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Inhibitory and antioxidant activities of enzymes involved in hyperglycemia of *Chrysanthellumamericanum(L.)*Vatke extracts

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Abstract:
Chrysanthellum americanum (L.)VATKE (Asteraceae) is a herbaceous plant native of South America (Bolivia and Peru). It is also found in Africa, particularly in Mali. It has a yellow flower reminding that of the Roman chamomile. The objective of this study was to evaluate the flavonoid contents, to determine the antioxidant and enzyme inhibitory activities of ethyl acetate (ACC) and aqueous (AQC) extracts of C. americanum. The flavonoidlevels were determined by spectrophotometric method, antioxidant activity by DPPH, ABTS and FRAP methods. As for the inhibitory activity of α-amylase, α-glucosidase and β-glucosidase, it was evaluated using in vitro model. The ACC and AQC fractions contained flavonoid (1028.33±8.84 and 308.33±11.46 mg ER/100g extract), respectively. With all systems used, both extracts showed good antiradical activity. The ethyl acetate fraction showed greater inhibitory activity on α-glucosidase (IC_{50}=164.4±13.3 µg/mL) than the other enzymes; α- amylase (IC_{50}=2036±0.00 µg/mL) and β-galactosidase (IC_{50}= 2436±109 µg/mL). These results show that the ACC extract with its richness in flavonoidcould have therapeutic potential against oxidative stress-related diseases and therefore could be useful in the management of hyperglycemia.
Keywords: *Chrysanthellum americanum, activités antioxydante, activité hypoglycémiante, flavonoïdes*

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I. Introduction

The traditional medicine remains the main recourse of a large majority of populations to solve their health problems, not only because it is an important part of cultural heritage, but also because of limited financial means in the face of conventional products [1]. Even today a majority of the world's population, especially in developing countries, treat themselves solely with traditional herbal remedies [2]. Oxidative stress is implicated in a wide spectrum of diseases that have a huge impact on the health of populations, among these pathologies the diabetes occupy an important place. Diabetes is a disorder of lipid, carbohydrate, and protein metabolism attributed to decreased production of insulin or abnormal resistance to this hormone that results in increased glucose levels [3].

According to the International Diabetes Federation, diabetes is a global health problem affecting 425 million people and is expected to reach 629 million by 2045 [4].

One therapeutic approach to decrease hyperglycemia is to delay and reduce the digestion and absorption of ingested carbohydrates by enzymes (such as α-amylase and/or α-glucosidase) in the gastrointestinal tract [5, 6, 7]. Inhibitors of these enzymes delay carbohydrate digestion and prolong the overall time of carbohydrate digestion, causing a reduction in the rate of glucose uptake and consequently blunting postprandial plasma glucose levels [7]*Chrysanthellumamericanum* (Linn.) Vatke, belonging to the Asteraceae family, is an aromatic herb that is widely found in tropical regions of Africa and America. It is known by common names such as: Chrysanthellum or golden chamomile.

Its nutritional richness in flavonoids and saponosides gives it a beneficial action on the circulatory system. It relieves heavy legs and helps to relieve the liver after certain food excesses [8]. In Burkina-Faso, it is known for its antioxidant properties, recent studies have shown that the plant is used in the treatment of kidney diseases due to oxidative stress [9]. An ethnobotanical survey conducted in Mali, showed that the plant is little known by traditional practitioners and that it is used against certain diseases such as anemia, stomach aches and iron deficiency [10]. To our knowledge, no study on the inhibition of the different enzymes responsible for hyperglycemia in certain people has yet been carried out on this species, which justifies this study.

II. Materialsand Methods

2.1. Plant material

It was consisted of the whole plant of *C. americanum*, collected in 2015 in Mali, at Ouéléssébougou, region of Koulikoro, located at 70 km south of Bamako. The sample was identified at the Department of Traditional Medicine and the botany laboratory of the Faculty of Sciences and Techniques of the University of Sciences, Techniques and Technologies of Bamako (USTTB). It is registered under N°3001. The plant was dried in the laboratory of organic chemistry and natural substances of the same faculty.

2.2. Flavonoid extraction

The extraction of flavonoids is performed according to the method of[11] with some modifications. It is based on the degree of solubility of flavonoids in organic solvents. This method is based on two main steps: the first phase consists of a reflux extraction which is done with methanol-water (70/30: v/v) to solubilize the flavonoids. Thus, to 50 g of plant powder, 500 mL of solvent were added. The second phase was carried out by liquid/liquid separation with solvents of increasing polarity: petroleum ether (waxes, lipids and chlorophyll); diethyl ether (extraction of free genins) and ethyl acetate (extraction of monoglycosides). The ethyl acetate fraction (ACC) and the aqueous residue (AQC) were assayed for flavonoids and biological tests.

2.3. Dosage of total flavonoids

The estimation of total flavonoids was carried out according to the method described by[12]. To 1000 µL of each extract to be analyzed, 1500 µL of 95% methanol, 100 µL of 10% $AlCl_3$ (w/v), 100 µL of 1 M sodium acetate and 2.8 mL of distilled water were added. The mixture is stirred and incubated in the dark at room temperature for 30 min. The blank is made by replacing the extract with 95 % methanol and the absorbance is measured at 415 nm using a UV spectrophotometer. The results are expressed in mg rutin equivalent per gram of dry weight.

2.4. Antioxidant activity *in vitro*

2.4.1. DPPH assay

The ability to trap the stable free radical 2,2-diphenyl 1-picrylhyorazyl (DPPH) of *Chrysanthellum americanum* extracts was evaluated using the spectrophotometric method described by [13]. One (1) mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of extract at various concentrations (1,66-16,67 µg/mL). At the same time, a mixture of 1mL methanol and 1mL DPPH solutions was used as a control. The reaction was performed in triplicate and the decrease in absorbance was measured at 517 nm after 30 minutes incubation of the samples in the dark. The positive control was BHTwhose absorbance was measured under the same conditions as the samples. The antioxidant activity related to the scavenging effect of the DPPH radical is expressed as percentage inhibition (PI) calculated from the absorbances obtained according to the following formula:

$$[PI] = \frac{A0-A1}{A0} \text{ x } 100$$

A0 = DPPH absorbance; A1: sample absorbance.
IC_{50} (concentrations that inhibit 50% of the DPPH radical) were inferred from the linear regression line obtained from the graph representing the percentage inhibition of DPPH.

2.4.2. ABTS assay

The 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) cation radical is generated by mixing equal volume of a 2.45 mM solution of potassium persulfate K2S2O8 and a 7 mM stock solution of ABTS, all of which is kept protected from light and at room temperature for 16 h before use [14]. The resulting solution is diluted with methanol to obtain an absorbance of 0.700 at 734 nm. A volume of 200 µL of each extract was added into 2 mL of the ABTS solution. After mixing the two volumes, we let the mixture stand for one minute. Then the absorbances were read at 734 nm. Three absorbance measurements were made for each concentration tested (n=3). The results were expressed as Trolox equivalent per gram of extract (mg TE/g).

2.4.3. FRAPassay

The reducing power of the extracts is determined according to the method described by[15]. To a test tube containing 0.1mL of sample solution are added 2 mL of phosphate buffer (0.2M, pH 6.6) and then 2 mL of potassium hexacyanoferrate [K3Fe(CN)6] (10g/L). The mixture is heated to 50°C in a water bath for 20 minutes. A volume of 2 mL of trichloroacetic acid (100 g/L) is then added to stop the reaction. The mixture is centrifuged at 3000 rpm for 10 minutes. Finally, 2 mL of the supernatant were mixed with 2 mL of distilled water and 0.4 mL of ferric chloride (FeCl3) (1 g/L). A blank without extract was prepared in the same conditions. The reading was taken at 700 nm. Ascorbic acid was used as a positive control. Three absorbance measurements were performed (n=3).

2.5. Inhibitory activities of enzymes involved in hyperglycemia

2.5.1. Inhibition of α-amylase

Following the procedure described by[16] with slight modifications, the inhibitory potentials of α amylase were investigated by reacting different concentrations of the extracts with the enzyme-amylase and starch solution. A mixture of 250 μL of sample and 250 µL of 0.02 M sodium phosphate buffer (pH = 6.9) containing the enzyme α-amylase (240U/mL) was incubated at 37° C for 20 min. Then, 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH = 6.9) was added to the reaction mixture. Therefore, the reaction mixture was incubated at 37 ° C for 15 min. Subsequently, 1 mL of Dinitro Salicylic Acid (DNS) was added and the reaction mixture was incubated in a boiling water bath (100°C) for 10 min. Then, the reaction mixture was diluted by adding 2 mL of distilled water. The absorbance readings were taken at 540 nm. Acarbose was used as a positive control. The percentage of inhibition was determined at different concentrations to obtain a 50% inhibitory concentration (IC_{50}). Three absorbance measurements were performed for each concentration tested (n=3).

2.5.2. Inhibition of α-glucosidase

The inhibitory activity of the extracts on this enzyme was performed using the method described by[17]. A solution of pNPG substrate (paraNitrophenyl-α-D- glucopyranosidase, 0.1M) and enzyme (α-glucosidase) was prepared in phosphate buffer solution (0.1M at pH=6.7), along with our extracts at 1mg/ml. A sodium bicarbonate solution (0.1M) was also prepared. In each test tube, 150µL of extracts was put (stock solution 1mg/ml) to which was added 100 µL of enzyme (10mL). For each tube containing an extract, a control tube was made (150 µL extract and 100 µL buffer solution). Also two types of blank were prepared: one blank tube contains (150 µL enzyme and 100 µL buffer), and the other blank tube contains only 250 µL of the buffer solution. The whole is incubated in the oven for 10 min at 37 °C. After this incubation, 200 µL of pNPG substrate was added to all test tubes, after which another incubation in the oven was performed for 30 min at 37 °C. The absorbance was measured at 405 nm using a spectrophotometer. Three absorbance measurements were performed for each concentration tested (n=3). The percentage of inhibition was determined at different concentrations to obtain a 50% inhibitory concentration (IC_{50}).

2.5.3. Inhibition of β-galactosidase

The method used was the one described by[18]. It has the same principle as α-amylase, only the substrate is β-D-galactose. The reading was taken with a spectrophotometer at 410 nm. Three absorbance measurements were made for each concentration tested (n=3).

The results were expressed as percent inhibition using the following formula.

$$Inhibition(\%) = \frac{(\mathrm{At} - \mathrm{Abc}) - (\mathrm{Ae} - \mathrm{Abe})}{(\mathrm{At} - \mathrm{Abc})} \times 100$$

where At is the absorbance of the control (enzyme and buffer); Abc is the absorbance of the control blank (buffer without enzyme); Ae is the absorbance of the sample (enzyme and inhibitor); and Abe is the absorbance of the sample blank (inhibitor without enzyme).

2.6. Data analysis

Excel 2010 software was used to analyze the data, which are expressed as the mean ± standard deviation.

III. Results and discussion

The data for flavonoid, ABTS, FRAP and DPPH assays are summarized in Table 1.

Table 1: Flavonoid contents and antioxidant activities of ethyl acetate and aqueous extracts.

	Flavonoïdes (mgRE/100g)	ABTS (mgTE/g)	FRAP (mgAAE/g)	DPPH (µg/mL)
ACC	1028,33±8,84	56,86±0,06	289,93±50,3	5,42±0,28
AQC	308,33±11,46	46,91±0,01	197,4±4,39	8,17±1,2

The ACC fraction is richer in flavonoids than the AQC one, with a content of 1028.33±8.84 mg Eq of Rutin/ 100 g of extract (Table 1). These data are in agreement of those of[18]who reported thatthe ethyl acetate is the best extractive solvent for polyphenols and flavonoids. But, our resultsarehigher than those recorded by[9], who had reported a level of 4.22 ± 0.06 mg EQ/100mg with methanolic extract.

The antioxidant activity of a compound corresponds to its capacity to resist oxidation. We studied the antioxidant and free radical activity of the two fractions on DPPH free radical scavenging, ABTS and FRAP. The results obtained are presented in the table 1. Our two fractions show a very good antiradical and antioxidant activity on free radicals. ACC has presented a good inhibitory activity on the DPPH radical than AQC (Table 1). Our fractions possess good activities on free radicals. [19] working on different solvent have found the best antioxidant power with ethyl acetate one due to its levels of polyphenols and flavonoids. Previous work on methanolic extract of the same plant showed that it possesses activity on free radicals [9]. This activity could be related to the presence of polyphenols such as flavonoids. Indeed, most synthetic or naturally occurring antioxidants have hydroxyphenolic groups in their structures and the antioxidant properties are attributed in part to the ability of these natural compounds to scavenge free radicals [20, 21, 22, 23].
According to the same authors flavonoids would be very good secondary metabolites to fight against free radicals and prevent diseases that are related.

Table 2: IC_{50} values of the different tests for *in vitro* antidiabetic activity.

	IC_{50} (µg/mL)		
	α-glucosidase	**α-amylase**	**β-galactosidase**
ACC	164,4±13,3	203,64±0,08	243,6±10,9
AQC	195,4±9,93	ND	259,4±64
Acarbose	18,01±2,01	311,2±1,38	-
Quercitine	-	-	246,9±1,09

The inhibitory concentration 50% (IC_{50}) corresponds to the effective concentration that allows to inhibit the enzyme responsible for the degradation and the increase of sugar in a patient. The ACC fraction presented an inhibitory activity on the three enzymes, but not less important than the references which are Acarbose and Quercitin. These different IC_{50} values obtained are presented in Table 2. It appears that the AQC fraction has no activity on the α-amylase enzyme. The plant has been the subject of several studies on different pharmacological activities including antibacterial activity on the volatile and non-volatile part [9, 24]. To our knowledge, this is the first time that its anti-diabetic activity has been evaluated *in vitro* on enzymes responsible for the degradation of polyholosides into monosides and promote postprandial glucose increase.

IV. CONCLUSION

The ethyl acetate and aqueous extracts of *C. americanum* show good antioxidant activity on DPPH, ABTS and FRAP radicals. This could be explained by its richness in phenolic compounds, especially flavonoids. Thus this plant could be used in the treatment of diseases related to free radicals. The ethyl acetate fraction seems to have an anti-diabetic activity *in vitro* contrary to the aqueous one which is not active onα-amylase. It would be important to investigate the structural identification (GC/MS, IRand NMR) of the flavonoid types and to conduct further biological studies in order to make it a phytomedicine.

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