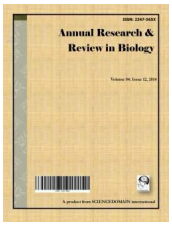
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|---|---|
| Item Type | info:eu-repo/semantics/article |
| Authors | Herrera-Calderon, Oscar; Arroyo-Acevedo, Jorge; Rojas-Armas, Juan; Chumpitaz-Cerrate, Victor; Figueroa-Salvador, Linder; Enciso-Roca, Edwin; Tinco-Jayo, Johnny |
| Citation | Phytochemical Screening, Total Phenolic Content, Antioxidant and Cytotoxic Activity of Chromolaena laevigata on Human Tumor Cell Lines 2017, 21 (1):1 Annual Research & Review in Biology |
| DOI | 10.9734/ARRB/2017/37045 |
| Publisher | SCIENCEDOMAIN international |
| Journal | Annual Research & Review in Biology |
| Rights | info:eu-repo/semantics/openAccess |
| Download date | 16/08/2022 13:51:00 |
| Link to Item | http://hdl.handle.net/10757/622501 |



*Annual Research & Review in Biology*

***21(1): 1-9, 2017; Article no.ARRB.37045***
***ISSN: 2347-565X, NLM ID: 101632869***

# Phytochemical Screening, Total Phenolic Content, Antioxidant and Cytotoxic Activity of *Chromolaena laevigata* on Human Tumor Cell Lines

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors OHC, JLAA and JRA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VCC and LFS managed the analyses of the study. Authors EER and JATJ managed the literature searches. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/ARRB/2017/37045
Editor(s):
(1) Gunanidhi Dhangadamajhi, Department of Biotechnology, North Orissa University, Baripada, Odisha, India.
(2) Md. Torequl Islam, Nuclear of Pharmaceutical Technology (NTF), Postgraduate Program in Pharmaceutical Sciences, Federal University of Piaui, Brazil.
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Reviewers:
(1) Divya S. Rajan, Christian College, Kerala University, India.
(2) Md. Abu Sayed, Hajee Mohammad Danesh Science and Technology University, Bangladesh.
Complete Peer review History: http://www.sciencedomain.org/review-history/22330

***Original Research Article***

***Received 28th September 2017***
***Accepted 9th November 2017***
***Published 16th December 2017***

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## ABSTRACT

**Aims:** Cancer is the first cause of death in the Peruvian population; searching alternative treatments of medicinal plants constitute a promissory field to find new anticancer drugs. The main objective in this study was to evaluate the phytochemical screening, total phenolic content, antioxidant and cytotoxic activity of ethanol extract of *Chromolaena laevigata* (*C. laevigata*) on human tumor cell lines.
**Study Design:** The fresh leaves of *C. laevigata* were soaked with ethanol followed by phytochemical screening using standard methods.
**Place and Duration of Study:** Laboratory of Applied Chemistry, Faculty of Pharmacy and Biochemistry, Universidad Nacional San Luis Gonzaga de Ica, Ica, Peru; Laboratory "Abraham Vaisberg Wolach", Universidad Peruana Cayetano Heredia, Lima, Peru.
**Methodology:** Phytochemical screening was assessed by using chemical reactives. Total phenolic content (TPC) was developed using Folin Ciocalteu reactive and the antioxidant activity was determined against DPPH and 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 ethanol extract. TPC resulted 45.21 ± 3.5 mg of gallic acid equivalent/g of dried extract. The highest antioxidant extract for DPPH and ABTS radical scavenging tests were $IC_{50}$ = 11.66 ± 1.0 µg/mL, $IC_{50}$= 12.45 ± 0.50 µg/mL respectively. Ethanolic extracts (µg/mL) showed a low cytotoxicity on human tumor cell lines ($CI_{50}$ > 20 µg/mL) for DU-145, HT-29, MCF-7 and M-14. Whereas, for H-460, and K562 tumor cell lines showed high cytotoxicity.
**Conclusion:** In our findings, *C. laevigata* demonstrated a high antioxidant and total phenolic content. The ethanol extract exhibited better cytotoxic effect compared with 5-FU. Hence, This medicinal plant could be effective to prevent chronical diseases as cancer and oxidative stress disorders.


*Keywords: Chromolaena laevigata; phytochemical; antioxidant; flavonoids; oxidative stress; cytotoxicity; anticancer.*

## ABBREVIATIONS

*Ch laevigata : Chromolaena laevigata;*
*5-FU : 5-Fluoro-uracil;*
*ABTS : 2,2´-azino-bis (3ethylbenzothiazoline-6-sulfonic acid;*
*DPPH : 2,2-diphenyl-1-picrylhydrazyl;*
*Trolox : 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid;*

## 1. INTRODUCTION

Cancer is considered as a serious public health problem worldwide that leads to search for antitumor agents, especially from natural sources, which have become an important research field in the scientific community [1]. Cancer is characterized by alteration of gene expressions, cell proliferation, and caused by exposure to carcinogenic agents, mutations and others [2].

Oxidative stress is an imbalance between the production of free radicals and antioxidant system, these generated radicals impairs 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 DNA (8 hydroxyl-2′-deoxyguanosine) causing transversions of GC (guanine-cytosine) to TA (thymine-adenine) [4].

Antioxidants are chemical compounds from natural or synthetic sources that can act as chain breakers on radicals such as peroxyl, alkoxyl or hydroxyl, chelating pro-oxidative metal ions and quenching singlet oxygen [5]. New natural products have been studied on many chronical diseases as cancer, diabetes, hypertension and overall those related with oxidative stress process [6,7].

Many medicinal plants are used by patients with cancer due to numerous positive effects on pain relief, vomiting, nauseas, depression, and anxiety [8]. Bioactive compounds such as polyphenols, tannins, flavonoids have antioxidant capacity with potential benefits for health and

could reduce cancer risk, it has been demonstrated that the anti-inflammatory effect of these compounds inhibit inflammatory cytokines, arachidonic acid (AA), prostaglandins, and leukotrienes [9]. Polyphenols are chemoprotectives that act by blocking the initiation stage of carcinogenesis, free radicals and activation of glutathione peroxidase, glutathione reductase and inactivation of glutathione S transferase [10].

Medicinal plants are the best sources of natural medicine in the world [11]. In Peru, there exists various vegetables species with potential use for commercial purposes and they have been studied by its therapeutic effect for the treatment in various diseases such as cancer, diabetes, and hypertension. *Chromolaena laevigata* is a species within the family Asteraceae called "chilca". The complementary medicine plays a promising role in cancer symptoms management by facilitating its easy access and administration in-developing countries. These kind of natural supplements are less toxic and provide fewer side effects compared to conventional medications. Therefore, it is important to establish a scientific validation of this species for cancer prevention. Hence, the main objective in this research was to evaluate the preliminary phytochemical screening, total phenolic content, antioxidant and cytotoxic activity of ethanol extract of *Chromolaena laevigata* on human tumor cell lines.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

5-Fluorouracil (5-FU), Folin-Ciocalteu reagent (FC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium peroxydisulfate, 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), were purchased from Sigma-Aldrich. Other chemicals used were of analytical grade.

### 2.2 Plant Material

Aerial parts of *Chromolaena laevigata* were collected, in January, 2016, from Chalhuanca, department of Apurimac, Peru, and identified by a botanic. Voucher specimen identified as 272-USM-2015 was deposited at the National Herbarium of Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru.

### 2.3 Extraction of Plant Material

Collected samples were dried at room temperature, at the Chemical Laboratory, Faculty of Pharmacy and Biochemistry, Universidad Nacional San Luis Gonzaga de Ica (UNICA), the powder material was exhaustively macerated with 96% ethanol for 7 days. The extract was filtered and evaporated by using a rotavap with 80 rpm and 40ºC.

### 2.4 Phytochemical Screening

Ethanol extract was evaluated by using specific chemical reactives for each secondary metabolite such as phenolic compounds, flavonoids, quinone, triterpenes, flavonoids, tannins, saponins, steroids and alkaloids [12].

### 2.5 Total Phenolic Content (TPC)

The total phenolic content was determined by Folin-Ciocalteu method in according to Singleton and Rossi [13]. Extract was diluted with distilled water. Next, 100 µL of the diluted samples, 150 µL of Folin-Ciocalteu's reagent (1/8 diluted in distilled water) and 150 µL 20% $Na_2CO_3$ were added and mixed. After standing for 5 minutes, 600 µL distilled water were added. The absorbance was measured at 760 nm after 90 min with distilled water as blank sample. Total phenolic content 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.

### 2.6 DPPH Radical Scavenging Assay

In according with the method of Okawa et al., with minor modifications, DPPH radical scavenging activity of extract was determined [14]. To 100 µL of different extracts at numerous concentrations (1 to 50 µg/mL) were added to 1900 µL 0.01mM 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 trolox 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. $IC_{50}$ value is the concentration of extract at which DPPH radicals

are scavenged by 50%. The lower $IC_{50}$ value indicates higher radical scavenging capacity.

## 2.7 ABTS 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 reaction mixture and incubated in the dark at room temperature for 7 min; the absorbance was read at 734 nm using an UV-VIS Spectrophotometer. The ABTS scavenging activity of the extracts was compared with trolox and the percentage inhibition was calculated as:

ABTS radical scavenging activity (%) = [(Abs control − Abs sample)]/(Abs control) × 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 $IC_{50}$ value as mentioned above in DPPH assay.

## 2.8 Cytotoxic Activity

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 ((non-tumorogenic, BALB/c mouse embryo cells) cell lines were obtained from the American Type Culture Collection (ATCC). The tumor cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 50 $\mu$g/mL gentamycin in humidified 5% $CO_2$/95% air at 37℃ for 24 h.

In according to Skehan et al. [16] Different dilutions of extract and 5-FU (0-250 µg/mL) were added to different plates containing the human tumor cell lines and incubated for 48 hours. Sulforhodamine B (SRB) was used as 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 by using a microplate reader. The results were expressed as half inhibitory concentration ($IC_{50}$) and were found by linear regression analysis.

## 2.9 Statistical Analysis

The antioxidant and cytotoxic activity were expressed as mean ± standard deviation (SD) from three observations. The statistical and graphical analysis was performed by linear regression in order to determine TPC, antioxidant and cytotoxic activity. Microsoft Excel Program 2016 was used for Rho`s Spearman test considering P values less than 0.05 as significant.

# 3. RESULTS AND DISCUSSION

## 3.1 Determination of Phytochemical Constituents

Phytochemical analysis is a qualitative test which indicates the presence of groups of compounds in a sample by using formation of a precipitate or a color change. The extract of *Ch. laevigata* indicated various classes of secondary metabolites such as tannins, phenolic compounds and flavonoids; however, quinone was positive in according to Table 1.

**Table 1. Phytochemical constituents of the ethanolic extract of *Ch. laevigata* leaves**

| Constituents | Test | Result |
|---|---|---|
| | Mayer | + |
| Alkaloids | Dragendorff | + |
| | Wagner | + |
| Flavonoid | Shinoda | + |
| Quinone | Bornträger | - |
| Phenols compounds | Ferric chloride | + |
| Saponins | frothing | + |
| Tannins | Gelatin | + |
| Terpenes and steroids | Liebermann–Burchard | + |

*Notes: (+) positive, (-) negative*

### 3.2 Determination of TPC

Content of total phenolic compounds was detected in the ethanolic extract (45.21 ± 3.5 mg GAE/g extract). Phenolic content was calculated from the standard curve of gallic acid ($y = 0.0150x + 0.0435$; $R^2 = 0.991$).

### 3.3 Determination of DPPH Radical Scavenging Activity

The $IC_{50}$ values against DPPH radical are shown in Fig. 1A. Compared to trolox the $IC_{50}$ value of *Ch. laevigata* was statistically significant ($IC_{50}$ = 11.66 ± 1.0; $P < 0.001$).

### 3.4 Determination of ABTS Radical Scavenging Activity

In Fig. 1B, significant differences ($P < 0.001$) were revealed between ABTS scavenging capacities of extract and trolox. *Ch. laevigata* extract showed a $IC_{50}$ value (12.45 ± 0.50 µg/mL) and was not statistically significant ($P = 0.211$) compared to trolox.

### 3.5 Cytotoxicity Assay

Based on the results by linear regression, inhibitory concentration ($IC_{50}$) was calculated and is represented in Table 2. According to the National Cancer Institute (USA), a half inhibitory concentration ($IC_{50}$) less than 20 µg/mL is considered as promising for an extract. The correlation response-concentration was calculated by using Rho`s Spearman test, these values were between 0.99 and -0.98 ($P < 0.001$) for *Ch. laevigata* extract and 0.98 ($P < 0.001$) for 5-FU.

## 4. DISCUSSION

The increasing interest for searching natural treatment in cancer disease has led to the evaluation of a number of natural sources. In the present study, *Ch. laevigata* extract was evaluated in order to determine its potential effect as antioxidant and cytotoxic. To achieve this purpose, the phytochemical analysis, total phenolic content, antioxidant and cytotoxic activity on various tumor cell lines were investigated. The data presented in this study revealed that the ethanol extract presented a good antioxidant activity. This suggests that the extract could protect *in vivo* systems against oxidative damage and free radical genesis 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. In this test, the ethanol extract of *Ch. laevigata* presented phenolic groups, flavonoids, alkaloids, triterpenes and saponins as secondary metabolites; these results were confirmed by Herrera et al. in previous studies [5].

Reactives oxygen species (ROS) and reactives nitrogen species (RNS) play an essential role in the oxidative and nitrosative stress as specified previously. Recently, there has been an upsurge of interest in the therapeutic potential of medicinal plants [17]. The phytochemical products such as phenolic compounds, flavonoids, tannins and terpenoids are responsible for antioxidant effects *in vitro* and *in vivo* studies [18]. Furthermore, it is known that flavonoids like rutin and quercetin are antioxidants used to protect the 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 in 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 a high phenolic content was reported for *Ch. laevigata* [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, hypoglycaemic, hypocholesterolemic, antibacterial, antifungal, antiviral activity and analgesic activities [22]. The electron gifting capability of the medicinal plant is most widely determined by DPPH and ABTS radical scavenging tests owing to its reliability. [23] In DPPH assay, purple-colored solution of DPPH radical by accepting electron was converted to discoloured 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 decrease in the absorbance of the reaction mixture indicates free

radical scavenging action [24]. This research 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 National Cancer Institute (NCI) to considerer a plant extract as potential cytotoxic agent, $IC_{50}$ values should be less than 20 µg/mL and for isolated compounds less than 4µg/mL [26]. The cytotoxic effect of many plant extracts could be related to its antioxidant effect; it has been demonstrated 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, triterpenos 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 or 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 was evidenced that at low concentrations antioxidants have many roles like preventing oxidation, which induces apoptosis. However, at high content can increase the production of ATP (generated by mitochondria) inducing apoptosis in tumor cell lines, via a pro-oxidant mechanism [30].

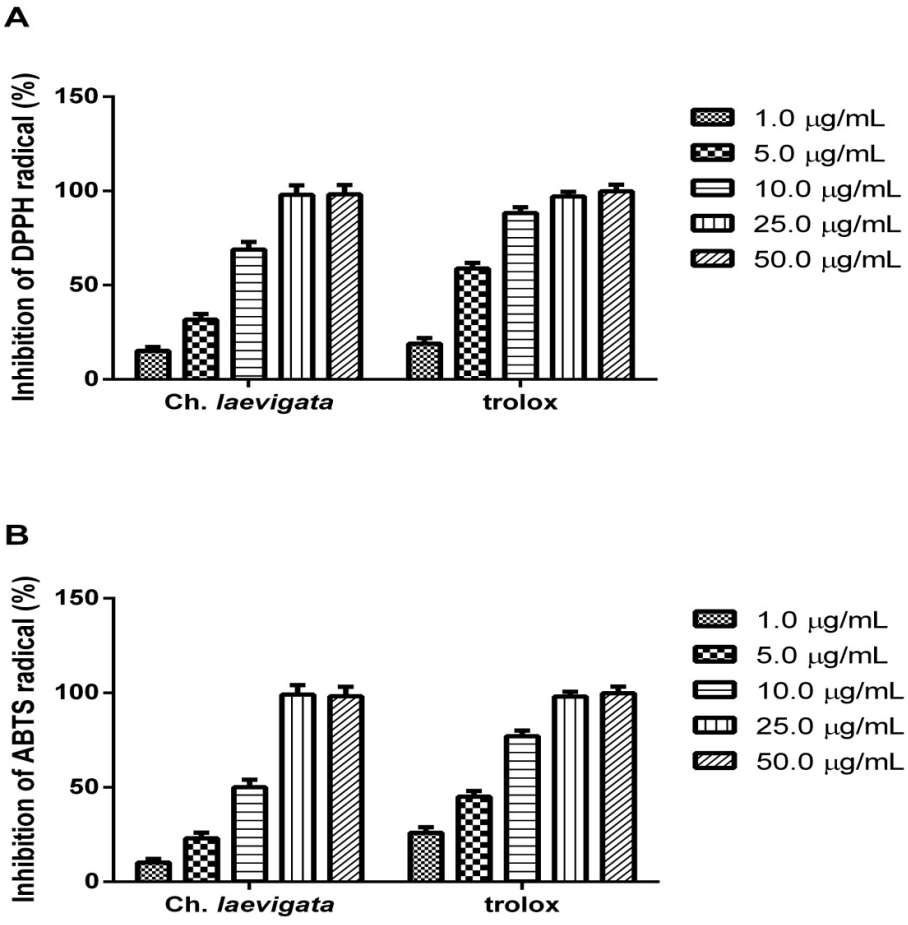



**Fig. 1. Antioxidant activity of *Ch. laevigata* extract and trolox (standard antioxidant) at various concentrations. Values were expressed as mean ± SD (n = 3). A: Percentage of antioxidant activity on DPPH radical. B: Percentage of antioxidant activity on ABTS radical**

**Table 2. Cytotoxicity of *Ch. laevigata* on different human tumor cell lines**

| Cytotoxic samples | Values of $IC_{50}$ (µg/mL) † | | | | | | Mouse embryo normal cells |
|---|---|---|---|---|---|---|---|
| | Tumor cell lines | | | | | | |
| | MCF-7 | K-562 | HT-29 | H-460 | M-14 | DU-145 | 3T3 |
| ***Ch. laevigata*** | 35.25± 0.81 | 7.34 ± 1.00 * | 78.52 ± 2.69 | 55.32 ± 1.5 | 112.3 ± 0.60 | 97.09 ± 0.09 | 18.8 ± 2.10 |
| **5-FU*** | 0.645 ± 0.05 | 4.08 ± 0.54 | 0.33± 0.01 | 0.35 ± 0.02 | 1.1 7± 0.09 | > 15.63 | < 0.24 |

*†Values were expressed as mean ± SD (n = 3).*

**P < 0.001 significant difference as compared to standard*

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

## 5. CONCLUSION

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

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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