



Journal Homepage: - www.journalijar.com
**INTERNATIONAL JOURNAL OF
 ADVANCED RESEARCH (IJAR)**

Article DOI: 10.21474/IJAR01/3546
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/3546>



RESEARCH ARTICLE

POLYPHENOLS CONTENT AND EVALUATION OF ANTIOXIDANT ACTIVITY OF ANACYCLUSPYRETHRUM(L.) LAG. FROM TIMAHDITE A MOROCCAN MIDDLE ATLAS REGION.

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Manuscript Info

Manuscript History

Received: 01 January 2017
 Final Accepted: 05 February 2017
 Published: March 2017

Key words:-

Anacyclus pyrethrum, Timahdite, polyphenolics content, Antioxidant activity.

Abstract

As part of valorisation of medicinal and aromatic plants from Timahdite, a region located in Moroccan Middle Atlas, we focused our study on an endemic species which belongs to Asteraceae family: *Anacyclus pyrethrum* L. This species, widely used by local people in traditional pharmacopeia, was chosen to conduct phytochemical studies and to assess the antioxidant activity of its roots, leaves and flowers extracts.

Thus, the plant was harvested in May 2014. The selected organs (roots, leaves and flowers) of *Anacyclus pyrethrum* were sorted, cleaned and dried separately. Then, they were reduced into powder and stored away from light in a cool and dry place until use. Each organ was subjected to a phytochemical screening in order to qualitatively highlight its secondary metabolites.

This qualitative analysis shows that roots are rich in alkaloids while the aerial parts (leaves and flowers) are rich in tannins and flavonoids. Extraction of total polyphenols was performed by maceration using methanol / water (70/30) as solvent. Yields were 2.30% for roots, 3.3% for leaves and 3.8% for flowers.

Then, fractionation of hydro-methanol crude extract was carried out using successively three organic solvents of increasing polarity: chloroform, ethyl acetate and butanol. Quantitative analysis of total polyphenols conducted with Folin-Ciocalteu reagent showed that hydro-methanol crude extracts are richer in phenolic compounds than other fractions.

Antioxidant activity of crude extracts determined through DPPH test was spectrophotometrically quantified and values of the half-maximal inhibitory concentrations (IC₅₀) were graphically determined. These values are 77.62 μg gallic acid equivalent (GAE) / ml for flowers, 94.18 μg / ml for leaves and 145.81 μg / ml for roots, while 46.79 μg / ml was found for ascorbic acid which was used as standard.

Finally, a correlation was observed between total polyphenols content and antioxidant capacity determined by DPPH method: Aerial parts

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(leafy stem and flowers) richer in polyphenols exert a better antioxidant activity compared to roots.

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Introduction:-

These last decades have been marked by the widespread use of synthetic products in various industrial fields. Through years, this practice in the case of synthetic antioxidant molecules has often led to potential toxicological risks. Now, their use is reconsidered by international organizations and new plants are sought as a source of natural antioxidants [1,2]. Thanks to their properties, these natural antioxidants attract more interest in various fields. They are used to prevent and to treat cancer, inflammatory and cardiovascular diseases [3]. They are also used as additives in food, pharmaceutical and cosmetic products [1]. Thus, scientific research has been developed with the aim to extract, identify and quantify antioxidant compounds from natural sources such as medicinal plants and food products. In this quest for safe natural antioxidants, polyphenolics, which are widespread natural plant compounds, are of great interest because of their beneficial effects on health [4].

With the aim to find promising phenolic compounds, our research focuses on *Anacyclus pyrethrum* which is a Moroccan and Algerian endemic species belonging to *Asteraceae* family [5]. This perennial plant is widely prevalent in Moroccan Middle Atlas [6]. It is a 40 to 60 cm-height species which is characterized by long and thick roots. Its numerous simple or ramified stems lie on the ground and bear pinnate, alternate, finely cut, delicate and pubescent leaves. Its yellow-heart flowers are composed of white petals inside and purple petals outside. The species is well appreciated as a remedy against toothache [5], digestive disorders [8]. It also acts as a warming agent to enhance women fecundity and constitute a tonic agent for the nervous system [9]. *Anacyclus pyrethrum* is also used to treat cough, cold as well as heart, lung and digestive diseases [10]. For this medicinal value, this plant was selected for the present study. Moreover, review on this species showed that its chemistry remains insufficiently studied. According to our knowledge, no research work was conducted on the aerial parts of the plant. Thus, our work aims to contribute to a complete phytochemical study of *A. pyrethrum* and to contribute to the explanation of its various activities. We focused our interest on the wild species which particularly grows in Timahdite's forests, at altitudes ranging from 400 to 3100m. [11].

The aim of our work is to quantify *Anacyclus pyrethrum*'s phenolics in the extracts of three organs and to assess their antioxidant power by DPPH method.

Material and methods:-

plant material

Anacyclus pyrethrum was harvested in May 2014 at 1800m of altitude in Timahdite, a region located at 30km south from Azrou. Samples were harvested with their roots, stems, leaves and flowers in order to make their identification easier. This plant identification was conducted in the laboratory of Botany and Plant Ecology at the Scientific Institute of Rabat by Professor MOHAMED IBN TATOU. Samples of different organs (roots, flowers and leaves) were cleaned, shade dried at room temperature and then stored away from light until use.

phytochemical screening :-

Plants organs (roots, leaves and flowers) were powdered with an electric grinder. After this step, chemical composition of the drug was determined by a phytochemical study based on solubility tests, colour and precipitation reactions and also by observations under ultraviolet light.

The target phytochemical groups in these tests include: alkaloids, polyphenols (flavonoids, anthocyanins, tannins, ...), saponins, coumarins, sterols, terpenes, mucilage, carotenoids, reducing sugars (oses and holosides)

- Research on alkaloids was performed using Dragendorff and Mayer's reagents which are reagents for general characterization of alkaloids [12].
- Characterization of tannins was carried out with Iron chloride reagent. The differentiation between gallic and catechic tannins was shown thanks to Stiasny's reaction [13].
- Identification of flavonoids was performed by cyanidin test [14].
- Research of saponosides was quantitatively determined by calculating the foam index after shaking the aqueous extract [15].
- Anthraquinones were identified by Borntrager's test [16].

- Sterols and terpenes were highlighted by Liebermann-Burchard's test [17].
- Coumarins were demonstrated by UV fluorescence at 365 nm [13].
- Mucilages were indicated by the presence of a flocculent precipitate after adding ethyl ether in a test tube containing plant's decoction [18].

Phytochemical screening was carried out separately on plant's leaves, flowers and roots.

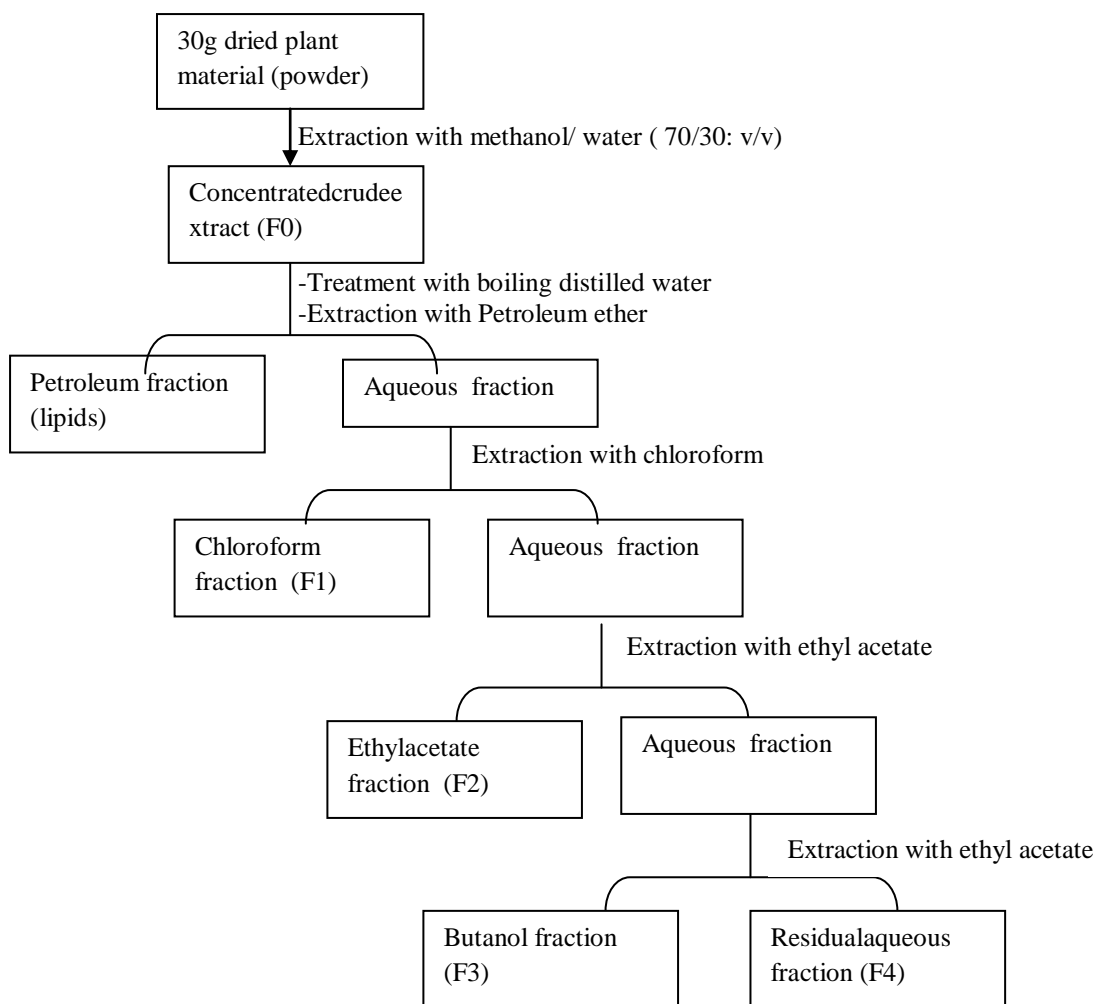
Preparation of extracts:-

Test samples composed of 30 g powder of root, leaves and flowers of *Anacyclus pyrethrum* was macerated in a hydro-alcoholic mixture (methanol / water 70/30: v / v) with magnetic stirring for 30 min. The mixture was then stored in the dark for 24 hours at room temperature. The obtained solution was filtered on filter paper and the filtrate was concentrated under vacuum at 50 ° C with a Büchi-type rotary evaporator [19]. The dry extract was then weighed, labelled and stored at 4 ° C until use.

Fractionation:-

Polyphenols extraction was performed according to Bruneton's method [15] with a slight modification. It is based on polyphenols' degree of solubility in organic solvents. Fractionation of crude hydro-methanol extracts was carried out by successive use of four organic solvents with different polarity: petroleum ether, chloroform, ethyl acetate and butanol.

In addition to the hydro-methanol crude extract (F0), four fractions were selected for further work: chloroform fraction (F1), ethyl acetate fraction (F2), butanol fraction (F3) and residual aqueous fraction (F4). The different extracts were kept cold away from light until use. Picture 1 summarises fractionation steps of the crude extracts.



Picture 1:- Fractionation steps of crude extracts.

Determination of Total Phenols Content :-

Total polyphenol content of *A. pyrethrum*'s crude extracts was determined by Folin-Ciocalteu's method [20]. In a 100 ml-volumetric flask, 40 ml of each extract were mixed with 1.5 ml of an aqueous solution of sodium bicarbonate (Na_2CO_3) at 10 % and 1.5 ml of Folin-Ciocalteu reagent (10fold diluted). Then the flask was supplemented with distilled water. The mixture was stirred and incubated in the dark at room temperature for 30 minutes. The reading step was performed against a blank using a spectrophotometer (UV mini-1240) at 765 nm. A standard curve with gallic acid as positive control was established in parallel under the same operating conditions. Results are expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE / g).

Determination of flavonoids contents:-

Total flavonoids content of *A. pyrethrum*'s extracts was determined according to aluminumtrichloride method. The mixture was stirred and then incubated in the dark at room temperature for 30 minutes. A blank is prepared by replacing the extracts by methanol and the absorbance is read at 433 nm with a UV spectrophotometer. Results are expressed as mg of quercetin equivalent / gram of dry plant material. Quercetin calibration curve served as a standard.

Assessment of antioxidant activity :-**Dpph Test:-****Principle:-**

DPPH (2,2-diphenyl-1-picrylhydrazyl) is generally the most widely used substrate for rapid and direct assessment of antioxidant activity thanks to the stability of its free radical form and the simplicity of the analysis. This method is based on the measurement of antioxidants' ability to scavenge the DPPH radical [21]. Indeed, the latter is characterized by its ability to generate stable free radicals. This stability is due to the relocation of free electrons in the molecule, this relocation leads to a violet blue colour which is characteristic of the DPPH solution. Evaluation of antioxidant effectiveness is spectrophotometrically assessed at 515 nm through the decrease of the blue colour due to recombination of DPPH radicals [22]. In the presence of free radical scavengers, purple-coloured DPPH (2,2-diphenyl-1-picrylhydrazyl), is reduced into 2,2-diphenyl hydrazine -1-picryl which presents a yellow colour in the presence of free radical compounds [23].

Protocole:-

Ethanol solutions of ethyl acetate fraction were prepared. The following concentrations were obtained: 16; 32; 48; 64; 80; 96; 112; 128; 144; 160; 176 and 192 $\mu\text{g} / \text{ml}$. 200 μl of each ethanol solution were added to 1.8 ml of DPPH* solution which is also prepared in ethanol (0.024g / l). In parallel, a negative control was prepared by mixing 200 μl of ethanol with 1.8 ml of ethanol DPPH* solution. Absorbance was read against a blank at 515 nm after 30 min of incubation in the dark at room temperature. Ascorbic acid solution was used as a positive control. Absorbance was read in the same conditions for samples and controls. The test is repeated three times. Results are expressed as DPPH* reduction percentage. This percentage is calculated using the following formula:

$$\% P = [(A_{bl} - A_s) / A_{bl}] \times 100$$

P : DPPH* reduction percentage; A_{bl} : blank absorbance; A_s : sample absorbance

Results and discussion:-**phytochemical tests :-**

Results of phytochemical tests are gathered in table 1.

Table1:- Results of phytochemical screening of *Anacyclus pyrethrum*'s hydro-methanol extracts

| Chemical class | Organs | | |
|--------------------------------|-----------|-------------|-------------|
| | Roots (R) | Leaves (LS) | Flowers (F) |
| Alkaloids | +++ | + | + |
| Gallic tannins | + | + | + |
| Catechic tannins | + | ++ | +++ |
| Flavonoids | - | ++ | +++ |
| Anthocyanins | - | - | - |
| Leucoanthocyanins | +++ | ++ | ++ |
| Saponosides | + | + | - |
| Free anthracenic compounds | - | - | - |
| Combined anthracenic compounds | +++ | ++ | ++ |

| | | | |
|-------------------------|-----|----|----|
| Coumarins | + | ++ | + |
| Carotenoids | - | - | - |
| Sterols and triterpenes | + | ++ | + |
| Reducing compound | +++ | ++ | ++ |
| Oses et holosids | +++ | ++ | ++ |
| Mucilages | +++ | + | + |
| Narcotics | - | - | - |

(+++): strong reaction; (++): moderate reaction; (+): weak reaction ;(-): no reaction

Results of phytochemical tests have shown that:

- Alkaloids are present in all three parts of the plant, but in small quantities in the aerial parts (leaves and flowers) compared to roots.
- Flavonoids are not detected in roots, but leaves contain mainly flavanols and flavanonols (red colour) while flowers essentially contain flavones (orange-pink colour).
- Tannins: Based on the colour obtained with FeCl₃ reagent, different parts of this species contain gallic tannin. Catechic tannins are less important in roots compared to leaves and flowers. Their presence is confirmed by Stiasny's reaction.
- Saponosides were detected and foams indices show that saponins content is low (1.1 cm) in leaves and roots. They are clearly absent in flowers.
- Sterols and terpenes are detected in a higher content in leaves compared to flowers and roots. A violet colour appears and then turns green.
- Coumarins presence is remarked thanks to fluorescent spots observed during qualitative analysis.
- Reducing compounds are present in large amount in the three parts of the plant.
- Finally, it should be noted that carotenoids, narcotics, free anthracenic compounds (anthraquinone) and anthocyanins are absent or rare in all studied parts of the plant.

The roots of *A. pyrethrum* mainly contain alkaloids and tannins. Similar results have been shown by Bellakhdar, 1996 [24], Hmamouchi, 1999,[25],Center for Mediterranean Cooperation, 2005[26], Selles et al., 2012[27], Limbachia&Jadhav, 2012 [28]). They also contain mucilage. The presence of mucilage in the roots of *A. pyrethrum* allowed us to note the viscous character of the extracts. During our phytochemical tests, we have demonstrated the presence of coumarins, sterols and triterpenes in the roots of *A. pyrethrum* but in small amount. These results are consistent with the previous works of Hamimed, 2009 [29]; Sellese et al., 2012[30]. The roots of *A. pyrethrum* also contain oses,holosides and reducing compounds. This was highlighted by (Selles C., 2012) [30]. Hence, the chemical composition of the roots of *A. pyrethrum* has proved to be interesting and rich in compounds known as bioactive molecules (Hamimed, 2009 [29]; Christian, 2013[31]). The overall results justify the various activities of this plant including the use of part of its chemical compounds as alternative to synthetic antioxidants.....

Extraction yields :-

Yields of crude hydro-methanol extracts from roots, leaves and flowers are respectively: 2.3%, 3.3% and 3.3% (Table 2).

For crude extracts fractionation, results show that butanol fraction has the highest yields (1.93%, 3.2% and 3.7%) followed by the aqueous fraction (1.33%; 2.9% and 3.2%) and then the ethyl acetate fraction (1.23%, 2.23% and 2.4%). On the other side, the lowest yield was obtained with the chloroform fraction:0.76%, 1.8% and 2.1% respectively for roots, leaves and flowers(Table2).

Table 2:- Yields of polyphenol extracts and fractions obtained from *Anacyclus pyrethrum*'s organs

| Extracts and fractions from <i>A. pyrethrum</i> 's organs | Yields (%) | | |
|--|------------|-------------|-------------|
| | Roots (R) | leaves (LS) | Flowers (F) |
| Crude hydro-methanol extract | 2.30% | 3.3% | 3.8% |
| Chloroform fraction | 0.76% | 1.8% | 2.1% |
| Ethyl acetate fraction | 1.23% | 2.23% | 2.4% |
| Butanol fraction | 1.93% | 3.2% | 3.7% |
| Aqueous fraction | 1.33% | 2.9% | 3.2% |

total phénol content

UV / Visible spectrophotometry allowed to quantify polyphenols in all extracts and fractions. Results are shown in Table 3.

Table 3:- Polyphenol content of *A.pyrethrum*'s extracts and fractions expressed in milligrams equivalents gallic acid per gram of extract (mg GAE/g of extract)

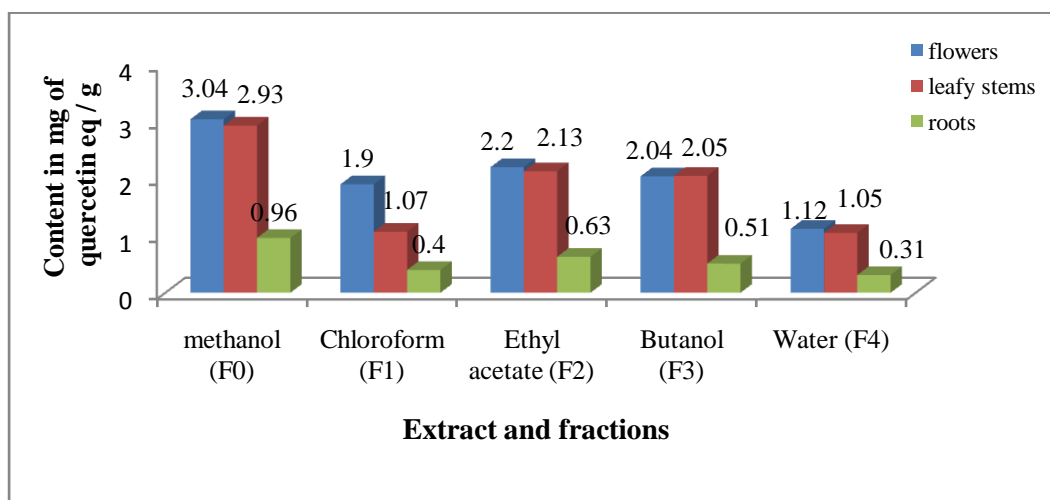
| Extracts | Polyphenol content in mg GA eq /g of extract) | | |
|--|---|--------------|-------------|
| | Flowers | Leaves | Roots |
| hydro-methanol extract (F ₀) | 21.84 ± 2.41 | 14.23 ± 1.44 | 7.83 ± 1.18 |
| Chloroform fraction (F ₁) | 13.49 ± 1.51 | 5.67 ± 0.48 | 4.94 ± 0.21 |
| ethylacetate fraction (F ₂) | 14.20 ± 1.40 | 10.23 ± 1.34 | 5.94 ± 1.21 |
| butanol fraction (F ₃) | 19.84 ± 1.81 | 12.15 ± 1.18 | 6.15 ± 1.70 |
| residual fraction (F ₄) | 16.12 ± 0.09 | 11.85 ± 1.09 | 5.88 ± 0.63 |

For all parts of the studied plant, we noticed the variability of total phenols contents (Table 3). The highest content was found in flowers. It varies between 13.49mg and 21.84mg of gallic acid equivalent per gram of extract followed by the leaves with a content ranging between 5.67 mg and 14.23mg GA eq/ g of extract. Polyphenol content of roots varies between 4.94 mg and 7.83mgGA eq / g of extract.

We also found that methanol crude extracts contain relatively high concentrations of polyphenols compared to other fractions. Phenol content of a plant depends on a number of factors such as climatic conditions, time of harvest, solvent of extraction, storage conditions [24].

flavonoids content:-

Flavonoid content in hydro-methanol extracts obtained from *A.pyrethrum*'s organs are recorded in picture 2.



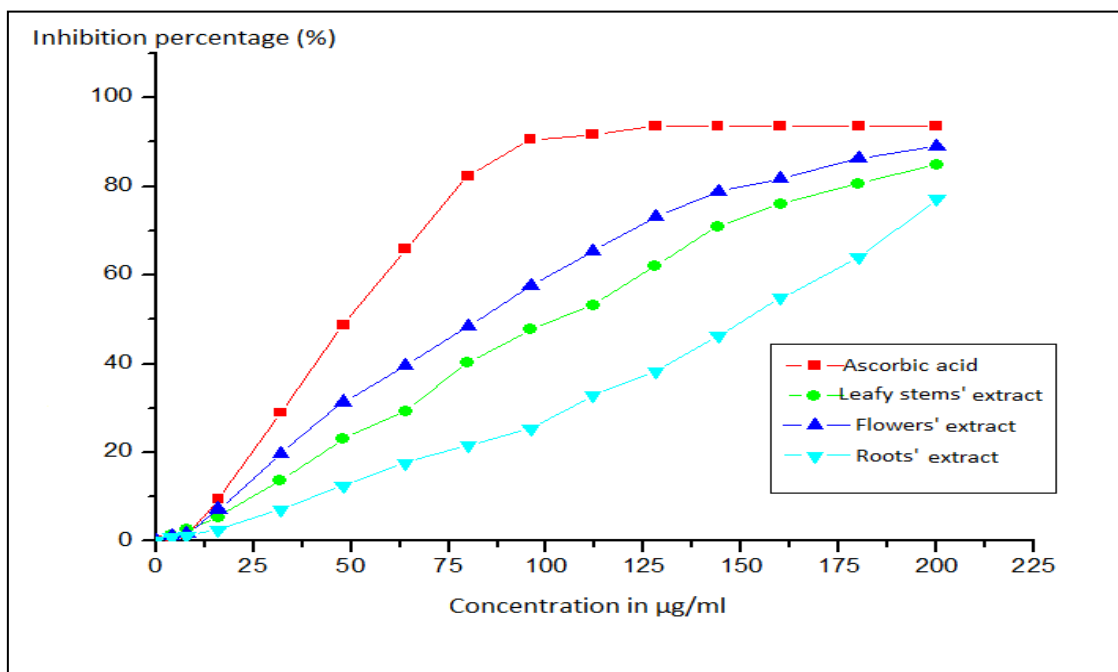
Picture 2:- Flavonoid contents in flowers', leaves' and roots' extracts and fractions of *Anacyclus pyrethrum*

Concerning flavonoid, the highest content is recorded in flowers' hydro-methanol extract with a value equal to 3.4 mg of quercetin eq / g of dried plant material followed by the leaves' hydro-methanol extract with 2.93 mg of quercetin eq / g and finally roots' hydro-methanol extract with 0.96 mg of quercetin eq / g dried plant material .

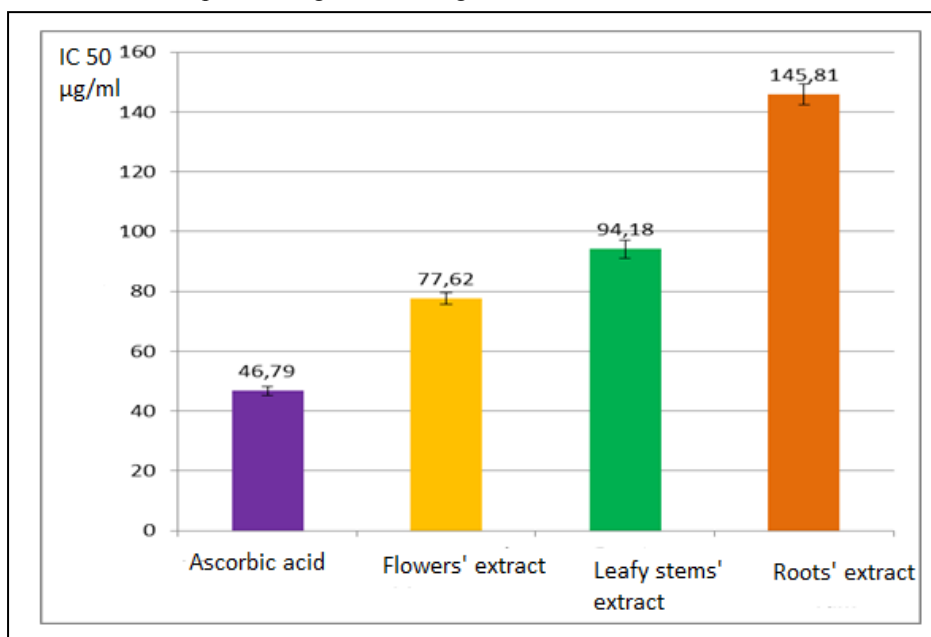
We can deduce that the crude extracts from flowers and leaves of *A. pyrethrum*. are the richest in flavonoids.

Antioxidant activity:-

Values obtained from DPPH test allowed to establish curves on picture 3.



Picture3:- DPPH d' inhibition pourcentages according to extracts concentrations



Picture4:- Half maximal inhibitory concentration (IC50) of hydro-methanol extracts towards DPPH

The results obtained show that hydro-methanol crude extracts of *A. pyrethrum*'s organs have very important antioxidative activities, with IC₅₀ 77.62 µg / ml for flowers, 94.18 µg / ml for leaves and 145.81 µg / ml for the roots. These levels are lower than that of ascorbic acid (IC₅₀ 46.79 µg / ml) used as a positive control (Picture 4).

On the other hand, we note that there is a correlation between the concentration of polyphenols and antioxidant activity, confirming that polyphenols are powerful antioxidants that inhibit the formation of free radicals and oppose oxidation of macromolecules.

The antioxidant activity depends not only of the concentration of polyphenols, but also on the structure and nature of antioxidants in the extract [13]. Generally, polyphenols with high number of hydroxyl groups show higher antioxidant activity [25].

Conclusion:-

The phytochemical study of three organs of *Anacyclus pyrethrum* shows that the leaves and flowers are rich in tannins and flavonoids, while the roots are richer in alkaloids than both of the other parts of the plant.

Concerning the phenolic composition of the extracts, flowers and leaves showed high total polyphenol content in comparison with the roots.

This in vitro study of the antioxidant activity performed with the method of scavenging DPPH free radical showed that methanol crude extracts have a high capacity to trap the DPPH radical with 50% inhibitory concentrations (IC₅₀) about 77.62 µg / ml for flowers, 94.18 µg / ml for leaves and 145.81 µg / ml for roots. These extracts may therefore be an alternative to some synthetic additives.

It is therefore important to characterise and identify the bioactive molecules in all fractions of the three organs of *A. pyrethrum* by HPLC analysis.

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