

Phytochemical Screening, Total Phenolic Content, Antioxidant, and Cytotoxic Activity of Five Peruvian Plants on Human Tumor Cell Lines

Oscar Herrera-Calderon, Corina Alvarado-Puray¹, Jorge Luis Arroyo-Acevedo², Juan Pedro Rojas-Armas², Victor Chumpitaz-Cerrate³, Renán Hañari-Quispe⁴, Rita Valenzuela-Herrera⁵

Laboratory of Pharmacognosy, Faculty of Pharmacy and Biochemistry, Universidad Nacional Mayor de San Marcos, ¹Department of Pharmacy, Hospital Carlos Lanfranco La Hoz, ²Academic Department of Dynamic Sciences, Laboratory of Experimental Pharmacology, Faculty of Medicine, Universidad Nacional Mayor de San Marcos, ³Laboratory of Physiology and Pharmacology, Faculty of Dentistry, Universidad Nacional Mayor de San Marcos, Lima, ⁴Laboratory of Animal Physiology, Universidad Andina Néstor Cáceres Velásquez, Puno, ⁵Faculty of Pharmacy and Biochemistry, Universidad Nacional San Luis Gonzaga de Ica, Ica, Peru

ABSTRACT

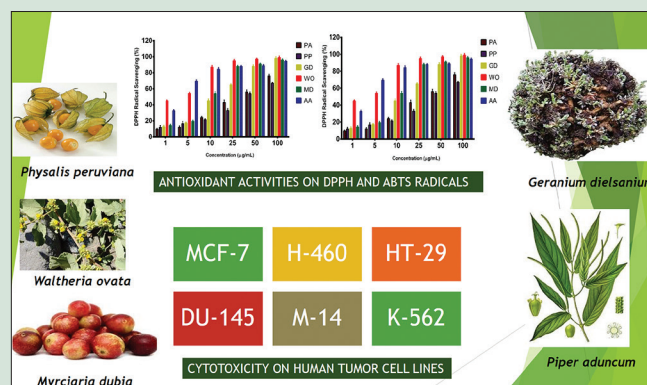
Background: Cancer is considered a serious public health problem in the world; searching alternative treatments of medicinal plants constitute a promissory field to find new anticancer drugs. **Objective:** The aim of this study is to evaluate the phytochemical screening, total phenolic content (TPC), antioxidant, and cytotoxic activity of ethanol extracts of *Waltheria ovata* (WO), *Piper aduncum* (PA), *Myrciaria dubia* (MD), *Physalis peruviana* (PP), and *Geranium dielsianum* (GD) on human tumor cell lines. **Materials and Methods:** Phytochemical screening was assessed using chemical reagents. TPC was developed using Folin Ciocalteu reagent, and the antioxidant activity was determined against 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals by spectrophotometry. The cytotoxic activity was determined on human tumor cell lines followed as: MCF-7, H-460, HT-29, M-14, K-562, and DU-145. **Results:** Phytochemical study confirmed flavonoids and phenolic compounds in all extracts. TPC was found to be the highest in WO extract (1250 mg of gallic acid equivalent/g of dried extract) rather than other extracts. The highest antioxidant activity was established in WO extract for DPPH and ABTS radical scavenging tests (inhibitory concentration [IC₅₀] = 0.89 ± 0.01 µg/mL, IC₅₀ = 4.20 ± 1.50 µg/mL). Ethanol extracts (µg/mL) showed low cytotoxicity on human tumor cell lines (CI₅₀ >20 µg/mL) for PA, PP, GD, MD. Meanwhile, WO presented cytotoxicity on H460, MCF-7, and K562 tumor cell lines. **Conclusion:** In our findings, WO and MD extracts revealed a high antioxidant and TPC. WO exhibited better cytotoxic effect compared with 5-FU. Hence, these medicinal plants could be effective to prevent cancer disease and oxidative stress.

Key words: Antioxidant activity, cancer, cytotoxicity, oxidative stress, phytochemical

SUMMARY

- The study investigated the total phenolic content, antioxidant activity and cytotoxic effect of five Peruvian plants on human tumor cell lines.
- These plants were *Waltheria ovata* (WO), *Piper aduncum* (PA), *Myrciaria dubia* (MD), *Physalis peruviana* (PP), and *Geranium dielsianum* (GD).

- Waltheria ovata* evidenced the best effect antioxidant in vitro against DPPH and ABTS radicals.
- The IC₅₀ for *Waltheria ovata* on H460, MCF-7, and K562 tumor cell lines was less than 20 µg/mL.



Abbreviations Used: IC50: Half inhibitory concentration. TPC: Total phenolic content. DPPH: ,2-Diphenyl-1-picrylhydrazyl. ABTS: 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). AA: Ascorbic acid.

Correspondence:

Dr. Oscar Herrera-Calderon,
Academic Department of Pharmaceutical Sciences, Faculty of Pharmacy and Biochemistry, Universidad Nacional San Luis Gonzaga de Ica, Ica, Peru.

E-mail: oherreracalderon@gmail.com

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INTRODUCTION

Cancer is considered the first cause of death in the world, resulting a serious public health problem that leads to a search for antitumor agents, especially from natural sources, which has become an important research field in the scientific community.^[1] Cancer process is characterized by the alteration of gene expression, cell proliferation, and can be modified by genomic and epigenetic factors.^[2]

Oxidative stress is an imbalance between the production of free radicals and antioxidant system, these generated radicals impair the essential biomolecules in the cell by oxidizing membrane lipids, enzymes, carbohydrates, cell proteins, and DNA.^[3] One example

is the hydroxyl radical (OH⁻) which can cause genetic mutation by forming adduct with guanine to form hydroxylated bases of

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DNA (8-hydroxyl-2'-deoxyguanosine) causing transversions of guanine-cytosine to thymine-adenine.^[4]

Antioxidants are chemical compounds from natural or synthetic sources that can act as chain breakers on radicals such as peroxy, alkoxy or hydroxyl, chelating pro-oxidative metal ions, and quenching singlet oxygen.^[5] New natural products have been studied on many chronically diseases like cancer, diabetes, hypertension overall those related to oxidative stress process.^[6] 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods were regarded as fast methods using both hydrogen and single electron transfer; furthermore, they are cheaper and easier to develop in the laboratory.^[7]

Many medicinal plants are used by patients with cancer due to numerous positive effects on pain relief, vomiting, nausea, depression, and anxiety.^[8] Bioactive compounds such as polyphenols, tannins, and flavonoids can have the antioxidant capacity with potential benefits for health and could reduce cancer risk, it has been revealed that the anti-inflammatory effect of these compounds inhibits arachidonic acid, prostaglandins, and leukotrienes.^[9] Polyphenols are chemoprotectives that act by blocking of initiation stage of carcinogenesis, free radicals and activation of glutathione peroxidase, glutathione reductase, and inactivation of glutathione S transferase.^[10]

Peruvian flora is one of the best sources of medicinal plants in the world.^[11] In Peru exists various species with potential use for commercial purposes and have been studied by its therapeutic effect for the treatment of various diseases, the main objective was to evaluate the preliminary phytochemical screening, total phenolic content (TPC), antioxidant, and cytotoxic activity of ethanol extract of five Peruvian medicinal plants as *Geranium dielsianum* (GD), *Myrciaria dubia* (MD), *Physalis peruviana* (PP), *Piper aduncum* (PA), and *Waltheria ovata* (WO) on human tumor cell lines.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, DPPH, potassium peroxydisulfate, ABTS diammonium salt, ascorbic acid, were purchased from Sigma-Aldrich (Lima, Peru) were purchased from Sigma-Aldrich, USA. Other chemicals used were of analytical grade.

Plant material

Aerial parts of GD, PA leaves, WO roots, PP fruits and MD fruits were collected, in December 2016, from Lima market, Peru, and identified by a botanic. Voucher specimens (272-USM-2015, 152-USM-2016, 05-USM-2014, 147-USM-2016, respectively) were deposited at the National Herbarium of National University of San Marcos (UNMSM), Lima, Peru.

Extraction of plant materials

Collected samples were dried at room temperature, except for fruits which were dehydrated and dried by using controlled temperature in a ventilated oven at 100°C to be pulverized subsequently at the Chemical Laboratory, Faculty of Pharmacy and Biochemistry, Universidad Nacional San Luis Gonzaga de Ica, the powder material of each species was exhaustively macerated with 96% ethanol for 7 days. The extracts were filtered and evaporated by using a rotavap with 80 rpm and 40°C.

Phytochemical screening

Dried extracts were evaluated using specific chemical reagents for each secondary metabolite such as phenolic compounds, flavonoids, quinone, triterpenes, flavonoids, tannins, saponins, steroids, and alkaloids.^[12]

Total phenolic content

The TPC was determined by Folin-Ciocalteu method according to the procedure of Singleton and Rossi.^[13] Extracts were diluted with distilled water. Next, 100 µL of the diluted samples, 150 µL of Folin-Ciocalteu reagent (1/8 diluted in distilled water) and 150 µL 20% Na₂CO₃ were added and mixed. After standing for 5 min, 600 µL distilled water were added. The absorbance was measured at 760 nm after 90 min with distilled water as blank sample. TPC was determined in a standard curve with gallic acid as reference and results were expressed as mg gallic acid equivalents per gram of dried extract.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

In accordance with the method of Okawa *et al.*, with minor modifications, DPPH radical scavenging activity of extracts was determined.^[14] To 100 µL of different extracts at numerous concentrations (1–200 µg/mL) were added to 1900 µL 0.01 mM of DPPH into the test tubes. Next, samples were incubated for 30 min in a dark place. The absorbances were read by spectrophotometry at 517 nm using methanol and ascorbic acid (AA) as blank and standard, respectively. The following equation was used to calculate the percent scavenging of the DPPH radical: Antioxidant activity (%) = $[A_o - A_s / A_o] \times 100$. Where, A_s symbolizes the absorbance of the sample/standard solution and A_o symbolizes the absorbance of the control. Inhibitory concentration (IC₅₀) value is the concentration of extract at which DPPH radicals are scavenged by 50%. The lower IC₅₀ value indicates higher radical scavenging capacity.

2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

The antioxidant activity was determined on ABTS radical in according to Re *et al.*^[15] Briefly, the reaction mixture (4.3 mM potassium persulfate and 7 mM ABTS solution) was incubated for 12–16 h at room temperature, in dark and was diluted, before use, to obtain an absorbance at 734 nm of 0.7 ± 0.02 . Next, 40 µL of different samples solution were added to 1960 µL of the reaction mixture and incubated in the dark at room temperature for 7 min; the absorbance was read at 734 nm using an ultraviolet-visible spectrophotometer. The ABTS scavenging activity of the extracts was compared with ascorbic acid, and the percentage inhibition was calculated as.

ABTS radical scavenging activity (%) = $[(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100$.

Where Abs control is the absorbance of ABTS radical; Abs sample is the absorbance of ABTS radical + extract/standard. ABTS radical scavenging activity of extracts was determined by IC₅₀ value as mentioned above in DPPH assay.

Cytotoxic effect

M-14 (human amelanotic melanoma), DU-145 (prostate carcinoma), H-460 (lung large cell carcinoma), HT-29 (colon adenocarcinoma), MCF-7 (breast cancer), and K562 (chronic myelogenous leukemia), 3T3 (nontumorigenic, BALB/c mouse embryo cells) cell lines were obtained from the American Type Culture Collection. The tumor cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 50 µg/mL gentamycin in humidified 5% CO₂/95% air at 37°C for 24 h.

According to Hossain *et al.*^[16], different dilutions of extracts and 5-fluorouracil (0–250 µg/mL) were added to different plates containing the human tumor cell lines and incubated for 48 h. Sulforhodamine B was used as a dye to determine the cytotoxic activity. Plates were washed with 1% acetic acid. Subsequently, a solution 10 mM Tris buffer (pH 10.5) was

added to solubilize the protein-bound dye. The absorbance was read at 510 nm using a microplate reader. The results were expressed as half IC_{50} and were found by linear regression analysis.

Statistical analysis

The antioxidant and cytotoxic activity were expressed as a mean \pm standard deviation from three observations. The statistical and graphical analysis were performed using linear regression to determine TPC and TEAC. Microsoft Excel Program 2016 was used to Rho's Spearman test considering $P < 0.05$ considered statistically significant.

RESULTS

Determination of phytochemical constituents

The phytochemical analysis is a qualitative test which indicates the presence of groups of compounds in a sample by using the formation of a precipitate or a color change. These extracts of PA, WO, PP, GD, and MD indicated various classes of secondary metabolites such as tannins, phenolic compounds, and flavonoids. However, alkaloids were not found in WO, whereas quinone was positive for GD extract according to [Table 1].

Determination of total phenolic content

The highest content of total phenolic compounds was detected in the WO ethanolic extract (1250 mg GAE/g extract) followed by MD extract (570.0 mg GAE/g extract). Phenolic content was calculated from the standard curve of gallic acid ($y = 0.0152 \times +0.0445$; $R^2 = 0.992$). The following order was founded based on the outcomes of Figure 1: $PP < GD < PA < MD < WO$.

Determination of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The ranking order was established based on the outcomes of Figure 2: $PP < GD < PA < MD < WO < AA$. The IC_{50} values against DPPH radical are shown in Table 2. Compared to AA the IC_{50} values of WO and MD were statistically significant ($P < 0.01$; $P < 0.01$).

Determination of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

In Figure 3, significant differences ($P < 0.01$) were revealed between ABTS scavenging capacities of extracts measured as IC_{50} value and ascorbic acid (AA). ABTS radical scavenging activity of extracts is shown in the following order: $PP < PA < GD < MD < WO < AA$. WO extract showed the

highest ABTS scavenging activity and the IC_{50} value (4.20 ± 0.50) of this extract were not statistically significant ($P < 0.05$) compared to AA.

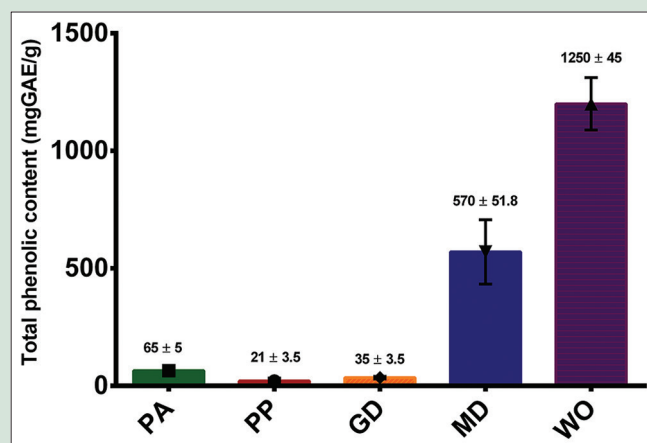


Figure 1: Total phenolic content of different extracts. Values were expressed as mean \pm standard deviation ($n = 3$). PP: *Piper aduncum*; GD: *Geranium dielsianum*; PA: *Piper aduncum*; MD: *Myrciaria dubia*; WO: *Waltheria ovata*

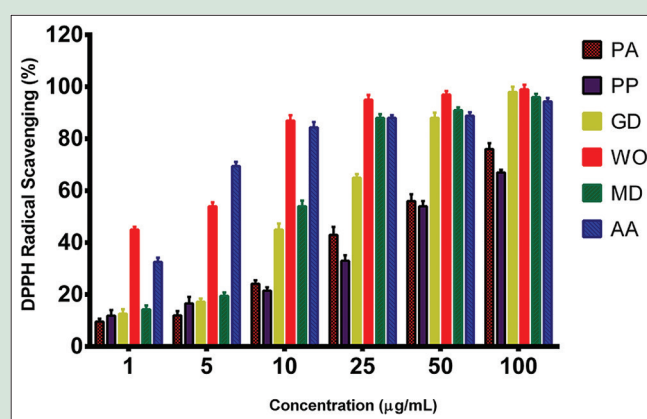


Figure 2: Antioxidants activity on 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical of different extracts. Values were expressed as mean \pm standard deviation ($n = 3$)

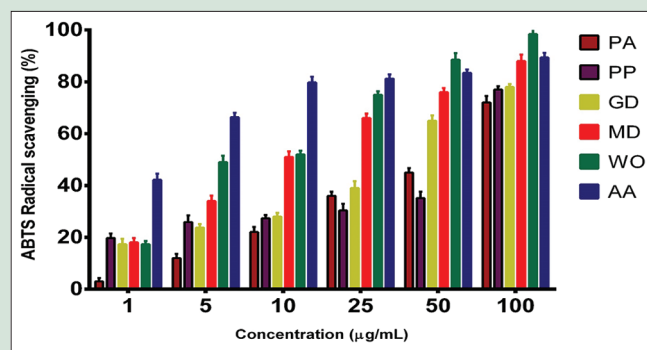


Figure 3: Antioxidant activity on 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical of different extracts. Values were expressed as mean \pm standard deviation ($n = 3$)

Table 1: Phytochemical constituents of ethanolic extracts of five Peruvian plants

Constituents	Test	GD	MD	PA	WO	PP
Alkaloids	Mayer	+	+	+	-	+
	Dragendorff	+	+	+	-	+
	Wagner	+	+	+	-	+
Flavonoid	Shinoda	+	+	+	+	+
	Bornträger	+	-	-	-	-
Phenols compounds	Ferric chloride	+	+	+	+	+
Saponins	frothing	+	-	+	+	+
Tannins	Gelatin	+	-	-	+	+
Terpenes and steroids	Liebermann-Burchard	+	+	+	+	+

+: Positive; -: Negative; GD: *Geranium dielsianum*; MD: *Myrciaria dubia*; PA: *Piper aduncum*; WO: *Waltheria ovata*; PP: *Physalis peruviana*

Cytotoxicity assay

Based on the results of linear regression, IC_{50} were calculated and are represented in Table 3. According to the National Cancer Institute (NCI) (USA), a half $IC_{50} < 20 \mu\text{g/mL}$ is considered as promising for an extract. The correlation response-concentration was calculated using Rho's Spearman test, these values were between 0.96 and -0.98 ($P < 0.01$) for all extracts $-0.97 y - 0.98$ ($P < 0.01$) for 5-FU.

DISCUSSION

The increasing interest for searching natural treatment in cancer disease has led to the evaluation of a number of plant sources. In the present study, extracts of MD, PP, GD, PA, WO were evaluated to determine its potential effect as antioxidant and cytotoxic. To achieve this purpose, the phytochemical analysis, TPC, antioxidant capacity, and cytotoxic activity on various tumor cell lines of ethanolic extracts of above-mentioned plants were investigated. The data presented in this study revealed that all extracts presented antioxidant activities. This suggests that the evaluated extracts could protect *in vivo* against oxidative damage and free radical occurring in different pathological mechanisms. The antioxidant activity of plant extracts is usually linked to their phenolic content. Furthermore, polar extracts have a high phenolic content due to its high rate of extractions.^[3] Preliminary phytochemical analysis is important to confirm the phytochemical characteristics in vegetable species. Regarding this test, all extracts have in common phenolic groups as secondary metabolite, these results were confirmed by and Herrera-Calderon *et al.*^[4,5]

Reactive oxygen species and reactive nitrogen species play an essential role in the oxidative and nitrosative stress as specified previously.

Table 2: Half inhibitory concentration values of five Peruvian plant extracts for 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

Samples	IC_{50} ($\mu\text{g/mL}$) [†]	
	DPPH radical	ABTS radical
GD	24.21±2.14	32.45±2.00
MD	10.21±2.50*	21.50±1.20**
PA	48.91±2.31	56.25±0.25
WO	0.89±0.01**	4.20±1.50*
PP	15.16±3.45*	68.25±2.00*
AA	4.01±1.26	5.00±0.80

[†]Values were expressed as mean±SD ($n=3$); * $P < 0.05$; ** $P < 0.01$ significant difference as compared to standard. IC_{50} : Half inhibitory concentration; AA: Arachidonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); GD: *Geranium dielsianum*; MD: *Myrciaria dubia*; PA: *Piper aduncum*; WO: *Waltheria ovata*; PP: *Physalis peruviana*; SD: Standard deviation

Recently, there has been an upsurge of interest in the therapeutic potential of medicinal plants.^[17] Among all phytochemicals, phenolic compounds, flavonoids, tannins, and terpenoids are responsible for antioxidant activities.^[18] Phenolic compounds are antioxidants for the protection of oxidative damage to crucial biomolecules (i.e., DNA, lipids, and proteins) involved in numerous diseases.^[19]

In the scavenging of various free radicals, flavonoids are highly effective by their redox potential. There has been increasing interest in the research on flavonoids obtained from plants since pharmacological properties of flavonoids are directly linked with their antioxidant potentiality.^[20] The results of this study endorsed that polyphenolic constituents (i.e., phenolic acids, and flavonoids) may be the foremost contributing agents for the antioxidant activity, in this study highest phenolic and flavonoid contents were reported for WO.^[21] An earlier study also exposed almost similar findings. Otherwise, this kind of metabolite with phenolic structure has been linked with multiple biological functions in human beings such as anti-inflammatory, anticancer, hypoglycemic, hypocholesterolemic, antibacterial, antifungal, antiviral activity, and analgesic activities.^[22] The electron gifting capability of the medicinal plant is most widely determined using DPPH and ABTS radical scavenging tests due to its reliability.^[23] In DPPH assay, a purple-colored solution of DPPH radical by accepting electron converted to discolor DPPH-H. In fact, the degree of color change is related to the concentration and effectiveness of the antioxidants. The degree of discoloration with respect to the decrease in the absorbance of the reaction mixture indicates free radical scavenging action.^[24] In the study among the extracts tested, WO and MD exhibited higher percentage of scavenging. This study suggests that the plant extract that contains flavonoid and related polyphenols are proficient for donating hydrogen to a free radical to prevent the disease related to free radical-mediated by oxidative stress.^[25] According to the US NCI to considered a plant extract as potential cytotoxic agent, IC_{50} values should be $< 20 \mu\text{g/mL}$ and for isolated compounds $< 4 \mu\text{g/mL}$.^[26] The cytotoxic effect of many plant extracts could be related to its antioxidant effect; it has been revealed that high doses of antioxidant on tumor cell lines leads to an apoptosis and cytotoxic activity, generally it is attributed to phenolic components, alkaloids, flavonoids, triterpenes, and others.^[27] However, further molecular mechanistic studies are required to understand the role of these extracts on cytotoxic and apoptotic activity.^[28] The cytotoxic effect showed on some tumor cell lines like H460 and K562 could be linked to one or more phytoconstituents. Cytotoxic agents may cause necrosis; cell membrane destruction leading to cell lysis or induce apoptosis cell death by activating numerous biochemical mechanisms.^[29] The low response of the evaluated extracts to induce cytotoxicity could be explained by its antioxidant effect, it has evidenced that at low concentrations an antioxidant has many roles like preventing oxidation, which induces apoptosis. However, at high content can increase the production of the ATP (generated by mitochondria) inducing apoptosis in tumor cell lines, through a pro-oxidant mechanism.^[30]

Table 3: Cytotoxicity of five Peruvian plants species on different human tumor cell lines

Cytotoxic samples	Values of IC_{50} ($\mu\text{g/mL}$) [†]						
	Tumor cell lines						Mouse embryo normal cells (3T3)
	MCF-7	K-562	HT-29	H-460	M-14	DU-145	
GD	112.25±3.50	189.44±2.48	196.54±1.32	75.13±1.25	175.5±1.60	127.03±3.20	58.12±3.10
MD	35.44±2.85	37.30±1.00	45.12±2.60	26.78±2.23	125.43±1.43	33.10±2.50	68.21±2.11
PA	79.25±1.65	167.34±1.88	155.52±2.50	93.12±2.00	>250.00	>250.00	53.11±1.14
WO	14.50±1.50	17.45±1.85	93.00±2.00	13.12±1.10	85.18±2.60	>250.00	125.14±2.10
PP	89.05±0.81	94.34±1.00	105.02±2.69	111.32±1.05	132.3±0.60	125.09±0.09	79.5±2.10
5-FU*	0.645±0.05	4.08±0.54	0.33±0.01	0.35±0.02	1.17±0.09	>15.63	<0.24

[†]Values were expressed as mean±SD ($n=3$); * $P < 0.05$; ** $P < 0.01$ significant difference as compared to standard. GD: *Geranium dielsianum*; MD: *Myrciaria dubia*; PA: *Piper aduncum*; WO: *Waltheria ovata*; PP: *Physalis peruviana*; SD: Standard deviation; IC_{50} : Half inhibitory concentration; 5-FU: 5-fluorouracil

Animals studies are necessary for assessing anticancer properties of these medicinal plants, but this research constitutes the first step to discover new alternatives and carry out future assays using *in vitro* models. On the other hand, isolating phytochemicals could explain the main role of each secondary metabolite in antioxidant and cytotoxic activities of these plants.

CONCLUSION

In this research, the TPC of five Peruvian plants showed a high content, furthermore, exhibited a strong free radical scavenging activity against DPPH and ABTS radicals. WO extract exhibited cytotoxic activity against H460 and K562 cell line, the other extracts presented low cytotoxicity. However, these plants could be good natural sources of antioxidants and alternative treatment for cancer.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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