	13.080	204	$C_{15}H_{24}$	
3	12.111	Alloaromadendrene; 1H-Cycloprop [e] azulene, decahydro-1,1,7-trimethyl-4-methylene-,[1aR-(1a.α.,4a.α., 7a.β.,7b.α)]-	11.601	204	$C_{15}H_{24}$	
4	12.451	Aromadendrene	3.369	204	$C_{15}H_{24}$	
5	12.994	Valencene; Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-,[1R-(1.α.,7.β,8a.α)]	1.845	204	$C_{15}H_{24}$	
6	13.387	δ-Cadinene; Naphthalene, 1,2,3,5,6 hexa,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis)-	3.712	204	$C_{15}H_{24}$	
7	14.406	Cyclopropa[d]naphthalene-2(4aH)-1, 1,1a,5,6,7,8-hexahydro-4a,8,8-trimethyl-,[1aR-(1a.α,4a.β,8aS*)]-	2.530	204	$C_{14}H_{20}O$	
8	27.623	Mono (2-ethylhexyl) phthalate; 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	52.137	278	$C_{16}H_{22}O_4$	

Anticancer activity of Methanolic extract of Psidium guajava

Methanol extract of PGM (50, 100 and 200 µg/mL) was used for the evaluation of anticancer activity against HeLa cell-line (ATCC # HTB-22). The standard drug doxorubicin (50 µg/mL) was employed as positive standard. The inhibition of cell-lines were 20.32%, 44.30% and 81.28% seen with 50, 100 and 200 µg/mL of extracts respectively as well as with doxorubicin (73.94%). The significant response was observed with 100 µg/mL while the PGM (200 µg/mL) extract showed the excellent sign of inhibition on comparison to doxorubicin a standard drug **(Figure 1)**.

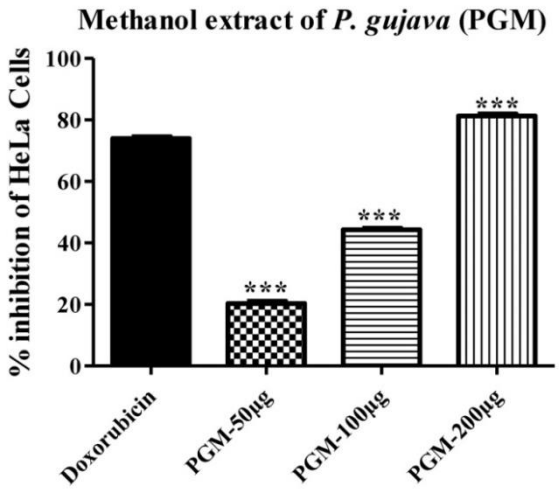


Fig. 1: The observation of significant suppression of HeLa cells with methanol extract of ***P. guajava***. Data presented the Mean ± SEM, where $^{*}P < 0.05$, $^{**}P < 0.01$and $^{***}P < 0.001$denoted the comparison of test groups with control

The anticancer potential of chloroform extract of P. guajava

The evaluation of anticancer activity of 50µg, 100µg and 200µg of chloroform extract of *P. guajava* was determined. The PGC extracts, 100µg and 200µg exhibited 52% and 91.67% suppression of HeLa cell-lines respectively. While doxorubicin showed 76% inhibition. We concluded that 200µg PGC extracts revealed a better antitumor potential when compared with standard drug **(Figure 2).**

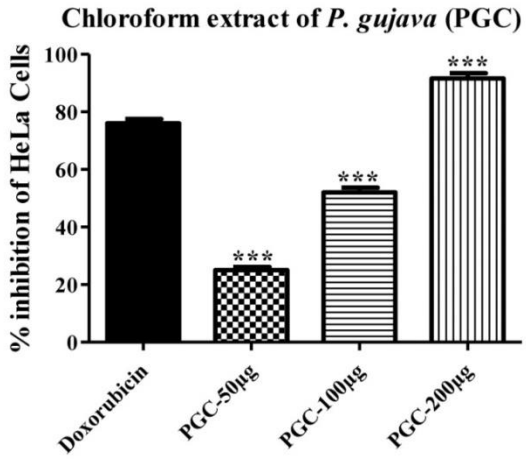


Fig. 2: The evaluation of chloroform extract of ***P. guajava*** against HeLa cell-lines. Data presented the Mean ± SEM, where $^{*}P < 0.05$, $^{**}P < 0.01$and $^{***}P < 0.001$showed the comparison of test groups with control

Comparative anticancer activity of methanol and chloroform extracts of P. guajava

When compared the anticancer activity of both extracts of *P. guajava*. Both extracts exhibited almost same rate of HeLa cells suppression. Chloroform extracts of *P. guajava* (PGC) with 50, 100 and 200µg/mL doses showed 25, 52 and 91% inhibition respectively. While methanol extract (PGM) with same concentrations showed 20, 44 and 81% suppression respectively. Hence, chloroform extracts of guava showed bit better response than the methanol extract **(Figure 3)**.

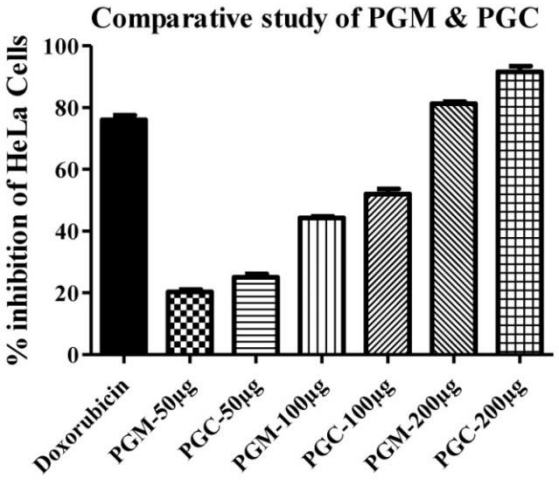


Fig. 3: The comparative anticancer activity of both plants evaluated and found PGC exhibited comparatively better response than methanolic extract

DISCUSSION

Thousands of herbal plants have been consuming all over the word to manage different diseases since ancient period. The current status of WHO report assumed that around 80% people worldwide are still relying on natural plant medicine on primary health-care levels. Phytochemical, chromatographic and spectroscopic screenings are always the initial steps on natural flora for the assessment of bioactivity and identification of bioactive compounds. Plants usually contained various kinds of compounds with diverse polarities relating to different rate of solubility. The methanol and chloroform extracts of *P. guajava* were selected for the qualitative phytochemical analysis and found different secondary metabolites such as; glycosides, flavonoids, alkaloids, saponins, anthraquinones and tannins. Secondary metabolites have a significant role to control various biological activities[15]. Likewise, it was also recognized that secondary-metabolite are effectively control the cancers and cancer related diseases[16,17]. The GC-MS analysis of methanol extract of *P. guajava* showed 08 different compounds. The recognized compounds in our extracts showed diverse proportions such as; Mono (2-ethylhexyl) phthalate found in highest concentration (52%) and 2nd higher percentage contents of Caryophyllene, (13%). The 3rd high share of Copaene (12%) and other big contents of Alloaromadendrene (11.6%) and Copaene (6%) were identified in PGM extract. In addition some other compounds were also found in appropriate concentrations in *P. guajava* that seemed to have therapeutic roles. The antitumor activity has been seen in different plants that had secondary metabolites and phytochemicals. This anticancer potential was assumed due to the presence of flavonoids, phenols, flavanols, flavone and alkaloids in medicinal plants[18].

The plant derived compounds usually exhibited their anti-malignant activity through either suppression of neo-vascularization or apoptosis[19]. The ideal antitumor drugs should be safe and non-toxic to the growth of normal cells in the body. Unfortunately, drugs generally used for the treatment of cancer, not only inhibit the cancerous cells but also have drastic effect on the normal cells' development. Therefore, scientists are continuously in race to find compounds from plants with solid anticancer capability and minor side effects. The extensive work on different parts of guava proved that *P. guajava* contained valuable therapeutic compounds. For instance *P. guajava* has employed as anti-oxidant, anti-bacterial, anti-fungal, anti-diarrheal, anti-hypertensive, leishmanicidal as well as used to treat obesity and Diabetes mellitus[20,21]. Relating to this concern, our current study was focused on the evaluation of anti-cancerous potential of methanol and chloroform extracts of *P. guajava* leaves of Sialkot (Punjab), Pakistan origin. The anti-tumor activity (IC_{50}) was pointed out by Braga et al., when alcoholic extract of *P. guajava* exhibited 15.6 ± 0.8 with HeLa cell-lines protocol. In addition, anti-neoplastic activity seen with comparatively high dose of guava extract (acetone)[22]. However, anticancer potential of methanol and chloroform extracts of *Psidium guajava* was evaluated through HeLa cell-line proliferation inhibition protocol. The results indicated that both extracts exhibited excellent anti-cancer activity when compared with other studies. Even better response of HeLa cells inhibition around 81% was observed with 200µg of PGM and PGC showed 91% better than doxorubicin which exhibited 73% suppression.

CONCLUSION

In conclusion the anti-tumor activity of methanol and chloroform extracts of *Psidium guajava* was primarily due to the presence of potent mediators in extracts. These mediators and phytochemical compounds have identified through GC-MS and phytochemical analysis. It was also concluded that HeLa cell-lines suppressive activity of PGM and PGC extracts was progressively amplified with increasing dose of extracts and at appropriate dose showed a significant response on comparison to other data. The PGC effect was found comparatively better than PGM regarding inhibition of cell-lines. As concerned the toxicity of recommended extracts on healthy cells, determined safe after giving 750 mg/mL dose to healthy rats. Further studies would also be required for the isolation and identification of active compounds from leaves of *P. guajava,* which aggressively involved in anti-cancerous activity.

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CONFLICT OF INTEREST

The authors declared no any conflict of interest.

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