ANTICANDIDAL, ANTIBACTERIAL, CYTOTOXIC AND ANTIOXIDANT ACTIVITIES OF CALENDULA ARVENSIS FLOWERS

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Abstract - Calendula arvensis (CA) is one of the important plants used in traditional medicine in Morocco, due to its interesting chemical composition. The present study aimed to determine the anticandidal, antioxidant and antibacterial activities, and the effects of extracts of CA flowers on the growth of myeloid cancer cells. Also, to characterize the chemical composition of the plant. Flowers of CA were collected based on ethnopharmacological information from the villages around the region Rabat-Khemisset, Moroccco. The hexane and methanol extracts were obtained by soxhlet extraction, while aqueous extracts was obtained by maceration in cold water. CA extracts were assessed for antioxidant activity using four different methods (DPPH, FRAP, TEAC, b-carotene bleaching test). Furthermore, the phenolic and flavonoid contents were measured; also the antimicrobial activity has been evaluated by the well diffusion method using several bacterial and fungal strains. Finally, extracts cytotoxicity was assessed using MTT test. Phytochemical quantification of the methanolic and aqueous extracts revealed that they were rich with flavonoid and phenolic content and were found to possess considerable antioxidant activities. MIC values of methanolic extracts were 12.5—25 mg/mL. While MIC values of hexanolic extracts were between 6.25—12.5 mg/mL and were bacteriostatic for all bacteria while methanolic and aqueous extracts were bactericidal. In addition, the extracts exhibited no activity on Candida species except the methanolic extract, which showed antifungal activity on Candida tropicalis 1 and Candida famata 1. The methanolic and aqueous extracts also exhibited anti- myeloid cancer activity (IC_{50} of 31 mg/mL). In our study, we conclude that the methanolic and aqueous extracts were a promising source of antioxidant, antimicrobial and cytotoxic agents.

Keywords - Calendula arvensis; Anticandidal, antibacterial; Cytotoxic.

I. INTRODUCTION

The World Health Organization (WHO) has catalogued 20,000 plant species studied for medicinal purposes [1]. Natural products from plants include phenolic compounds that could delay or inhibit the inception of infectious diseases [2]. Literature surveys show that plant-based drugs play a promising role in the treatment of infectious diseases [3]. Efficacy and safety of plant extracts against some several pathogens is also well-documented [4]. The use of plant derived natural compounds as part of herbal preparations and alternative sources of drugs continues to play major roles in the general wellness of people all over the world. The search for new antioxidant, antimicrobial and anticancer agents are becoming an important objective for scientists.

Calendula arvensis (CA) is an interesting medicinal plant, known as ‘‘Aljemrah’’ in Morocco; CA is abundant, spring, annual, weeds and is doubtfully native. It is found as gregarious in open places, wastelands, roadside, and graveyard and field margins [1]. CA is traditionally used as food dye, spice and tea as well as tincture, ointment or cosmetic cream [5]. Numerous investigations have proved that extracts of the aerial parts of CA are rich in sesquiterpene glycosides (based on their alloaromadendrane, eudesmane and cubebane skeletons) [6]. Also, some triterpenoid saponins (e.g., arvensoside A and B) were isolated [7]. Furthermore, phytochemical studies [8, 9] have shown that CA contains various families of compounds such as phenolic acids and flavonoids. In this study, we have explored the anticandidal and cytotoxic effect of the Calendula Arvensis flowers, to explore its proclaimed antibacterial and anticancer properties in traditional medicine.

The objectives of this study were:

- To investigate antioxidant activities using different tests.
- To quantify the phenolic and flavonoid contents present in CA flowers.
- To estimate the antimicrobial capacities against human pathogen strains;
- To evaluate the cytotoxic potential on acute myeloid leukemia cells.

II. MATERIALS AND METHODS

A. Sample collection and authentication

Flowers of CA were collected based on ethnopharmacological information from the villages

around the region of Rabat- Khemisset, with the agreement from the authorities and respecting the United Nations Convention of Biodiversity and with assistance of traditional medical practitioner. The plant material was authenticated by Pr. M. Fanan (botanist from Institute Scientific in Rabat) and a voucher specimen (N8RAB 78161) was deposited in the herbarium of the botany department. Samples preparation and extraction For the aqueous extract (AE), CA flowers powder (200 g) was extracted with boiling water (500 mL) for 30 min. The infusion was filtered and then freeze-dried (aqueous extract) [10]. For the organic extracts (OE), hexanolic and methanolic extracts were obtained by soxhlet extraction of 200 g of CA flowers for 6 h in about 500 mL of solvents. Then, the filtrated extracts were evaporated in vacuo until dryness using rotator evaporator. This extracts were concentrated to dryness and the residue was kept at 48C [10].

B. Phytochemical screening of the extracts

The amount of total phenolic contents was determined according to Folin—Ciocalteu method as described by Lister and Wilson [11]. The standard curve of Gallic acid is obtained under the same conditions as above. The total phenolic content was measured as Gallic acid equivalents (mg GAE/g edw). The total flavonoids in the extracts were determined using a colorimetric method by Zhishen and Mengcheng. (1999)[12].

The absorbance was recorded against a blank at 510 nm. The flavonoid content was determined as the rutin equivalent from the calibration curve of rutins. Antioxidant activity . The free radical scavenging activity of the extracts was measured by 1.1-diphenyl-2-picryl-hydrazil (DPPH) as described by Huang and Ke [13] with some modifications. Briefly, 0.2 mm solution of DPPH in methanol was prepared and 0.5 mL of this solution was added to 2.5 mL of plant extract and was allowed to stand at room temperature for 30 min, and then absorbance was read at 517 nm against blank samples. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration. Using the equation:

$$\% \text{ RSA} = [(\text{AD} _ \text{AE})/\text{AD}]*100$$

Where AD is the absorbance value of the DPPH blank sample and AE is the absorbance value of the test solution. AE was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank. The ferric ions (Fe3+) reducing antioxidant power (FRAP) method [14] was used to measure the reducing capacity of oat extracts with a slight modification, which involves the presence of extracts to reduce the ferricyanide complex to the ferrous form. The absorbance was measured at 700 nm at the reaction time of 30 min. The reducing power of the extracts was represented as ascorbic acid equivalent (mg AAE/g edw).

The Trolox equivalent of antioxidant capacity (TEAC) was used. The assay is based on the inhibition of the absorbance of the radical cation 2, 20-azino bis(3-ethylbenzothiazoline-6- sulfonate) (ABTS+) solution when it is exposed to an anti-oxidant as described by Re et al. [15].

The ABTS+ cation radical was produced by the reaction between 10 mL of 2 mm ABTS in H2O and 100 mL of 70 mm potassium persulphate, stored in the dark at room temperature for 24 h. The ABTS+ solution was then diluted with methanol to obtain an absorbance of 0.70 at k = 734 nm and equilibrated at 30 8C. Samples were prepared in triplicate by diluting 50 mL of extracts in 2 mL of the ABTS+ solution diluted with methanol and let to react for 1 min. Absorbances were recorded at 734 nm. The antioxidant activities samples were expressed as TEAC values, defined as the concentration of standard Trolox with the same antioxidant capacity of the extract under investigation. The antioxidant activity of the extracts was also evaluated using a b-carotene-linoleic acid (linoleate) model system [16] with slight modifications. The method works in an aqueous emulsion of linoleic acid and bC, which is discolored by the radicals generated by the spontaneous oxidation of the fatty acids [17] and spectrophotometrically followed at 470 nm.

C. Antimicrobial activity screening

For the screening, the hexane and methanol extracts were dissolved in dimethylsulfoxide (DMSO) solvent. As a precaution not to miss trace amount of antimicrobial agent for preliminary screening, a relatively high concentration (200 mg/mL) of each extract were prepared for the bioassay. The extracts of CA flowers were individually tested against seven bacteria strains and six species of Candida spp. including: Salmonella blockley, Salmonella aequatoria, Salmonella braenderup, E. coli enteropathogenes, E. coli, E. coli ATCC, E. coli MDR and 6 species of Candida spp. (2 Candida tropicalis, 2 Candida famata and 2 Candida glabrata).These strains were obtained from three different sources; the American Type Culture Collection (ATCC, USA), Bacteriology Lab of the Institute National of Health (INH, Morocco) and Institute of Agronomy and Veterinary (IAV, Morocco). All media used were manufactured by Oxoid (Basingstoke, Hampshire, England). All bacteria and fungi were stored in BHI (brain and heart infusion broth) containing 30% (v/v) glycerol (Sigma-Aldrich) at 20 8C. Prior to use bacterial strains were first grown in Muller Hinton agar (MHA) at 37 8C for 24 h.

Mueller-Hinton agar (MHA) and Sabouraud Dextrose agar (SDA) (Hi-Media, Bombay, India) were

respectively used for bacteria and yeasts growth. Microbial cultures, freshly grown at 37/30 8C were appropriately diluted in sterile normal saline solution to obtain the cell suspension at 10^5 CFU/ mL. To evaluate antimicrobial activity, an agar well diffusion method was used as described by Nongpanga and Aporn [18], the organisms were spread on MH and SD agar plates by cotton swab. Wells of 6 mm diameter were punched into the agar medium and filled with 50 mL/mL of plants extracts. The plates were incubated for 24 h at 37 8C for bacteria and 48 h at 30 8C for yeast. Antimicrobial activity was evaluated by measuring the inhibition zone diameter against the test organism. Determination of the minimum inhibitory concentration (MICs) MICs are defined as the lowest concentration of CA extracts that will inhibit the visible growth of a microorganism after overnight incubation, the determination of MICs of extracts of CA flowers against the target test microorganisms was performed by microtitration technique as recommended by CLSI guidelines. The 0.5 McFarland suspension of bacterial strain from 24 h culture in Muller Hinton agar was appropriate diluted in saline water solution (0.85% w/v) to obtain the final inoculums to 10^5 cfu/mL. Determination of the minimum bactericidal concentration (MBCs) MBCs are defined as the lowest concentration of CA extracts that will prevent the growth of an organism after subculture on to antibiotic-free media, minimum bactericidal concentration was carried out by subculturing 50 mL aliquots from each well. Least concentration of extract showing no visible growth on subculturing was taken as MBCs value.

D. Cytotoxicity effect

Cancer cells of myeloid cell lines were procured by the National Centre for Cell Sciences, Fes, Morocco. Culture medium MEC (minimum essential communications) was purchased from Biopharma Society, Morocco. Antibiotics and FBS (fetal bovine serum). Cells were grown in nutrient mixture F-12, 82.5% supplemented with 2.5% FBS, 0.2% sodium bicarbonate, antibiotic and antimycotic solution. The cells were grown in the following conditions: 5% CO2, 95% atmosphere in high humidity at 37 8C in a CO2 incubator.

E. Mitochondrial activity by MTT assay

Cells (1×106/well) were seeded in poly-l-lysine precoated 96 well tissue culture plates and allowed to adhere for 48 h in CO2 incubator at 37 8C. The medium was replaced with the serum free medium containing different concentrations of CA extracts (10—100 mg/mL) separately again for 48 h in CO2 incubator at 37 8C. Tetrazolium bromide salt solution (10 mL/ well) was added in cell suspension (100 mL), four hours prior to completion of incubation period. Periodically viewing the cells under an inverted microscope for the presence of intracellular punctuates purple precipitate, dimethyl sulfoxide (200 mL) added to each well by pipetting up and down several times unless the contents get homogenized. Plate was placed in the dark for four hours at room temperature. The plates were kept on rocker shaker for 10 min at room temperature and then read at 550 nm using Multiwell micro-plate reader (Synergy HT, Biotech, USA). The average values were determined from triplicate readings and substract from the average values of the blank. Percent of inhibition was calculated by using the formula: Percent of inhibition = (C _ T)/C _ 100

Where C = absorbance of control, T = absorbance of treatment.

III. RESULTS AND DISCUSSION

A. Phytochemical content

Antioxidants properties

In order to estimate the in vitro antioxidant scavenging activities of CA extracts, the DPPH free radical scavenging assay was used. Fig. 1 shows the percentage of scavenging activity of CA methanolic extract, aqueous extract and hexanolic extract on free radicals of DPPH. The radical scavenging effects of different concentrations of methanol, water and hexane extracts of CA and Gallic acid (GA) are demonstrated. The antioxidant activities of the extracts were compared with GA, which is a synthetic antioxidant. Both water and methanol extracts had radical scavenging effects. However, this effect was not as great as the radical scavenging effect of GA. The radical scavenging effect of the methanol extract appeared to be closer to that of GA at 332 mg/mL. The magnitude of the radical scavenging effect at all concentrations was ranked as GA > ME > AE > HE with EC50 of 20.9 _ 5.34 and 33.2 _ 2.12 mg/mL for methanolic and aqueous extracts respectively (Table 1). Results from ABTS assay (Fig. 3) were comparable to DPPH activity. Radical scavenging activity has been demonstrated before in numerous studies [20,21]. Manifestly, the extracts obtained from CA and C. officinalis growing in Serbia and Monte-negro scavenged all radicals in concentration dependant manner [21]. EC50 registered in our assay was lower and with potent antioxidant activities compared to previous studies by Ercetin et al. [20].

To confirm this result the reducing power of the extracts were assessed using ferric to ferrous reduction activity by Oyaizu's method [14] which is used to measure the reducing capacity of these extracts. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of the CA polar and a polar extracts, determined by the FRAP method are shown in Fig. 2. Significant differences were shown

by CA flowers in the following order ME > AE > HE. The reducing ability of CA was most potent among the three extract in methanol solvent (203.96 _ 1.92 mg AAE/g Ext). The beta-carotene bleaching activity test was also used to demonstrate the antioxidants properties of CA extracts. It was important to assess CA extracts in a b-carotene—linoleic acid system: water emulsion assay since free radical scavenging models don't use a food or biologically relevant, oxidisable substrate [22]. In this test, oxidation of linoleic acid produces hydroperoxide-derived free radicals that attack the eleven pairs of b-carotene double bonds, resulting in a bleaching of the reaction emulsion. An extract capable of retarding or inhibiting the oxidation b-carotene may be described as a free radical scavenger and primary antioxidant [16]. Inhibition of b-carotene bleaching activity of CA extracts is exposed in Fig. 4. As it is shown in Fig. 4, all of the flowers extracts inhibited the bleaching of b-carotene by scavenging linoleate-derived free radicals methanolic extract inhibit b-carotene with a comparable rate to BHT. Differences in b-carotene bleaching assay reflect clearly the phenolic content as shown in Table 1.

B. Antimicrobial activity

To demonstrate the antimicrobial activity of CA extract, Candida species, E. coli species and Salmonella species were used. Table 2 indicates that all extracts of CA flowers show inhibitory activity against Salmonella aequatoria and E. coli ATCC. Moreover, methanolic extract inhibited E. coli ATCC, E. coli and Salmonella braenderup. Additionally, there were no inhibitory effects of extracts against Salmonella blockley, E. coli enteropathogenes and E. coli MDR (Table 2). However, no effect of CA extracts was observed on fungi species except methanolic extracts which showed moderate activity against two strains tested namely Candida tropicalis 1 and Candida famata 1 with inhibition zones (IZ) of 14 and 20 mm respectively. The phenolic compounds and flavonoids found in CA could be responsible for its antimicrobial activity against Salmonella aequatoria, E. coli ATCC, Candida tropicalis 1 and Candida famata 1. Similarly, the aqueous-methanolic extracts from C. officinalis also showed anti-microbial activity against tested germs [23]. Following these results, we could conclude that aqueous-methanolic flower extract from CA has very interesting antibacterial and anti-fungal activities against the tested microorganisms. MIC values of methanolic and aqueous extracts were between 12.5—25 mg/mL and were bactericidal for all bacteria. While MICs of values of hexanolic extracts were between 6.25—12.5mg/mL and were bacteriostatic for all bacteria (Table 3) confirming our previously published results [4].

C. Cytotoxic activity

To test the potential anticancer properties of CA extracts, human cancer of myeloid cells were used for cytotoxicity assays. Cells were treated with twelve increasing concentrations (2—0.00093 mg/mL) for 48 h by MTT bioassay. Percent inhibition of methanolic and aqueous extracts of the CA flowers were calculated for cancer cells of myeloid cell and presented in subsequent (Fig. 5). A perusal indicates the presence of noticeable percent inhibition of methanolic and aqueous extracts against cancer cells of myeloid cell. MTT bioassay which provides a real picture of percent inhibition of herbal extract was used for the present study. Percent inhibition resulting MTT bioassay demonstrated that methanolic and aqueous extracts are the efficient candidates as cytotoxic bioagent (antimyloid cancer activity). The percent inhibition for these extracts showed more pronounced efficacy compared to hexanolic extract. It was also inferred that comparatively methanolic extract is more significant antimyloid cancer agent, showing maximum 89% inhibition at the concentration 100 mg/mL at 24 h ($P < 0.05$). Attempts were also made to present our findings in graphical forms highlighting the significant cytotoxicity of both extracts against myeloid cell line. Methanolic and aqueous extracts of the CA flowers being potent, therefore, was further studied for its percent inhibition as a function of time (24—96 h) and dose (20—100 mg/mL). This study is in accordance with previous reports [24] describing the isolation of flavonoids from different parts of A. nervosa, A. corniculatum, C. pulcherrima, C. viscosum, D. induce, D. peregrina, G. luteoalbum, G. oppositifolius, J. sambac, L. coromendalica and M. nagi [24]. It is likely that these constituents are also associated with the anticancer activity observed in this study [25—27]. The trend of the caused anticancer activity showed that 48 h is the optimum time for significant effect. On the basis of the observed encouraging cytotoxic effect by the in vitro bioassays, it can be revealed that methanolic and aqueous extracts of CA (flowers) have promising antimyloid cancer bioefficacy and must have some chemical moiety in the flowers part of this plant which are responsible for the observed beneficial effect. We suggest that detailed in vivo studies should be carried out to elucidate the mechanism. Screening of phytochemicals for anticancer activity is extremely important in today's context as a variety of tumors are getting resistant to conventional anticancer drugs. Structural elucidation of bioactive principle of methanolic and aqueous fraction of CA is to be worked.

Phyto constituents such as polyphenols, flavonoids and catechins have long been recognized as having potential anticancer, anti-inflammatory; antioxidant

and antimicrobial properties and efforts on the above lines are in progress in the Lab.

CONCLUSION

Our study demonstrates that antioxidant and cytotoxic activities were observed for methanol and aqueous CA flowers extracts, which might be correlated to its leading phenolic and flavonoid contents as compared to its hexanolic counterparts. All extracts of CA flowers show an inhibitory activity against most bacteria and fungi species. In fact, we were able to demonstrate the cytotoxic, antioxidant and anti-microbial activities of methanol and aqueous extracts. Future work is underway to isolate bioactive constituents of CA flowers extract to determine potential pharmacological agents.

Disclosure of interest

The authors declare that they have no competing interest.

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RESULTS

Tables and Figures

Extracts	PC[W]	FC[Y]	DPPH[Z]
HE	n.d	n.d	n.d
ME	47.89±2.34[a] 50.26±0.18[b]	74.93±1.50[a] 174.93±5.21[b]	33.2±2.12[b] 20.9±5.34[a]

Table 1 Phenolic, flavonoid contents and radical scaveng- ing activity of extracts from CA flowers.

PC: phenolic content; FC: flavonoid content; ME: methanolic
extract; HE: hexane extract; AE: aqueous extract; n.d: not determined. Data are reported to mean (n = 3) ± standard error. Values in the same column not sharing a common letter differ significantly at $P < 0.05$. [W]mg GAE/g dw. [y]mg RE/g dw. [z]EC_{50} expressed in mg/Ml

	ME	HE	AE
Candida tropicalis 1	14	n.d	n.d
Candida tropicalis 2	n.d	n.d	n.d
Candida famata 1	20	n.d	n.d
Candida famata 2	n.d	n.d	n.d
Candida glabatra 1	n.d	n.d	n.d
Candida glabatra 2	n.d	n.d	n.d
Salmonella blockley	n.d	n.d	n.d
Salmonella aequatoria	14	12	14
Salmonella braenderup	8	n.d	n.d
E. coli enteropathogenes	n.d	n.d	n.d
E. coli ATCC	14	8	12
E. coli	15	n.d	n.d
E. coli MDR	n.d	n.d	n.d

Table 2: Antifungal and antibacterial activities of different extracts from CA Flowers.

n.d: not determined.

Strains	Methanolic MIC	MBC	AA	Hexanolic MIC	MBC	AA	Aqueous MIC	MBC	AA
Salmonella aequatoria	12.5	12.5	+	6.25	6.25	-	12.5	12.5	+
Salmonella	12.5	12.5	+	-	-	-	-	-	-
E. coli ATCC	12.5	12.5	+	6.25	6.25	-	12.5	12.5	+
E. coli	25	25	+	12.5	12.5	-	25	25	+

Table 3 : Determination of MICS and MBCS of the CA flowers extracts.

(+): bactericidal; (-): bacteriostatic; AA: antibacterial activity

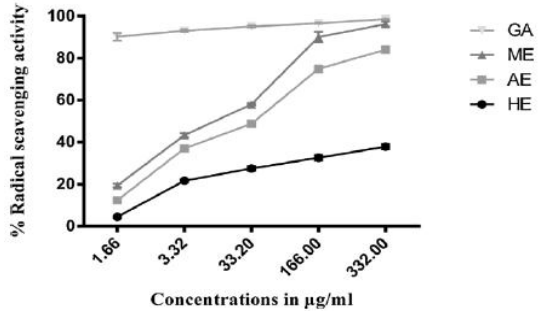


Figure 1 Radical scavenging activity of CA Flowers extracts. GA (gallic acid), ME (methanolic extract), AE (aqueous extract), HE (hexanolic extract). No significant differences have been registered between methanolic extract (ME) and the reference gallic acid (GA) at a concentration of 332 mg/mL.

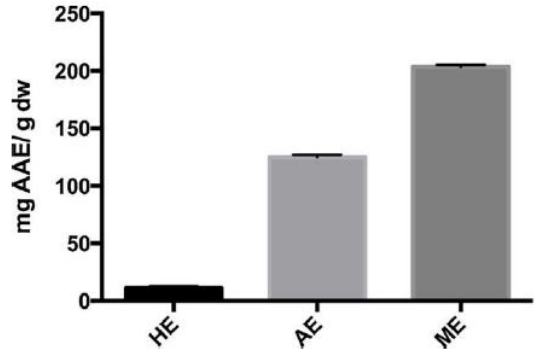


Figure 2 Ferric reducing antioxidant power of CA Flowers extracts. GA (gallic acid), ME (methanolic extract), AE (aqueous extract), HE (hexanolic extract). Data are reported to mean (n = 3) ± standard error. The results are expressed in mg ascor- bic Acid equivalent/g dry weight (mg AAE/g dw).

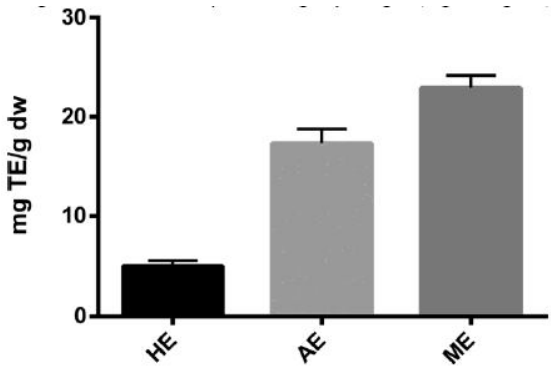


Figure 3 Trolox equivalent antioxidant capacity of CA Flowers extracts using ABTS. ME (methanolic extract), AE (aqueous extract), HE (hexanolic extract). Data are reported to mean (n = 3) ± standard error. The results are expressed in mg Trolox equivalent/g dry weight (mg TE/g dw).

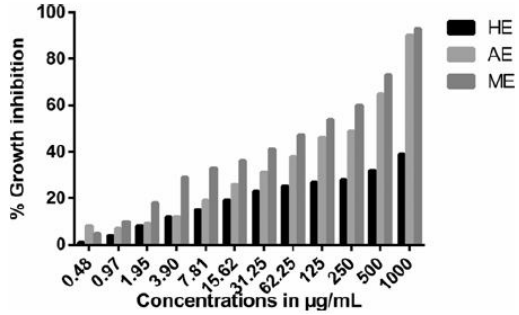


Figure 5 Cytotoxic activities of CA flowers extracts on acute myeloid cancer cells.

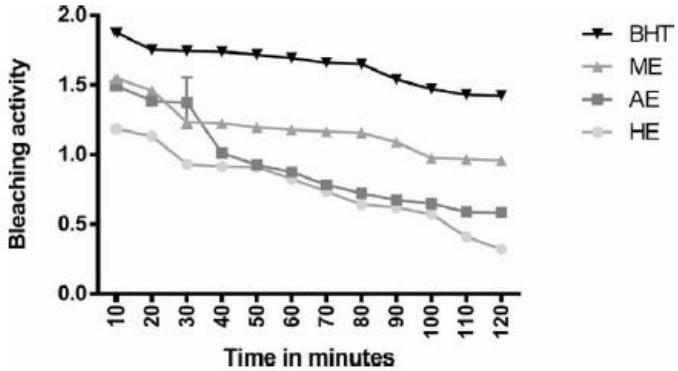


Figure 4 Beta-carotene bleaching activity of CA flowers extracts. ME (methanolic extract), AE (aqueous extract), HE (hexanolic extract). Bleaching activity of CA extracts is pre- sented by the emulsion absorbance.

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