

# Screening of antioxidant, anti-acetylcholinesterase and antifungal activities and HPLC-MS identification of the bioactive phenolics of *Eclipta alba* (L.) Hassk.

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## Abstract

*Eclipta alba* is commonly used in traditional medicine for several ethnobotanical properties. Researches have isolated several bioactive compounds of this plant. In Burkina Faso, this medicinal plant is mainly used for its antifungal properties.

Our study aimed to evaluate the antioxidant, acetylcholinesterase inhibitory, antifungal activities and to quantify polyphenols from *Eclipta alba* extracts using HPLC-MS. The crude extract (CE) and fractions of this plant were used for these activities through standard methods. Ethyl acetate fraction (EAF) and acetonitrile fraction (ACNF) have been the potential inhibitors of the liver lipids peroxidation (According to the thiobarbituric acid method) with the percentages of 65.48% and 65.13%, respectively. Concerning the ABTS radical cation scavenging capacity described by Guenné, ACNF (67.28 mg T/g of extract) and butanol fraction (BF) (66.03 mg T/g of extract) have presented the highest activities. BF has also presented an interesting acetylcholinesterase (AChE) inhibitory activity with a percentage of 55.82%. For antifungal activity, the crude extract and ACNF have given the better MIC values against *C. albican* and *T. beigeli* with a concentration of 1.25 mg/mL using microplate method described by Eloff. The strain *C. krusei* was only sensitive to butanol fraction. The polyphenols identified and quantified using HPLC-MS are: gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, apigenin, luteolin and quercetin.

The butanol fraction of *E. alba* presented the best activities for the tests performed; this fraction is a good way to isolate new anti-fungal and anti-acetylcholinesterase compounds.

**Keywords:** *E. alba*, Antioxidant, anti-acetylcholinesterase, Anti-fungal, Polyphenols HPLC-MS

## Introduction

*Eclipta alba* is an annual herb of *Asteraceae* family met in tropical and subtropical regions of the world. This plant is used in Africa and Asia in traditional medicine for its multiple therapeutic properties to treat leprosy, asthma, bronchitis and inflammatory diseases [1-3]. In Burkina Faso the plant is used to treat the children cough, the burns, the wounds and the fungal infections [1, 4]. Most of these diseases are linked to oxidative stress; this is also the case of Alzheimer's disease manifested by a decreased level of acetylcholine at nerve.

A phytochemical and pharmacological researches on the extracts of the plant have allowed to isolate several compounds with pharmacological interest from this plant. These compounds are diosmetin; 3'-hydroxybiochanin; A; 3'-O-methylorobol; Orobol; Ecliptal; Wedelolactone; Demethylwedelolactone; eclalbasaponin II-V; echinocystic acid [5-10]. These compounds are polyphenols, terpenes, alkaloids. It is shown that polyphenols have

antioxidant properties. Also, molecules that have antioxidant capacity and are able to pass the blood-brain barrier can improve nervous functions [11].

Of all these molecules isolated from extracts of *E. alba*, no study has been made in the direction to isolate compounds that are active inhibitor of acetylcholinesterase. The AChE is an enzyme involved in Alzheimer's disease. Thus, it is essential to evaluate the biological activities of the polyphenols resulting from extracts of this plant. Based on ethnobotanical data obtained in Burkina Faso; our study aimed to evaluate the antioxidant, acetylcholinesterase inhibitor, antifungal capacities of *Eclipta alba* extracts. Finally, with a view to justify these activities of the extract of the plant revealed by the surveys, we used the HPLC-MS to detect and quantify the polyphenol this plant extract.

## Materials and Methods

## Chemicals

To carry out our different activities, we used solvents, enzymes and various classic reagents. All reagents were of analytical grade. Sodium chloride, quercetin, hydrochloric acid, ethyl acetate, acetic acid, p-iodonitrotetrazolium chloride (INT), n-hexane, acetonitrile, magnesium chloride, Bovine Serum Albumin (BSA), Acetylcholinesterase (EC 232. 559. 3. 3) (AChE) from electric eel, acetylcholine iodide (ATCI), 5,5'-dithiobis-2-nitrobenzoic acid (DTN) and 2-thiobarbituric acid were purchased from Sigma Aldrich chemie (Steinheim, Germany); potassium persulfate, 2,2'-azinobis (3 ethylbenzothiazoline-6-sulphonate) ABTS and trichloroacetic acid were supplied by Fluka chemie (Buchs, Switzerland); dichloromethane, ferric dichloride, ethanol, methanol were sourced from Probalo (Paris, France); butanol was sourced from sds (Peyin, France). Standards: caftaric acid from Dalton (USA), gentisic acid, ferulic acid, sinapic acid, patuletin, luteolin from Roth (Germany), caffeic acid, chlorogenic acid, p-coumaric acid, hyperoside, isoquercitrin, rutoside, myricetol, fisetin, quercitrin, quercetol, kaempferol and apigenin were from Sigma (Germany).

## Plant material

*Eclipta alba* (L.) Hassk. specie was collected in August 2010 in Loumlila, 15 Km north of Ouagadougou, capital of Burkina Faso. The plant was identified by Prof. Millogo- Rasolodimby from the plants Biology Department of the University of Ouagadougou. A voucher specimen (ID-10476) was deposited at the Herbarium of the Laboratory of Vegetable Biology and Ecology, of the University of Ouagadougou.

## Preparation of extracts

*E. alba* whole-plant was dried at room temperature and ground to fine powder. 25g of powder was extracted with 250mL of aqueous ethanol (80%) at laboratory conditions during 24 hours. After, extract solutions were concentrated under reduced pressure in a rotary evaporator (BÜCHI, Rotavapor R-200, Switzerland) at approximately 40 C, frozen and lyophilized using a lyophilizer (Telstar-Cryodos 50, Spain). The extracts (crude) obtained were fractionated by solvents of increasing polarity (hexane, dichloromethane, acetonitrile, ethyl acetate, butanol and water residual). The aqueous fraction was also frozen and lyophilized. All obtained extracts were weighed before packed in waterproof plastic flasks and stored at 4 C until use.

## Microorganisms

Three microorganisms used in this study consisted of clinical isolates. These clinical isolates were obtained from biomedical laboratory. *Candida albican*, *Candida krusei* and *Trichosporon beigeli* were used. Before testing, pure cultures of these fungi were realized using sabouraud and Mueller Hinton (MH) tryptic soy Broth. Inoculate were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standard.

## Antioxidant tests

### Trolox Equivalent Antioxidant Capacity (TEAC)

The ABTS radical cation decolorization assay according to the procedure Guenné *et al.* [4] with some modifications was used to determine the antioxidant capacity of extracts. ABTS radical cation (ABTS<sup>+</sup>) was produced by reacting aqueous ABTS stock solution (7 mM) with 2.45mM potassium persulfate (final concentration). The mixture was put down in the dark at room temperature during 16h before use. This mixture was diluted with ethanol to give an absorbance of  $0.7 \pm 0.02$  units at 734 nm using microtitre plates UV/visible light spectrophotometer (Epoch 251465, Biotek Instruments, U.S.A.) reader. 50  $\mu$ L of the diluted sample (1g/mL in methanol) or identified polyphenols were added with 200  $\mu$ L of fresh ABTS<sup>+</sup> solution and the absorbance was taken 15 min exactly after initial mixing. Trolox was used to produce the calibration curve ( $R^2 = 0.99$ ) and the antioxidant capacity of extracts were expressed as mg Trolox Equivalent per g of extract.

### Rat liver lipid peroxidation inhibition

Extracts lipid peroxidation (LPO) inhibitory activities were determined according to the 2-thiobarbituric acid method [12, 13] Ferrous chloride (FeCl<sub>2</sub>) with H<sub>2</sub>O<sub>2</sub> was used to induce the liver homogenate peroxidation. In this method 0.2mL of extracts (1.5 mg mL<sup>-1</sup>) was mixed with 1.0 mL of 1% liver homogenate in Tris-HCl buffer, then 50  $\mu$ L of FeCl<sub>2</sub> (0.5 mM) and 50  $\mu$ L of H<sub>2</sub>O<sub>2</sub> (0.5 mM) were added. The mixture was incubated at 37 C for 60 min, then 1.0 mL of trichloroacetic acid (15%) and 1.0 mL of 2-thiobarbituric acid (0.67%) were added and the mixture was heated up in boiled water for 15 min. The absorbance was recorded at 532 nm using spectrophotometer. Quercetin was used as the positive controls.

### Acetylcholinesterase (AChE) inhibitory activity

Acetylcholinesterase inhibitory assay with inhibition kinetics analysis were conducted according to the protocol described by Kiendrebego *et al.* [14] with some modifications. 50  $\mu$ L of Tris- HCl buffer (50 mM, pH8, 0.1% BSA) was added with 25  $\mu$ L of extract or identified polyphenols (final concentration of 100  $\mu$ g/mL dissolved in MeOH buffer 10%) and 25  $\mu$ L of AChE (0.22 U/mL). The mixture was incubated at room temperature for 2 min before the addition of 125  $\mu$ L of DTNB (3 mM) and 25  $\mu$ L of substrate (ATCI 15 mM). The developing yellow color was measured at 405 nm after 4 min on a spectrophotometer. Galanthamine a reference compound use for Alzheimer cure was used as a positive control at a final concentration of 2 $\mu$ g/mL in the assay mixture. AChE inhibitory activity was expressed as inhibition percentage of AChE

## Antifungal tests

### Inhibition zone diameter (IZD) determination

Sensitivity of different fungus species to various extracts (dissolved in DMSO 5%) were measured by using disc diffusion method with

some modifications [15, 16]. Petri plates containing sabouraud/Nutrient agar were spread with 0.2 mL of the inoculum. 6 mm diameter of sterile Whatman filter paper were soaked with 10 $\mu$ L of extract or fractions (20 mg/mL) and deposited in plates. The plates inoculated with different yeasts were incubated at 33 C up to 48h-96h and diameter of any resultant zones of inhibition were measured. For each combination of extracts and the fungi, the experiment was performed in triplicate and repeated twice. The yeast with a clear zone of inhibition of more than 10 mm was considered to be sensitive. Sensitivity of different yeasts to DMSO was measured to evaluate this solvent toxicity (negative control).

### Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of antifungal (sensitive yeasts to extracts) activity was determinate with serial dilution technique, using 96-well microplates [17, 18]. In each colon of well 100 $\mu$ L of sterile MH broth and 100 $\mu$ L of sterile extract (20mg/mL) were put only in the first line. Successively dilutions permit to obtain extracts concentration between 20 and 0.325mg/mL. 10 $\mu$ L of each fungal culture was added singly to each well. The density of yeast was standardized using McFarland 0.5 turbidity standard. The plates were covered and incubated overnight (24 hours) at 33 C. To indicate fungal growth, 50  $\mu$ L of 0.2 mg/mL p-iodonitrotetrazolium (INT) was added to each well and the plates incubated at 33 C for 30 min. This assay was twice and repeated three times.

### HPLC-MS analysis

#### Standard and sample solutions preparation

Stock solutions (0.1g/mL in methanol) of the following standard compounds were prepared: caftaric acid, gentisic acid, ferulic acid, sinapic acid, patuletin, luteolin, caffeic acid, chlorogenic acid, p-coumaric acid, hyperoside, isoquercitrin, rutoside, myricetol, fisetin, quercitrin, quercetol, kaempferol and apigenin. All stock solutions were stored in the dark at 4 C, and an appropriate dilution of each of them was performed with double distilled water before analysis. The methanol extract was used to prepare sample stock solution of 10 mg/mL in 50% methanol. The non hydrolysed samples (NHS) were diluted at a concentration of 5 mg/mL in 50% methanol before injection. The hydrolyzed samples (HS) were prepared and adjusted to 10 mL with methanol (50%) to get the same concentration as the non hydrolyzed extract, before injection.

#### Apparatus and chromatographic conditions

The experiment was carried out using an Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser, binary gradient pump, column thermostat, auto sampler and UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18 100 x 3.0 mm i.d., 3.5  $\mu$ m particle); the work temperature was 48 C. The

detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min for phenolic acids (Caftaric acid, Gentisic acid, Caffeic acid, Chlorogenic acid, p-coumaric acid, Ferulic acid, Sinapic acid), then at 370 nm for flavonoids (Hyperoside, Isoquercitrin, Rutoside, Myricetol, Fisetin, Quercitrin, Quercetol, Patuletin, Luteolin, Kaempferol, Apigenin). The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using ChemStation and Data Analysis software from Agilent, USA.

The mobile phase was a binary gradient prepared from methanol and solution of acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; isocratic elution followed for the next 3 minutes with 42% methanol. The flow rate was 1 mL min<sup>-1</sup> and the injection volume was 5  $\mu$ L.

### Identification and quantitative determinations of polyphenols

The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library. Later, the MS traces/spectra of the analyzed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral match. The UV trace was used for quantification of identified compounds from MS detection. Using the chromatographic conditions described above, the polyphenols eluted in less than 35 minutes. Four polyphenols cannot be quantified in current chromatographic conditions due overlapping (caftaric acid with gentisic acid and caffeic acid with chlorogenic acid). However, all four compounds can be selectively identified in MS detection (qualitative analysis) based on differences between their molecular mass and MS spectra. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5-50  $\mu$ g mL<sup>-1</sup> range with good linearity ( $R^2 > 0.999$ ) for a five point plot were used to determine the concentration of polyphenols in plant samples.

### Statistical Analysis

Results were expressed as mean  $\pm$  standard deviations (SD); Tukey's test was used to determine level of significance of all results obtained on XLSTAT 7.1. Results were regarded as significant at  $p < 0.05$ .

## Results and Discussion

### Antioxidant capacities

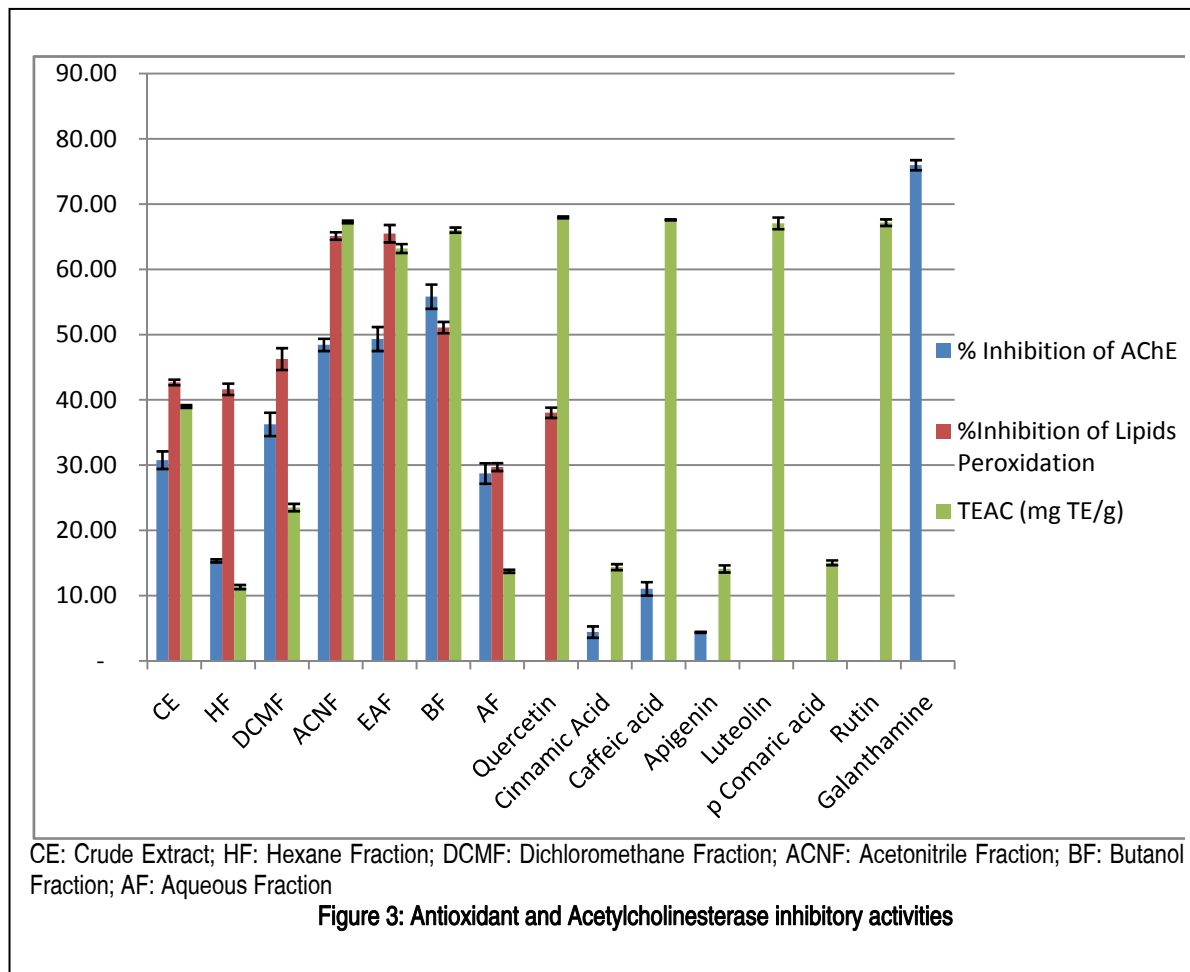
### Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox Equivalent Antioxidant Capacity of *E. alba* extracts are presented in the figure 3. The highest value was obtained with acetonitrile fraction (ACNF) giving  $67.28 \pm 0.20$  mg Trolox Equivalent per g of extract. Extracts capacities are in the following order ACNF > BF > EAF > AAE > DCMF > AF > HF and these results are significantly different ( $p < 0.05$ ). The antioxidant capacity of the aerial part of *E. alba* has been already evaluated [19]. In this study it's was found that the methanol and aqueous extracts exhibited an interesting activity with the values of 54.3 and  $148.1 \mu\text{mol}$  Trolox Equivalent/ 100g dry extract. These results are very low compared

to those we obtained. In their study these authors had attributed this antioxidant capacity in major part to phenolic compounds. The antioxidant capacities of polyphenols are well-known [11, 20]. In our last research, results obtained with methanolic extract using ascorbic acid as standard were in the same order [4]. Also, ABTS<sup>+</sup> scavenging activity of extracts of three *Asteraceae* species were positively correlated with polyphenols content of these plants.

### Rat liver lipid peroxidation inhibition

Liver lipids peroxidation inhibition potential of *E. alba* fractions were consigned in the figure 3.



The best result ( $65.48 \pm 1.34\%$ ) was obtained with EAF and ACNF. This antioxidant capacity of the plant fractions is approximately in the same order of their TEAC. Pearson correlation test between TEAC and liver lipids peroxidation potential gave a good correlation  $R = 0.847$  ( $p = 0.016$ ). Several studies have shown the correlation between antioxidant and hepatoprotector activities [21, 22]. Other studies have shown that the ethanol extract of *E. alba* presented a good hepatoprotector activity [23]. Furthermore, Kim and their collaborators have shown that butanol extract of *E. alba* reduce

significantly serum lipid levels. One could attribute this activity to liver peroxidation inhibition demonstrated in our study. Our study through antioxidant properties, contributes to explain the traditional uses of this plant in the treatment of atherosclerosis and hypercholesterolemia in human [24].

### Acetylcholinesterase (AChE) inhibitory activity

The AChE inhibition activities of fractions and crude extract from *E. alba* are presented in the figure 3.

The high inhibitory percentage was obtained with butanol fraction ( $55.82 \pm 1.86\%$ ) follow by EAF ( $49.33 \pm 1.84\%$ ) and ACNF ( $48.43 \pm 0.94\%$ ). The AChE inhibitory activity of these fraction are significantly lowest compared to the Galanthamine ( $75.97 \pm 0.77\%$ ), a pure compound used to treat Alzheimer diseases.

It has showed that the butanol fraction of *E. alba* increase AChE activity and increase brain acetylcholine level [24]. In their study the cause of this acetylcholine variation level is not explained. Our study could explain this plant fraction role in acetylcholine degradation by inhibition of acetylcholinesterase (AChE). Also, in the same order with these authors works, this plant AChE inhibitory activity was positively correlated ( $R = 0.913$ ;  $P = 0.004$ ) with antioxidant capacity using Pearson's correlation test.

### Antifungal activity

**Table 1: Antifungal capacity**

	<i>C. albican</i>		<i>C. krusei</i>		<i>T. beigellii</i>	
	ZID (mm)	MIC (mg/mL)	ZID (mm)	MIC (mg/mL)	ZID (mm)	MIC (mg/mL)
CE	$10.67 \pm 0.58^a$	1.25	$8.00 \pm 0.00^b$	20	$6.00 \pm 0.00^c$	20
HF	$11.67 \pm 0.58^a$	10	$8.00 \pm 0.00^b$	20	$6.00 \pm 0.00^c$	20
DCMF	$8.00 \pm 0.00^b$	20	$8.33 \pm 0.58^b$	20	$6.00 \pm 0.00^c$	20
ACNF	$8.00 \pm 0.00^b$	20	$8.00 \pm 0.00^b$	20	$10.33 \pm 0.58^a$	1.25
EAF	$8.00 \pm 0.00^b$	20	$8.00 \pm 0.00^b$	20	$8.00 \pm 0.00^b$	20
BF	$10.67 \pm 0.58^a$	2.5	$11.33 \pm 0.58^a$	10	$10.00 \pm 0.00^a$	10
AF	$11.67 \pm 1.15^a$	10	$8.00 \pm 0.00^b$	20	$8.00 \pm 0.00^b$	20

ZID: Inhibition Zone Diameter; MIC: Minimum Inhibition Concentration; CE: Crude Extract; HF: Hexane Fraction; DCMF: Dichloromethane Fraction; ACNF: Acetonitrile Fraction; BF: Butanol Fraction; AF: Aqueous Fraction

### Phenolic compound content

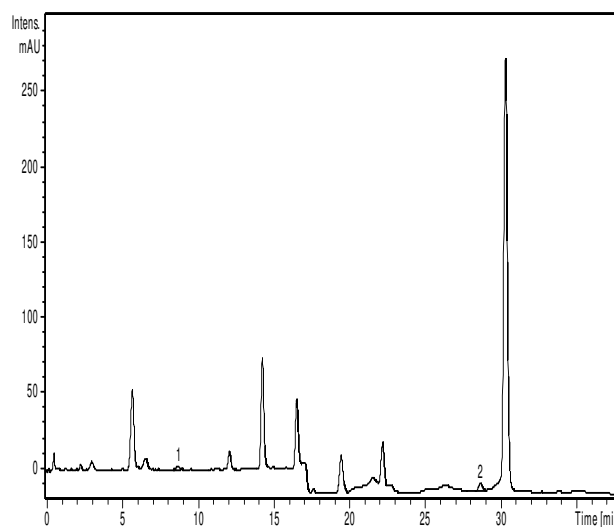
18 phenolic compounds have been investigated and the chromatographic profiles of phenolic acids and flavonoids of methanol extract were presented in the figure 1 and 2. After analysis, four (4) phenolic acids and three (3) flavonoids have been identified in the methanol extract of *E. alba* (Table 2).

One benzoic acid (gentisic acid) and three cinnamic acids (caffeic acid, chlorogenic acid and p-coumaric acid) were identified in the methanol extract. Only p-coumarin has been quantified with a content of  $774.4 \mu\text{g/g}$  in hydrolysed sample. Due to overlapping, gentisic acid, caffeic acid and chlorogenic acid have only been identified. These polyphenol compounds have presented good antioxidant capacities (figure 3). The best antioxidant capacities were obtained successively with quercetin, caffeic acid, rutin, luteolin. It is shown that polyphenol compounds have antioxidant properties [11]. Also, it is established that these compounds have properties to protect against neurodegenerative disorders so Alzheimer's disease (AD).

The table 1 indicates the antifungal activities of fractions and crude extract from *E. alba*.

About the three fungal strains used, the higher inhibition zone diameter was found with HF and AF ( $11.67 \text{ mm}$ ) against *C. albican*. *C. krusei* and *T. beigellii* were also sensitive to BF and ACNF with inhibition diameters of  $11.33 \text{ mm}$  and  $11.33 \text{ mm}$ , respectively. According to the MIC determination, our results show that *C. albican* and *T. beigellii* are the most susceptible fungals to crude extract and ACNF with a concentration of  $1.25 \text{ mg/mL}$ . This result could explain the use of crude (decoction) extract in traditionally medicine of Burkina Faso to cure some fungal diseases.

Also, Bioassay-guided research of methanol extract using *C. albican* has permitted to isolate a bioactive steroidal alkaloids compound (25 $\beta$ -hydroxyverazine) against this strain [26]. The activity of the extracts of the plant against *C. albican* could be due to this compound.



**Figure 1: NHS chromatographic profile**

1: p-Coumaric acid; 2: Luteolin



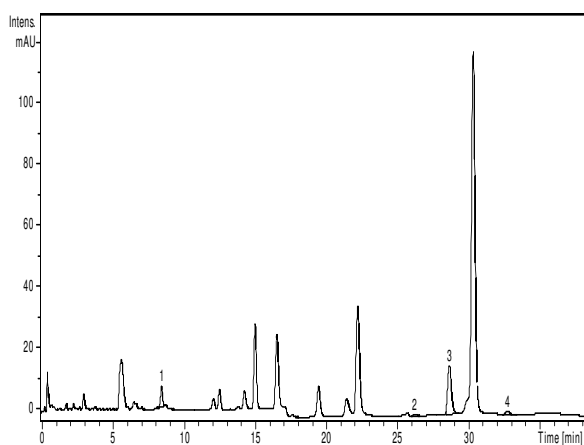


Figure 2: HS chromatographic profile

1: p-Coumaric acid; 2: Quercetin; 3: Luteolin;  
4: Apigenin

identified selectively in MS detection in the non-hydrolyzed and the hydrolyzed samples (HS). Concerning the pattern of flavonoids, quercetin, luteolin and apigenin have been identified. Apigenin (4117.4  $\mu\text{g/g}$ ) was the most abundant flavonoid in hydrolysed sample.

Table 2: Phenolic content of *E. alba* extract ( $\mu\text{g/g}$  of extract)

Polyphenols	Hydrolysed sample	Non-hydrolysed sample
Gentisic acid	lq	lq
Caffeic acid	lq	lq
Chlorogenic acid	lq	lq
p-Coumaric acid	774.4	341
Quercetin	111.8	---
Luteolin	4117.4	1172
Apigenin	488.4	---

---, not found; lq, low quantity.

and Parkinson's diseases (PD) through their antioxidant capacities [11, 27]. In our study the best percentage of AChE inhibition was obtained with butanol fraction, also we have obtained the high content in polyphenol with this fraction [28]. These results confirm the relation between polyphenol compounds- antioxidant capacity- neurodegenerative disorder protection. Nowadays, obtaining a polyphenol which has AChE inhibition property is very interesting. It is in this sense that huperzine A which is a polyphenol was discovered. In China, this molecule has been studied in phase IV clinical trials and revealed a significant improvement of memory of elder people, patients with AD [11].

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The phenolic compounds identified in *E. alba* extract have presented a low AChE inhibition activities (table 2) as compared to butanol fraction of plant extract activity (best inhibition percentage was obtained with caffeic acid of  $11.05 \pm 1.03$ ). The same result was obtained with quercetin by [29].

The capacity of *E. alba* extracts to increase brain acetylcholine level could be due by combination of these polyphenols or by their derivatives or by another compounds present in the plant extracts.

## Conclusion

Aqueous ethanol extract of *E. alba* and its fractions were analyzed for their antioxidant, acetylcholinesterase inhibitory and antifungal activities. Our finding was that among the extract and fractions used, ACNF, EAF and BF which have shown the best antioxidant capacities have also the most potential inhibitors of AChE. The ability of the plant extract to increase the level of brain AChE may be due to molecules endowed with antioxidant properties. The fungal strains used were weakly sensitive to the plant extract and fractions. *C. krusei* was only sensitive to the butanol fraction.

After HPLC-MS analysis, four (4) phenolic acids and three (3) flavonoids have been identified in this study. p-coumaric acid and luteolin were the major phenolic compounds in the plant extract. These compounds have given good antioxidant properties but low AChE inhibition potentials. Therefore, AChE inhibition activity of *E. alba* extracts could be attributed to the derivatives of these compounds, to the combination of these compounds or to compounds of other phytochemical groups.

In general, the butanol fraction of *E. alba* presented the best activities for the tests performed. For future research in Burkina Faso, bio-guided studies from this fraction are needed to isolate bioactive compounds.

## Authors' contributions

All authors have made substantial contributions and final approval of the conceptions, drafting and final version.

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