

Total phenolic and flavonoids contents and *in vitro* evaluation of antioxidant activity of several *Calendula officinalis* (Marigold) extracts

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Abstract

The search for natural antioxidants to replace synthetic antioxidants is one of humanity's health priorities. Medicinal and Aromatic Plants (MAP) contain phenolic compounds that act as natural antioxidants. The aim of this work is to study total phenols and flavonoids contents and evaluate the antioxidant power of various extracts of *Calendula officinalis*. Extracts were prepared in two steps: hot solid-liquid extraction, by Soxhlet and from the plant powder using a solvent (Methanol/water 70/30, Acetone/water 70/30) followed by a liquid-liquid extraction by splitting the obtained extract by two increasingly polarized solvents (Ethyl acetate and Butanol). Total phenols and flavonoids were evaluated using gallic acid and quercetin as standards, respectively. The antioxidant activity of extracts was determined by DPPH (2,2-diphenyl 1-picrylhydrazyle) free radical reduction method. The two crude fractions of Methanol and Acetone presented the highest levels of total phenols (7,58±0,38 mg GAE/g dm and 6,80±0,34 mg GAE/g dm respectively), and flavonoids

(7,37±0,37 mg QE/g dm and 7,93±0,40 mg QE/g dm respectively). Ethyl acetate fractions showed a high antioxidant activity. This study demonstrated that extracts from *Calendula officinalis* flowers present a promising source of natural antioxidant.

Introduction

Medicinal and Aromatic Plants (MAP) and other natural products contain hundreds of compounds that act as natural antioxidants.¹ Plants contain a variety of molecules capable of trapping free radicals such as phenolic compounds, nitrogen compounds, vitamins and more.² These natural antioxidants have recently attracted much attention due to their health benefits as an effective way to eliminate and reduce the action of free radicals that cause oxidative stress, and as protective agents against it in biological systems.³ In addition, natural antioxidants offer alternatives to synthetic antioxidants widespread in food and pharmaceuticals and that can be toxic and harmful to health.⁴

The introduction of natural antioxidants in cosmetics and especially in photoprotective products (sunscreen) is also one of the axes of recent research in dermatology and cosmetics. The exposure of the skin to the sun even with the application of photoprotective products, allows penetration of some of the ultraviolet rays. These cause the formation of free radicals that can damage membranes, DNA or proteins in the body and cause serious skin damages such as premature aging and, in the most serious cases, skin cancers.⁵ The formulation of new sunscreens that also contain antioxidants will fight the effect of penetrated UV rays and neutralize the formed free radicals.

Calendula officinalis, known as Marigold, is a plant of the family Asteraceae, native to the Mediterranean region, and several properties have been attributed to it. *Calendula* extracts have shown antioxidant, anti-inflammatory, analgesic, antibacterial and antiviral powers.⁶ It has also been reported to have tumour inhibition activity as well as protective activities from cancer development and adverse effects of radiotherapy and chemotherapy.⁷

Organic extract from *Calendula* flowers showed inhibitory properties against Human Immunodeficiency Virus (HIV) and has shown promise in the treatment of AIDS to replace anti-HIV drugs that present several adverse effects.⁸

Calendula officinalis is also known for its virtues of skin treatment and its use in cosmetics and dermatology is widespread,⁹ this is due to the terpenoids carotenoids, flavonoids and volatile oils content in its extracts, and that have appreciable cosmetic activities for the skin. *Calendula officinalis* is used in cosmetics for its

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toning, antiseptic and anti-irritant properties, it is also used for healing wounds and skin rashes,¹⁰ some extracts of *Calendula officinalis* are applied externally to treat skin ulcers, eczema and conjunctivitis,⁷ and also to remove blue spots on the skin.¹¹

Akhtar *et al.*¹⁰ evaluated the effect of creams formulated from emulsions (Water/Oil) and containing *Calendula* extracts on the skin, the study showed that these *Calendula* creams have a whitening power by producing a decrease in the content of melanin in the skin, anti-inflammatory effects as they are able to decrease cutaneous erythema, in addition to the hydration effects of the skin and the anti-wrinkle effects. But *Calendula* extract is not suitable for people with oily skin as it increases the sebum content in the skin.¹⁰ The addition of *Calendula officinalis* extracts in cosmetic emulsions can contribute to improving their stability without the use of chemical stabilizers.⁶

Calendula officinalis's dry flower is also used in food as a spice and orange dye because of its carotenoid content, it is sometimes called «fake saffron», its use is generally recognized to be safe on the part of the FDA (Food and Drug Administration) Set FEMA (Flavors and Extracts Manufacturers Association), (number 2658 in the Known Safe Substances table).¹²

Calendula officinalis is a good source of natural antioxidants, particularly phenolic substances,⁷ and other compounds that trap harmful free radicals,¹³ but studies of its phenolic content and antioxidant potency of its extracts have been considered rare^{14,15} and only a few studies have addressed this problem.¹⁴⁻¹⁶

Calendula officinalis is part of the rich and diverse Moroccan flora, it is called in Moroccan dialect *Jemmra* and is used in traditional Moroccan medicine.^{17,18} In this work, we present our study carried out on spontaneous *Calendula officinalis* and originating from the El Hammam region in Khenifra Morocco. The study consists first of identifying the important families of secondary metabolites using a phytochemical screening of the flowers powder, then to prepare various extracts and then to determine the content of these extracts in total polyphenols and flavonoids and evaluate their antioxidant power by the free radical DPPH (2,2-diphenyl 1-picrylhydrazyle) trapping test.

Materials and Methods

Plant material

The flowers of *Calendula officinalis* studied were collected in full bloom in May 2019 in the region EL Hammam Khénifra Morocco, where they grow spontaneously at an altitude of 1250 m. Identification of species was confirmed in the Scientific Institute of Rabat. The samples were dried in darkness and at room temperature.

Phytochemical screening

It aims to highlight the important families of secondary metabolites contained in the plant. This is a qualitative study based on colouring and/or precipitation reactions from different plant extracts. These extracts are obtained by decoction, infusion or maceration by solvents. Phytochemical screening is also based on the use of several reagents.

The characterization tests of different chemical groups were carried out according to Bruneton (2009) and N'Guessan *et al.* (2009).^{19,20}

Family of Alkaloids was searched by Dragendorff reagent. Catchic Tanins was searched by Isoamyl alcohol and hydrochloric acid while Gallic Tanins was searched by Stiasny reagent, sodium

acetate and iron chloride. Acetic anhydride and concentrated sulphuric acid were used to look for Sterols and Triterpenes. Dilute hydrochloric alcohol, magnesium shavings and iso-amylic alcohol were used to search Flavonoids family. Quinonic substances were searched by Chloroform, dilute ammonia and hydrochloric acid.

Extraction of polyphenols

The extraction of phenolic compounds was carried out in two steps: first a hot solid-liquid extraction, by Soxhlet and from the plant powder using a solvent, followed by a liquid-liquid extraction by splitting the obtained extract by two solvents with increasing polarity.

Solid-liquid extraction was carried out by two solvents, Methanol/Water 70/30 (Me) and Acetone/Water 70/30 (Ac), 30 g of the plant powder is placed in a filter paper cartridge and introduced into a Soxhlet, the flask is filled with 350 mL of a solvent hydrolic solution at 70%, after several cycles, until the plant is exhausted and the colouring disappears, the solvent is evaporated and the extract is recovered with warm distilled water.

The crude extract thus obtained is subjected to liquid-liquid extraction (the splitting) by two increasingly polarized solvents, ethyl acetate and butanol, to obtain four fractions: crude fraction (F0), ethyl acetate fraction (F1), butanol fraction (F2) and aqueous fraction (F3) (Figure 1). The four fractions finally obtained from the hydro-methanol solution are then indexed; Me (F0), Me (F1), Me (F2), Me (F3), and those from the hydro acetone solution are indexed Ac (F0), Ac (F1), Ac (F2), Ac (F3).

Determination of total polyphenols contents in *Calendula officinalis*

The total phenol content of the extracts was determined by Folin-Ciocalteu method.²¹ In 100 mL volumetric flask, 5 µL of each extract is mixed with 1,5 mL of Folin-Ciocalteu reagent (10%) and 1,5 mL of sodium carbonate (Na₂CO₃) at 7,5% (m/v). Then the flask is completed with distilled water. The whole is left for 30 minutes at room temperature. The absorbance is measured at 760 nm.

Gallic acid is used as a positive control. The polyphenol content of the studied extracts is calculated from the regression equation of gallic acid calibration (). The results are expressed in milligram of Gallic Acid Equivalent per gram of dry matter (mg GAE/g dm). The polyphenol content is calculated using the following formula:

$$T = \frac{C \times V}{m \text{ (dry matter)}} \times D$$

- C: The concentration measured by the regression equation of gallic acid calibration
- V: Sample volume
- D: Dilution factor

Determination of flavonoids contents in *Calendula officinalis*

Flavonoids content was estimated by the Aluminum Trichloride AlCl₃ method.²² Ten µL of each fraction was mixed with 0,1 mL of aluminum trichloride 10%, followed by 20 mL of distilled water and supplemented at 50 mL with absolute methanol. The whole is incubated in darkness at room temperature for two hours, the absorbance is measured at 430 nm. Flavonoids are quantified using a calibration curve performed with Quercetin ($y = 0,073X - 0,081$). The flavonoid content is expressed in milligrams of Quercetin Equivalent per gram of dry matter (mg QE/g dm).

Antioxidant power: DPPH radical scavenging activity

The antioxidant activity was evaluated by measuring the trapping power of the DPPH radical (2,2-diphényl 1-picrylhydrazyle). For this purpose, an ethanolic solution of DPPH is prepared by dissolving 2,4 mg of DPPH in 100 mL of ethanol.

A series of dilutions of each extract were prepared with concentrations ranging from 0,125 mg/mL to 5 mg/mL. The tests are performed by mixing 200 μ L of each extract solution at different concentrations with 2,8 mL of the DPPH ethanolic solution. After 30 min in darkness, the absorbance is measured at 517 nm. Ascorbic acid inhibition of the free radical DPPH was also analyzed at the same concentrations for comparison.

Antioxidant activity is determined by two parameters: i) The percentage of DPPH inhibition (I%) calculated from sample absorbance A_{sample} as follows: $I\% = (A_{control} - A_{sample})/A_{control}$. $A_{control}$: absorbance of the solution containing only DPPH radical solution and A_{sample} : absorbance of sample solution to be tested in the presence of DPPH; ii) IC_{50} : The antioxidant concentration required to decrease the initial concentration by 50%, it is inversely related to the antioxidant capacity. This parameter is defined graphically from the absorbance curve as function of concentrations.

Results and Discussion

Phytochemical screening

The phytochemical characterisation tests carried out on the extracts from *Calandula officinalis* flowers showed the presence of the following chemical families: tannins, flavonoids, saponosides, triterpene and quinones. On the other hand, we note the absence of alkaloids. These constituents have been cited in other studies as bioactive secondary metabolites present in *Calendula officinalis* flowers,^{6,23} in addition to other chemical compounds such as phenolic acids and coumarin,²⁴ flavonols glycosides.²⁵ Other active constituents are included such as mucilages and carotenoids to which the flower owes its orange colour.^{24,26}

Extractions yields

The yield of the crude extracts (F0) is calculated in relation to the mass of the dry matter at which the solid-liquid extraction was carried out.

$$R\% (F0) = \frac{\text{Mass of crude extract}}{\text{Mass of dry matter powder}} \times 100 = \frac{m_0}{30} \times 100$$

The yield of the other fractions (F1), (F2) and (F3) is calculated in relation to the mass of the crude extract to which the liquid-liquid extraction was carried out.

$$R\% (F1,2,3) = \frac{\text{Mass of the fraction}}{\text{Mass of crude extract}} \times 100 = \frac{m_{1,2,3}}{m_0} \times 100$$

The yield differs from one extraction solvent to another and is also dependent on the splitting solvent. This is due to the different polarity of solvents which allows the separation of different metabolites because of their solubility in the solvent and their structural complexity.²⁷

The highest yield is that of the butanolic fraction of the crude acetonic extract Ac (F2), while the low yield is that of the ethyl acetate fraction of the crude methanolic extract Me (F1). Hot solid-liquid extraction by Methanol has a better yield than by Acetone, and the yield of liquid-liquid extraction by Butanol is higher than that of ethyl acetate (Figure 2).

Determination of total polyphenols and Flavonoids contents in *Calendula officinalis*

The total polyphenols contents of different extracts are presented in Table 1. The results show that all fractions present significant contents of polyphenols, but they differ according to the splitting solvents. The extraction solvents Methanol and Acetone do not have much influence on the content. The two crude fractions, fraction of Methanol Me (F0) and fraction of Acetone Ac (F0), had the highest levels of 7,58 \pm 0,38 mg QE/g dm and 6,80 \pm 0,34 mg GAE/g dm, respectively (Table 1).

The lowest levels of polyphenols are those for aqueous extracts, 1,75 \pm 0,09 mg GAE/g dm and 0,73 \pm 0,04 mg GAE/g dm

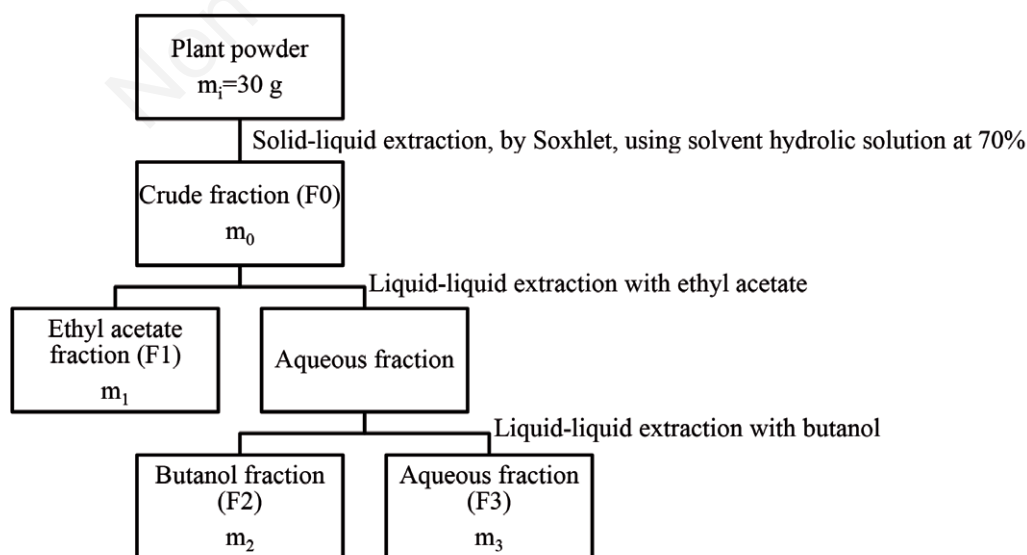


Figure 1. The fractionation process to obtain the four fractions: crude fraction (F0), ethyl acetate fraction (F1), butanol fraction (F2) and aqueous fraction (F3).

for aqueous methanol extract Me(F3) and aqueous acetone extract Ac(F3) respectively (Table 1). The low polyphenol content of aqueous extracts compared to crude extracts shows that the fractionation (liquid-liquid extraction by ethyl acetate and butanol) was able to extract a large quantity of polyphenols from the plant.

Flavonoid content of extracts ranges from $1,24 \pm 0,06$ to $7,93 \pm 0,40$ mg QE/g dm (Table 1), the crude methanol and acetone fractions, Me (F0) and Ac (F0), represent the highest content of Flavonoids ($7,37 \pm 0,37$ and $7,93 \pm 0,40$ mg EQ/g dm respectively) with a small gap between them in favour of Acetone extract. Aqueous fractions represent the lowest contents (Table 1).

The phenol content values found are much lower than those found by Jasmina and al. on three extracts of *Calendula officinalis* (Water, Ethanol and Solution 50/50 hydroethanolic) and which were of the order of 45,13 mg GAE/g dm, 31,86 mg GAE/g dm and 29,79 mg GAE/g dm respectively. The high phenol content of the aqueous extract is due to the solubility of phenols, flavonoids and their glycosides in water. As for the flavonoid content values, they are higher than those found for these three extracts (Water, Ethanol and Solution 50/50 hydroethanolic) and which are 0,12 QE/g dm, 0,10 QE/g dm and 0,17 QE/g dm respectively.¹⁵

The polyphenol content of *Calendula officinalis* flowers found by Četković, using the UV spectrophotometric method, are in the order of 15,12 mg/g of the sample, and the flavonoid content was 5,13 mg/g of the sample.²⁶

The difference in phenol content of the same plant depends, in addition to the extraction solvent, on several factors such as the environmental conditions of the plant (altitude, temperature, light) as well as soil conditions and the state of maturation of the plant.²⁸

A study comparing three different extracts of *Calendula offic-*

inalis, by ethanol, ethyl acetate and n-hexane, Ethanol was confirmed to be the best solvent for the extraction of phenols and flavonoids from the plant *Calendula officinalis*.²⁹

Antioxidant power: DPPH radical scavenging activity

We evaluated the antioxidant power for all Methanol and Acetone fractions except aqueous fractions since their polyphenols and flavonoids were the lowest. The results obtained expressing the percentages of antioxidant activity by concentrations are presented in Figures 3 and 4.

The antioxidant tests showed that the six fractions Me (F0), Me (F1), Me (F2), Ac (F0), Ac (F1) and Ac (F2) show an antioxidant activity that increases with the increase in concentration. All fractions reach a maximum inhibition percentage between 82% and 93%. The potency of ethyl acetate fractions appears to be highest, followed by crude fractions and finally butanolic fractions.

To better characterize this antiradical power, we determined the IC₅₀ values (concentration of the extract needed to inhibit the activity of DPPH by 50%). The results obtained are shown in Table 1. The extracts Ac (F1) and Me (F1) present the strong antioxidant activities relative to the lowest values of the IC₅₀ parameter and which are of the order of $0,649 \pm 0,03$ mg/mL and $0,723 \pm 0,04$ mg/mL respectively, followed by the values of the extracts Me (F0) and Ac (F0) ($0,926 \pm 0,05$ mg/mL and $1,088 \pm 0,05$ mg/mL respectively) and finally the values of the extracts Ac (F2) and Me (F2) whose values are $1,679 \pm 0,08$ mg/mL and $2,697 \pm 0,13$ mg/mL respectively.

Although the butanolic fractions had a high content of total polyphenols and flavonoids compared to the ethyl acetate fractions, they did not have a high antioxidant activity, it means that

Table 1. Numerical values of total phenols and flavonoids contents and the antioxidant power parameter IC₅₀ of various *Calendula officinalis* extracts.

	Me (F0)	Ac (F0)	Me (F1)	Ac (F1)	Me (F2)	Ac (F2)	Me (F3)	Ac (F3)
Total phenols content (mg GAE/g dm)	7.58 ± 0.38	6.80 ± 0.34	2.16 ± 0.11	2.59 ± 0.13	3.05 ± 0.15	4.67 ± 0.23	1.75 ± 0.09	0.73 ± 0.04
Flavonoids content (mg QE/g dm)	7.37 ± 0.37	7.93 ± 0.40	3.21 ± 0.16	3.86 ± 0.19	5.25 ± 0.26	5.38 ± 0.27	1.24 ± 0.06	1.25 ± 0.06
Antioxidant power parameter IC ₅₀ (mg/mL)	0.926 ± 0.05	1.088 ± 0.05	0.723 ± 0.04	0.649 ± 0.03	2.697 ± 0.13	1.679 ± 0.08	-	-

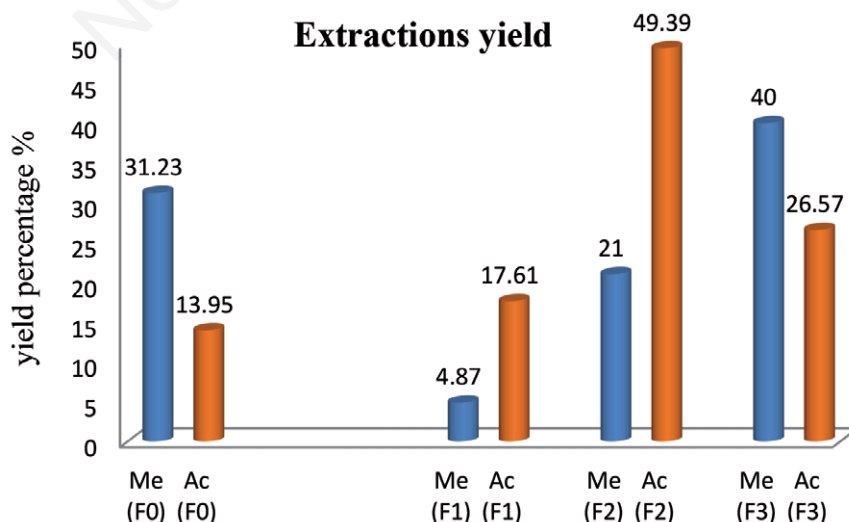


Figure 2. Extractions yield of *Calendula officinalis* different extracts.

the phenols they contain are not the main polyphenols responsible for antiradical power. It is deduced that the polyphenols responsible for antioxidant potency are contained in the crude fractions and have been well extracted in the fractions of ethyl acetate, This explains the poverty of butanolic extracts of these phenols and subsequently their low antioxidant capacity.

The antioxidant power of *Calendula officinalis* flower extracts has been demonstrated in other *in vitro* and *in vivo* studies. A comparison of the potency of three extracts (ethanol, ethyl acetate and n-hexane) showed that the ethanolic extract had the highest antiox-

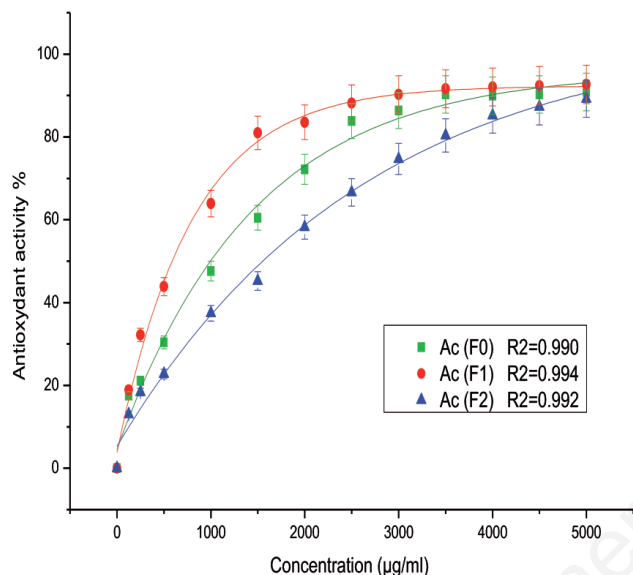


Figure 3. Antioxidant activity at varying concentrations of 3 hydro-acetone fractions : crude fraction Ac (F0), ethyl acetate fraction Ac (F1) and butanol fraction Ac (F2).

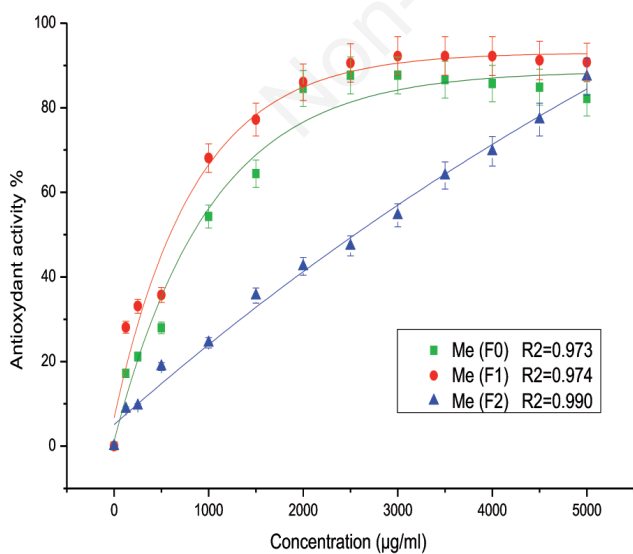


Figure 4. Antioxidant activity at varying concentrations of 3 hydro-methanol fractions: crude fraction Me (F0), ethyl acetate fraction Me (F1) and butanol fraction Me (F2).

idant activity compared to the other two extracts.²⁹ Hydroethanolic extract (50/50) of *Calendula officinalis* from south-eastern Serbia showed strong antiradical potency tested on DPPH free radicals and exceeded 96%.¹⁵

Alnuqaydan *et al.* studied *in vitro* the antioxidant power of *Calendula officinalis* flowers extracts on human skin cells. They were exposed to these extracts before being exposed to oxidizing attacks by the H₂O₂ hydrogen peroxide capable of killing them. The study showed that these extracts contain compounds that can remove free radicals and subsequently protect against oxidative stress in human skin cells.²³ The protective power of *Calendula* extract has also been demonstrated *in vitro* against nitrogen monoxide free radicals.³⁰

The antioxidant activity of *Calendula officinalis* was demonstrated *in vivo* by Preethi *et al.*, *Calendula* extract was given orally to rats and the results showed inhibition of superoxide production in macrophage and increased reduced glutathione levels in blood and liver.¹⁴

The antioxidant power of *Calendula officinalis* extracts is related to their content of polyphenols and flavonoids, which are the most important group of natural antioxidants. The biological effects of these antioxidants are mainly attributed to their action to reduce free radicals such as hydroxyl and superoxide radicals by producing low reactivity phenoxylic radicals. The antioxidant power of *Calendula officinalis* is at the origin of its therapeutic properties and it can be a promising source of antioxidant in the treatment of serious diseases such as cardiovascular disease, inflammation and cancer.

Conclusions

This study on *Calendula officinalis* from Morocco revealed its content in secondary metabolites (tanins, flavonoids, saponosite, triterpene and quinone). Extracts obtained from the flowers plant contain total phenolic compounds and exhibit antioxidant activity. The difference in phenol content and antioxidant power between the extracts of the hydromethanolic solvent (30/70) and the hydroacetonetic solvent (30/70) is not very great, however the antioxidant power is strongest for the fractions of ethyl acetate. *Calendula officinalis* can be considered as a source of natural antioxidants and used in the development of biomedical and cosmetic products, but for safe use of this plant further studies must be conducted to investigate its heavy metal content, toxicity and biological properties.

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