Antioxidant and anti-cancer activities of *Anacyclus pyrethrum* root extracts

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**ABSTRACT**

This study aims to investigate the antioxidant and cytotoxic activities of two ecotypes of *Anacyclus pyrethrum*, an indigenous medicinal plant prized for its roots for various medicinal virtues. Plant samples were collected from the Central Mid-Atlas Mountains of Morocco and root extracts were prepared. Total polyphenols and total flavonoid contents were determined in the extracts and antioxidant activity was measured using DPPH free radical scavenging method. Cytotoxicity effect was assessed for two cell lines, HeLa (human cervical cancer) and N2a (mouse neuroblastoma) against increasing concentrations of test extracts of 12.5, 25, 50, 100, 150 and 200 µg/ml and cell viability was quantified using ATP assay. The results obtained showed that total polyphenols were relatively high, with 83.9 mg GAeq/g for ecotype-1 as compared with 57.3 mg GAeq/g for ecotype-2. Total flavonoids presented moderate values of about 19.3 mg Qeq/g for ecotype-1, but low of about 2.3 mg Qeq/g for ecotype-2. Both ecotypes expressed a strong antioxidant effect against DPPH radicals, with comparable IC\textsubscript{50} values of 44 and 46 µg/ml for ecotype-1 and ecotype-2, respectively as compared with 11.7 µg/ml for ascorbic acid used as the reference antioxidant. The cytotoxicity assay revealed a strong inhibitory effect of root extracts of both ecotypes against the two cancer cell lines evaluated. The IC\textsubscript{50} values were 106.7 and 165.7 µg/ml, respectively for HeLa and 34.1 and 73.7 µg/ml for N2a, indicating a higher potency of pyrethrum extracts against N2a Cells as compared with HeLa Cells, with a higher effect of ecotype-1 as compared with ecotype-2. These findings highlight the pharmaco-therapeutic properties of *A. pyrethrum* roots as an important and natural source of polyphenols with important antioxidant activity and high cytotoxicity effect against cancer cells.

**Key words:** *Anacyclus pyrethrum*, polyphenols, flavonoids, antioxidant activity, cytotoxicity.

**INTRODUCTION**

Plants have been used for their medicinal and aromatic virtues for centuries and represent the foundation of traditional medicine in many parts of the world (Farnsworth et al., 1985; Van Wyk, and Wink, 2004; Lev and Amar, 2008; Hye-Lim and Hun-Soo, 2012; Adhikari and Paul, 2018). They are inexhaustible sources of naturally occurring bioactive compounds. A renewed interest in natural products has emerged to find safe and effective alternatives to chemical drugs. Plant extracts stand their effects from individual compounds, but synergetic effects from various constituents are most likely to explain their benefits.

Anti-oxidant activities of phytochemical compounds from a variety of plants are considered to have protective and
curative effects on many health ailments. Phenolic compounds, particularly the flavonoids, have been established to possess a wide range of biochemical activities, including antioxidant, antmutagenic, anticarcinogenic, antimicrobial, antiarthritic, protein-kinase inhibition, immune-stimulant, etc (Bahri et al, 2018; Zaidi, 2013; Bendjeddou et al, 2003; Ren et al, 2003; Dalila et al, 2010; Pahuja et al., 2012; Sulaiman and Balachandran, 2012; Jalayer-Naderi et al., 2016; Kumar, 2017; Tungmunithum et al., 2018). Cancer is a major health concern, and is largely due to lifestyle and environmental factors (Gonzalez and Riboli, 2010). Cancer problems could find answers in yet undiscovered virtues of many plants. Recent studies are discovering important inhibitory effects of extracts from many plants species on various cancer cell lines (Mohammadi et al, 2016; Mohammadi et al, 2017).

Anacyclus pyrethrum L, is among the plant species highly valued for the medicinal properties of its roots. This plant, endemic to Morocco and Algeria (Dobignard, A. 1989; Bellakhdar et al, 1991; Bellakhdar, 1997; Benabid 2002; Batanouny, 2005; Fennane and Ibn Tatou, 2008; Dobignard and Chatelain, 2010), also occurs in many other countries around the world (Iran, India, Pakistan, etc). Medicinal use of root extracts from this species include mouth dryness, gum problems, pharyngitis, rheumatism, face palsy, epilepsy, seizure and skin pigmentation disorders (Rani et al, 2013; Daoudi et al, 2017; Tauheed et al, 2017). Recent studies explored the potential effects of this plant on other aspects such as memory enhancement, sexual impotency, rejuvenation and cancer (Puri, 2003; Sharma et al., 2009; Badhe et al, 2010; Tyagi et al., 2011; Annalakshmi et al., 2012; Sharma et al, 2013; Sharma et al., 2013; Shahraki et al, 2014; Usmani et al., 2016; Mohammadi et al., 2016). The chemical composition of A. pyrethrum root extracts have been reported to contain a variety of compounds such phenols, flavonoids, alkylamides, alkaloids, tannins, saponin, terpenes and sterols (Crombie, 1954; Burden and Crombie, 1969; Jente et al, 1972; Harald 1978; Boonen et al, 2012; El Azzouzi et al, 2014; Usmani et al, 2016; Subasri and John, 2016; Althaus et al., 2017; Daoudi et al, 2017; Hosseini et al, 2018). 

The roots virtues of this plant could be attributed to any of the individual compounds (molecules), but more likely to synergistic effects of several of these compounds. In Morocco, two main ecotypes of A. pyrethrum are present and are distinctly known among the population that collects them in the wild. They are locally referred to as Tiguendezt and Iguendez. Tiguendezt, the more abundant ecotype, presents a small (8-12 cm) and thin (3-8 mm Ø) root and a relatively small flower heads. Iguendez, the less abundant, but the most prized, is characterized by a longer (12-18 cm) and thicker (7-15 mm Ø) root and a larger flower heads. The aim of this study was to investigate the antioxidant and anti-cancer activities of roots extracts from these two ecotypes of A. pyrethrum found in Morocco.

MATERIALS AND METHODS

Plant collection and preparation

Plant materials of the two ecotypes described above were collected from an open grazing land in the Middle Atlas mountain area of Morocco, about 15 km southeast of the city of Azrou, at an altitude of about 1790 m. This region is among the main habitats of this species. Plant samples were collected at flowering stage in early spring. The roots were separated, washed, air-dried, finely grounded and sieved to 1 mm. The two ecotypes are designated in this paper as ecotype-1 and ecotype-2 for Tiguendezt and Iguendez types, respectively.

Antioxidant activity assessment

Preparation of methanolic root extracts

Root extracts were prepared according to Li et al. (2008). A 0.5 g of root powder was macerated in 20 ml methanol at 40°C for 24 h and centrifuged at 4500 tr/min for 15 min. The supernatant was filtered and evaporated under reduced pressure at 40°C, weighed and then dissolved in methanol to a final concentration of 10 mg/ml and kept at 4°C.

Total polyphenols and total flavonoids

Total polyphenols (TP) content was determined according to the Folin-Ciocalteu reagent method (Folin and Ciocalteu, 1927; Singleton and Rossi, 1965; Singleton et al, 1999). 2 ml of the Folin-Ciocalteu reagent were diluted 10 fold in distilled water and added to 0.4 ml of root extracts at a concentration of 2.5 mg/ml. After 4 min, 3.2 ml of sodium carbonate solution (75 mg/ml) was added and the mixture was incubated in the dark for 2 h. Polyphenol content was measured at 765 nm using a UV-VIS spectrophotometer. Gallic acid, with concentrations ranging from 25 to 200 µg/ml was used to make a standard calibration curve. TP contents were expressed as Gallic acid equivalent (GAEg).

Total flavonoids (TF) content was determined according to Baharun et al. (1996) using the Ammonium Chloride method. 2 ml of root extracts at 2.5 mg/ml were mixed with a 2% ammonium chloride solution and incubated for 15 min at room temperature. Absorbance was measured at 430 nm using a UV-VIS spectrophotometer. Quercetin, with concentrations ranging from 0 to 35µg/ml was used to make a standard calibration curve. TF contents were expressed as Quercetin equivalent (Qeq).

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity

DPPH radicals scavenging activity of pyrethrum root
Table 1: Total polyphenols (TPC) and total flavonoids (TFC) contents and antioxidant activity of the two pyrethrum ecotypes (mean ± SD; n=3, and p<0.05).

<table>
<thead>
<tr>
<th>A. pyrethrum ecotypes</th>
<th>TP (mg GAeq/g)</th>
<th>TFC (mg Qeq/g)</th>
<th>Antioxidant activity (IC-50) µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecotype-1</td>
<td>83.9 ± 3.0</td>
<td>19.3 ± 0.5</td>
<td>36.4</td>
</tr>
<tr>
<td>Ecotype-2</td>
<td>57.3 ± 0.2</td>
<td>2.3 ± 0.02</td>
<td>38.7</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td></td>
<td>11.7</td>
</tr>
</tbody>
</table>

extracts was evaluated according to the method of Brand-Williams et al. (1995). 3.75 ml root extracts at varying concentrations (0.01, 0.05, 0.1, 0.15 and 0.2 mg/ml) were mixed to 0.25 ml of a 0.8 mM Methanolic DPPH solution. The mixture was incubated for 30 min in the dark at room temperature. Absorbance was measured at 517 nm against a blank with no root extract added. Ascorbic acid, with concentrations ranging from 1 to 0.4 mg /ml, was used to make a standard calibration curve.

DPPH radical scavenging activity was expressed as percent inhibition relative to the blank (negative control) as:

% Inhibition = [(Abs. C – Abs. T)/Abs. C]*100

Where, Abs. C=Absorbance of the negative control, Abs. T=Absorbance of the test sample.

DPPH inhibition values were plotted against the Log of the concentrations of the extracts and IC50 values were derived using GraphPad Prism 8.

Cytotoxicity assay

Preparation of ethanolic root extract

Pyrethrum ethanolic extracts were obtained by mixing 10 g of ground roots with 100 ml ethanol at 70°C for 24 h, then evaporating under vacuum. The yielded extract was dissolved in dimethyl sulfoxide (DMSO) with appropriate volumes to prepare a stock solution at the concentration of 400 µg/ml root extracts.

Cells and culture protocol

Two cancer cell types were used: HeLa, a human cervical adenocarcinoma and N2a, a mouse Neuroblastoma cancer cell. The two Cancer cell lines were obtained from the Department of Biology of the College of Staten Island, City University of New York.

Cancer cells were grown in Eagle's MEM (EMEM) supplemented with 10% fetal calf serum (FCS) in deionized water and 1% streptomycin. The growth medium was renewed 3 times per week and cells were observed under microscope to evaluate cell confluence. Cell count was performed with trypan blue solution. The pH of the medium was adjusted to 7.4 and kept at 4°C before use.

Cell viability against pyrethrum root extracts

Cancer cell viability was evaluated against increasing concentrations of pyrethrum root extracts in comparison with the negative control (blank with no addition of extract). 200 µl of cell lines in media were seeded in 24-microwell plates at a density of 10^4 and root extracts were added to the wells in appropriate dilutions with media in order to achieve six test concentration of 200, 150, 100, 50, 25 and 12.5 µl. Final DMSO concentration was less than 0.2%. The plates were incubated at 37°C with 95% O2 and 5% CO2 for 48 h, then cell viability was assessed using the ATP-assay. The plates were allowed to equilibrate to ambient temperature for 30 min, and Cell Titer-Glo® reagent was added to all wells (v:v) and mixed for 2 min on an orbital shaker to induce cell lysis. The plates were allowed to incubate for 10 min. Luminescence was immediately measured in relative light units (RLU) using a multimode microplate reader. The results were expressed in percent inhibition relative to the control. The percent inhibition of cell lines against log of concentrations of test extract was fitted using GraphPad Prism 8, and the corresponding IC50 was derived.

RESULTS AND DISCUSSION

Total Polyphenols (TP) and total flavonoids (TF)

The results obtained showed that the two pyrethrum ecotypes have important TP levels, with 83.9 mg GAeq/g for ecotype-1 and 57.3 mg GAeq/g for ecotype-2 (Table 1). TF values were moderate for ecotype-1 with 19.3 µg Qeq/mg, but they were comparatively low for ecotype-2 with 2.3µg Qeq/mg (Table 1). The contents of TP of ecotype-1 are close to those reported by Daoudi et al. (2017) for A. pyrethrum from Morocco who reported values ranging from 66.5 to 97.6 mg GAeq/g with different water extraction methods. Cherrat et al. (2017) reported on the other hand lower values of TP and TF using various extraction methods, and the highest values obtained were those with water-methanol extraction, being 21.8 and 9.6 mg Qeq/g of root extracts respectively. Using methanolic
extration, Kalim et al. (2010) found values of 63.8 and 32.9 mg Qeq/g for TP and TF in root extracts of *Anacyclus pyrethrum* from India, respectively.

Total polyphenols contents found for both ecotypes are considered high as compared with other plant extracts reputed rich in these compounds. Nicolì et al. (2019) reported values for olive leaves from different varieties to range from 11.4 to 48.6 mg GAeq/g. John et al. (2014) reported values of TP not exceeding 17.2 mg GAeq/g for several medicinal plants.

**Antioxidant activity**

The antioxidant activity of *A. pyrethrum* root extracts, as measured by the ability to scavenge DPPH free radicals, were compared with that of Quercetin. IC$_{50}$ corresponds to the concentration at which 50% inhibition of DPPH occurs. The lower the IC$_{50}$ the stronger the scavenging activity of the extracts. Antioxidant activity is considered high when IC$_{50}$ is less than 50 µg/ml, moderate when IC50 is between 50 and 100 µg/ml, low for IC$_{50}$ between 100 and 200 µg/ml, and negligible when IC50 is greater than 200 µg/ml (Simirgiotis et al., 2013). The DPPH inhibition for the two ecotypes studied is shown in Figure 1. The results showed that both ecotypes exhibited high dose-inhibition relationship with R$^2$ of 0.99. The extracts exhibited strong antioxidant activity, with maxima of about 91% with 100 µg/ml. The IC$_{50}$ values were relatively close, with 36.4 and 38.5 µg/ml for ecotype-1 and ecotype-2, respectively. The IC$_{50}$ value for ascorbic acid was 11.7 µg/ml.

Sujith et al. (2011) found that ethanolic root extracts of *A. pyrethrum* from India showed a maximum inhibition beyond a concentration of 400 µg/ml. The dose-inhibition relationship gave an IC$_{50}$ of 55.8 µg/ml. Cherrat et al. (2017) reported a higher IC$_{50}$ value (that is, a lesser antioxidant activity) for water-methanolic root extracts of *A. pyrethrum* from Middle Atlas Mountains of Morocco (145.8 µg/ml). The IC$_{50}$ they obtained for ascorbic acid was also high (46.8 µg/ml) as compared with those reported in other studies (usually in the range of 10 to 15 µg/ml). Kalim et al. (2010) found an even higher DPPH IC$_{50}$ of 467 µg/ml for *A. pyrethrum* from Kolkata, India, which means a weak antioxidantative activity. On the other hand, Manouze et al. (2017) reported very high oxidative activity for methanolic root extracts of *A. pyrethrum* from the High Atlas Mountains of Morocco, with IC$_{50}$ about 12.4 µg/ml, very close to those of reference antioxidant compounds such as ascorbic acid. The high antioxidant activity of the root extracts of *A. pyrethrum* can be attributed to its high contents in polyphenols and alkaloids.

**Cytotoxicity on HeLa cells**

Cancer cell viability against increasing concentrations of *A. pyrethrum* root extracts assessed using the ATP assay is shown in Figure 2. The results show that the two ecotypes
expressed an important dose-dependent cytotoxic effect on HeLa cells. Significant differences in terms of inhibition were observed starting from the 50 µg/ml test extract concentration for both ecotypes. Furthermore, the increasing extract concentrations exerted a more depressive effect on HeLa cells in the case of ecotype-1 as compared with ecotype-2. The 200 µg/ml extract concentration exerted an inhibition effect almost six-fold higher for the case of ecotype-1, in comparison with the control, while the effect was only about three-fold for ecotype-2.

The curve fitting of the percent inhibition of cell lines against increasing concentrations of test extract on a Log scale is shown in Figure 3. IC50 values for Hela cell line were 106.7 and 165.7 µg/ml for ecotype-1 and ecotype-2 extracts, respectively indicating that both ecotypes had a high inhibitory effects with ecotype-1 more inhibiting than ecotype-2.

Cytotoxicity on N2A cells

Pyrethrum root extracts exerted a very pronounced and dose dependent cytotoxic effect on N2A cell viability (Figure 4). Significant differences in terms of inhibition were obtained starting from the extract
concentration of 12.5 µg/ml for both ecotypes, and a more marked effect is observed above 50 µg/ml. In the case of ecotype-1, the 200 µg/ml test extract reduced N2a cells about six-fold higher as compared with the control, while in the case of ecotype-2, this same concentration induced a more important reduction of about ten-fold.

The IC$_{50}$ values derived from % inhibition in relation to Log concentrations were 34.1 and 73.7 µg/ml for ecotype-1 and ecotype-2 respectively (Figure 5). These values indicate that ecotype-1 is more depressive on N2a as compared with ecotype-2. The higher inhibitory effect of ecotype-1 can be attributed to its richness in phenolic compounds (Table 1).

Cytotoxicity results reported by Mohammadi et al. (2017)
for Pyrethrum root extract from Iran tested on Human Colorectal Cancer Cell Line (HCT) gave an IC$_{50}$ of 64.8 µg/ml for a comparable exposure time of 48 h similar to that adopted in the present study. However, for a shorter exposure time of 24 h, they obtained an IC$_{50}$ value of 105 µg/ml.

The results of the above cytotoxic effect of the root extracts from the two ecotypes evaluated against two cancer cells, highlights on the one hand, the important cytotoxic effect of *A. pyrethrum* root extracts exert against the two cancer cell lines evaluated with a more potent effect against N2a cells as compared with HeLa cells. On the other hand, the results indicate a variable potency among ecotypes, with ecotype-1 presenting a strongest cytotoxic effect against both cell lines.

Studies of the cytotoxicity effect of *A. pyrethrum* are limited. Kalim et al. (2010) reported that *A. pyrethrum* was among medicinal plants used in Unani medicine with important oxidative DNA damage preventive activity. A recent study by Mohammadi et al. (2017) evaluated the effect of *A. pyrethrum* on HCT cancer cells by looking at the induction of apoptosis and the inhibition of metastasis. They reported that exposure of HCT cells to IC$_{50}$ dose of *A. pyrethrum* root extract markedly increased the mRNA levels of the caspase-3 gene and reduced that of Bcl-2, which consequently was in favor of inducing apoptosis. On the other hand, the exposure to the IC$_{50}$ dose markedly reduced the mRNA levels of the MMP1 and Vimentin genes and consequently inhibited metastasis. The inhibition effect was also attributed to changes in the cell cycle progression where breaking due to *A. pyrethrum* roots extract compounds can occur at different phases of cancer cells multiplication. Hammed et al. (2018) conducted a cytotoxicity bioassay of *A. pyrethrum* from Algeria on Brine shrimp nauplii (a microorganism commonly used in biological toxicity assays) to assess the fraction most active in cytotoxicity using chromatographic fractionation and multiple purification. The results showed that four alkyamids of *A. pyrethrum* ethyl acetate extracts (including pellitorine) were the most involved in the lethal effect on the nauplii, with an IC$_{50}$ of 42.5 µg/mL. Subasri and John (2017) fabricated gold nano drug by root aqueous extract of *A. pyrethrum* (AuNPs) and assessed them for cytotoxicity on Hela cancer cells. They found that a concentration of 50 µg/ml was lethal to Hela cancer cells.

**Conclusions**

The results obtained showed that *A. pyrethrum* roots from Morocco presented important levels of total polyphenols and relatively moderate amounts of total flavonoids. The two ecotypes evaluated showed different levels for these phenolic compounds, with higher contents obtained for ecotype-1 (Tiguendez) as compared with ecotype-2 (Iguendez). Both ecotypes expressed strong and comparable antioxidant activity against DPPH radicals, with IC$_{50}$ values of 36.4 and 38.5 µg/ml for ecotype-1 and ecotype-2, respectively. Furthermore, the two ecotypes exhibited important dose dependent cytotoxic effect against the two cancer cell lines Hela and N2a. The IC$_{50}$ values were 106.7 and 165.7 µg/ml, respectively for Hela and 34.1 and 73.7 µg/ml for N2a, indicating a higher potency of pyrethrum extracts against N2a Cells as compared with HeLa Cells, with a higher effect of ecotype-1 as compared with ecotype-2. The significant differences obtained for the two ecotypes in terms of their polyphenolic compounds as well as in terms of their anticancer activity calls for more investigations of the local *A. pyrethrum* biodiversity in different parts of the country. The screening and chemical profiling will contribute to their thorough characterization and to the identification of most potent ecotypes in view of their valorization. Moreover, the anarchic collections of the plants of this species are causing a decrease of its biomass from the wild, and some ecotypes are already about to extinct. This raises the urgent need for the conservation of this precious species against genetic erosion, while some of its medicinal secrets are yet to be unveiled.

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