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INHIBITION OF ACETYLCHOLINESTERASE AND GLUTATHIONE-S-TRANFERASE ACTIVITIES BY METHANOLIC EXTRACT OF *Lippia chevalieri* MOLDENKE

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RESUMEN

El objetivo del presente estudio fue evaluar la capacidad inhibidora de acetilcolinesterasa (AChE) y glutation-S-transferasa (GST) de extractos metanólicos (100 µg/mL) de *Lippia chevalieri* de Burkina Faso. Se determinaron también, por métodos espectrométricos las concentraciones de flavonoides, flavonoles y taninos totales. *Lippia chevalieri* inhibió ambas enzimas en más del 35%. GST fue la enzima que sufrió mayor efecto de inhibición. El contenido de fenoles totales fue de 17.88 mg de equivalentes de ácido gálico (GAE)/100 mg de extracto seco. Dado que los taninos fueron los principales compuestos fenólicos en los extractos, su contribución a la inhibición enzimática podría representar el 42.61%. Los resultados de este trabajo sugieren que el extracto metanólico de *L. chevalieri* es un inhibidor importante de AChE y GST y confirman su uso tradicional en el tratamiento de desordenes mentales, respiratorios, y de enfermedades cardiovasculares.

PALABRAS CLAVE: Glutation-S-transferasa, acetilcolinesterasa, extracto metanólico, *Lippia chevalieri*.

ABSTRACT

The aim of the present study was to evaluate acetylcholinesterase (AChE) and glutathione-S-transferase (GST) inhibition against methanolic extract (100 µg/mL) of *Lippia chevalieri* from Burkina Faso. The total phenolics, flavonoids, flavonols and tannins were also determined spectrophometrically. *Lippia chevalieri* inhibited both enzymes at more 35 %. The best inhibition activity was found on GST. The total phenolics content was 17.88 mg gallic acid equivalents (GAE)/100 mg dry extract. As tannins were the major phenolic compounds in the extracts, their contribution to inhibition activity could represent 42.61 %. The present findings suggest that the methanolic extract of *L. chevalieri* is a relevant inhibitor of AChE and GST and confirm its traditional use in the treatment of mental and respiratory disorders and cardiovascular diseases.

KEY WORDS: Glutathione-S-tranferase, acetylcholinesterase, methanolic extract, *Lippia chevalieri.*

INTRODUCTION

Acetylcholinesterase and glutathione-s-transferase are involved in the development of Alzheimer's disease (AD), cancer and cardiovascular diseases (Hayeshi *et al*., 2007; Bangou *et al*., 2011a). These diseases are becoming now-a-day a threat to public health (Orhan *et al*., 2004; Wu and Ng, 2008). It has been demonstrated that medicinal plants are a promising way for obtaining compounds with a broad range of biological activities (Djeridane *et al*., 2006). According to the World Health Organization (WHO, 2002), traditional medicine is largely more available than modern medicine. According to Gurib-Fakim (2006), approximately 50 drugs result from the tropical plants. Fifty percent of the products prescribed in several countries of Europe and America are natural products or their derivatives (Newman *et al*., 2003; Krzaczkowski, 2008). In Africa, WHO (2002) estimated that 80% of the population used traditional medicine to meet its needs for health. *Lippia chevalieri*, which belongs to the Verbenaceae family, is widespread in the world, and is used to treat several diseases such as cancer and mental disorders (Nacoulma, 1996; Pascual *et al*., 2001). Previous biological investigation showed that this specie possess antimicrobial and antibacterial activities (Bangou *et al*., 2012a). Former phytochemistry studies demonstrated that this plant species synthesize many essential oils (Pascual *et al*., 2001) and recently, 20 phenolic compounds were detected using HPLC-DAD (Bangou *et al*., 2012a); these same authors showed the presence of cafeic acid and rutin in a methanol extract. The aim of the present paper was to evaluate *in vitro* the glutathione-s-transferase and acetylcholinesterase inhibition activities against total phenolic, flavonoid, flavonol and tannin contents of methanolic extracts of *Lippia chevalieri.*

MATERIALS AND METHODS

Chemicals

Reagents came from Sigma Aldrich Chemie GmbH, Germany: L-glutathione reduced (GSH), glutathione-s-transferase (GST) from rate liver, 1-chloro-2,4-dinitrobenzene (CDNB), albumin from bovine serum (BSA), potassium phosphate monobasic (KH_2PO_4) and dibasic K_2HPO_4). Acetylcholinesterase (AChE) from electric eel, acetylcholine iodide (ATCI), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), tannic acid, gallic acid, and quercetin were provided from Sigma-Germany. HCl and sodium carbonate were from Labosi-France. Folin-Ciocalteu reagent was obtained from Sigma, USA. Dimethylsulphoxyde (DMSO) and tween were purchased from Sigma-Aldrich Chemie GmbH (Germany). Aluminium trichloride ($AlCl_3$), galanthamine, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Plant materials

Plant material, constituted of *Lippia chevalieri* from Burkina Faso was collected in Ouagadougou in June 2006. This plant species was botanicaly idenfied by Professor Millogo-Rasolodimby from the Ecology Laboratory of the University of Ouagadougou. Voucher specimen (BK-la2775) was deposited in the OUA herbarium of the CIB (Centre d'Information sur la Biodiversité), UFR-SVT of the University of Ouagadougou.

Preparation of plant extracts

Stems-leaves of *Lippia chevalieri* were dried at room temperature and ground to fine powder, using a grinder. The extraction was carried out using 10 g (3x100 mL) after overnight maceration. The extract was filtered and evaporated to dryness.

Acetylcholinesterase activity

The AChE inhibition was conduct according to the protocol described by Lopez *et al*. (2002) with some modifications. Briefly described, the assay mixture consisted of 200 µL of Tris-HCl 50 mM pH 8, 0.1 % BSA buffer, 100 µL of extracts solution (final concentration: 100 µg/mL), and 100 µL of AChE (0.22 U/mL). The mixture was incubated at room temperature for 2 min before adding 500 µL of DTNB (3 mM) and 100 µL of substrate (ATCI 15 mM). The developing yellow color was measured at 405 nm after 4 min (Cecil CE2041, England). Galanthamine was used as a positive control at a final concentration of 0.2 µg/mL in the assay mixture. AChE inhibitory activity was expressed as inhibition percentage and it was calculated as:

$$\text{Inhibition percentage of AChE(\%)} = \frac{[(A-B)x100]}{A}$$

Where, A is the absorbance of the assay without the plant extract and B is the absorbance of the assay with the plant extract.

The principle of this method is based on acetylcholinesterase hydrolysis (ATCI) in thiocholine and acetate, products not coloured. The thiocholine in the presence of the DTNB gives a yellow product, the 5-thio-2-nitrobenzoate, which makes it possible to follow the kinetics by registering the absorbance at 405 nm.

Acetylthiocholine + H_2O ⟶ AChE acetate + thiocholine

Thiocholine + DTNB ⟶ 5-thio-2-nitrobenzoate + 2-nitrobenzoate-5-mercaptothiocholine

Inhibition of glutathione-S-transferases

GST inhibition assay were conducted as described by Habdous *et al*. (2002) with some modifications. The reaction mixture was formed by 200 µL of phosphate buffer 100 mM (pH 6), 100 µL of enzyme (1 U/mL), and 100 µL of extract (100 µg/mL). The reaction was initiated with 100 µL of GSH (5 mM) and 500 µL CDNB (1 mM). GST inhibitory activity was expressed as inhibition percentage and it was calculated as:

$$\text{Inhibition percentage of GST(\%)} = \frac{[(A-B)x100]}{A}$$

Where, A is the absorbance of the assay without the plant extract and B is the absorbance of the assay with the plant extract.

This procedure described by Habdous *et al*.(2002), inspired of the method of Habig *et al*. (1974), was slightly modified for this purpose (Figure 1). At the step 1, there is ionization of the reduced glutathion in proton (H+) and thiolate anion [GS]-. Step 2 : nucleophilic attack is carried out by CDNB on the thiolate anion in the C1 of its aromatic nucleus. Then, a Mesenheimer complex 1-(S-glutathionyl-2,4-dinitrobenzen) (Gs-DNB) is formed and it can be followed by the absorbance registers at 340nm (way 1). The electrophilic can be inactivated at the time of the conjugation with the GSH is formed and this unit will inhibit the action of the enzyme (way 2). Or the electrophilic can cling to the enzyme and prevent its action (way 3).

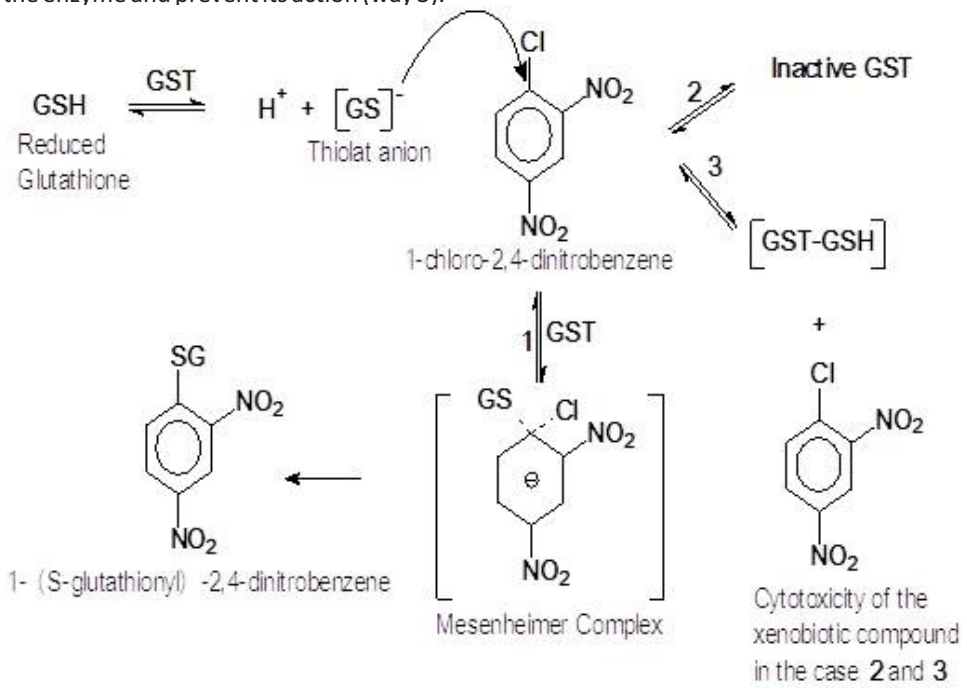

Figure 1. Reaction of catalysis of the GST (Habdous *et al*., 2002; Ibarra *et al.*, 2003; Bangou, 2012b).

Determination of the total phenolic content

The total phenolics of the plant extract were determined by the Folin-Ciocalteu method (Bangou *et al*., 2011b).

Determination of tannin content

Tannin content was determined according to the European Commission (2000) as adapted by Bangou *et al*. (2011b).

Determination of the total flavonoid content

The total flavonoids were estimated according to the Dowd method as adapted by Bangou *et al*. (2011b).

Determination of the total flavonols content

Flavonol content was determined according to Almaraz-Abarca *et al*., (2007) method as adapted by Bangou *et al.* (2012c).

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean ± standard deviation (SD) calculated with Excel 2007. Statistical comparisons were done with the XLSTAT7.5.2, using Spearman correlation. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

AChE inhibitory activity

The methanolic extract of *Lippia chevalieri* inhibitory activity on AChE was 39.26 % ± 2.18 (Figure 2). This result can be compared to the galanthamine (50.76 ± 0.68%), which is used like reference and also in the treatment of Alzheimer's disease (Lopez *et al.*, 2002). According to the cholinergic hypothesis, memory impairment in patients suffering from Alzheimer's disease is a result of decreased levels of the neurotransmitter acetylcholine (ACh) in the cortex. In the healthy brain AChE is the most important enzyme regulating the ACh level (Adsersen *et al*., 2007). In this way, it would be relevant to search for more substance capable of inhibiting AChE activity in Alzheimer's disease patients to increase their ACh levels besides galanthamine, which is used in the treatment of this disease.

AChE is found in the insects and by its action of hydrolysis of acetylcholine helps the insects to resist insecticides (Yi *et al*., 2006). In *Drosophila melanogaster*, several mutations of AChE were identified around the action site of insecticides, such as: I161V, G265A, F330Y and G368A (Shi *et al.,* 2004). These mutations compromise the attack of the insecticide molecules. Each one of these four mutations enzymes, can only or in association to hydrolize acetylcholine contents varying between 2 µM and 300 mM (Shi *et al.*, 2004). Organophosphorous and the carbamates act by inhibiting the catalytic activity of the AChE. These compounds are fixed on the active site of the enzyme, in the place of acetylcholine. Then, the accumulation of ACh in the synaptic area causes a hyperexcitation of the cholinergic connections causing the death of the insect finally. On the other hand, several studies showed that the GST was able to degrade organophosphorous and to confer a resistance to the insects (Bangou, 2012c).

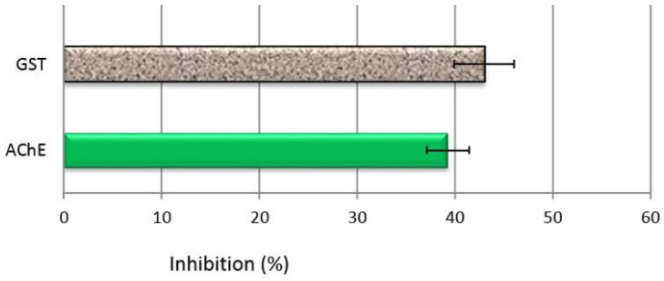

Figure 2. Enzyme inhibition percentage of the methanolic extract of *Lippia chevaliery* on acetylcholinesterase (AChE) and glutathione-S-transferase (GST).

GST inhibitoryactivity

The result of GST inhibition activity by the methanolic extract of *Lippia chevalieri* was 42.99 ± 3.03% (Figure 2). This inhibition was higher than that observed for the ACh by the same extract. Several investigation showed that overexpression of GST is associated with multidrug resistance of tumor cells (Zanden *et al*., 2004; Hayeshi *et al.*, 2007). Thus, the inhibition of GST activity become a promising way to develop antitumoral drugs, particulary, drugs from medicinal plant (Bangou *et al.*, 2011a). It is important to mention that there are several types of GST according to their origin (animal, insect, or plant) and according to their location inside the cell (microsomal, cytosolic, mitochondrial or peroxisomal) (Sheehan *et al*.,2001; Enayati *et al*., 2005). They play a central role in the endogenous detoxication of the xenobiotic compounds, facilitate intracell transport, biosynthesis of hormones and protection against the oxidative stress (Enayati *et al.*, 2005). Figure 1 gives detail of its mechanism.

Relationship between enzyme inhibition activities and polyphenolic contents

In order to determine the implication of polyphenolic compound (phenols, flavonoids, tannins and flavonols) in the inhibition activities measured, several determinations were carried out. Firstly, total phenolic content was 17.88 ± 0.90 mg GAE/100 mg dry extract (Figure 3), which is lower than the phenolic contents found in other species, like *Cassia mimosoides* (Ceasalpiniaceae) and *Sclerocarya birrea* (Anecardiaceae) in which yields of 51.3 ± 0.49 and 56.10 ± 1.16 mg GAE/100 mg dry extract, respectively were found (Bangou *et al*., 2011a), those values are around three fold the content found for *Lippia chevalieri* in the present study; however, those species showed inhibition activities, which are comparable to those found here for *L. chevalieri*. The results of the present study suggest that the type of compounds present in the *L. chevalieri* extract could have a higher impact on the inhibition of AChE and GST than the phenolic content. Our research group have obtained for *Sclerocarya birrea* total extract, 41.12% AChE inhibition and 44.34 % for GST; and for *Cassia mimosoides* 39.12 % and 20.04%, in the same order (Bangou *et al*., 2011a; Bangou *et al.*, 2011b). Concerning the total flavonoids, total tannins and total flavonols we found 3.19, 7.62, and 0.8 mg/100 g dry extract, respectively (Figure 3). According to those values, tannins could do the highest contribution (42.6%) to the enzyme inhibition of the *L. chevalieri* extract. Several research had shown that AChE inhibition is sometimes correlated with tannin content and hydroquinones (Pithayanukul *et al*., 2005; Wang *et al*., 2007), and sometimes with total flavonoids (Ji and Zhang, 2006), while GST inhibition has been correlated with total flavonoids (Van Zanden *et al*., 2004). Recently, 20 polyphenolic compound have been found in *L. chevalieri* by using HPLC-DAD methods (Bangou *et al*., 2012a), and, with thin layer chromatography. Bangou *et al.* (2012c) detected rutin and cafeic acid in a ethyl acetate fraction of that species. Others researchers had shown that this species content several essentials oils (Pascual *et al.*, 2001). The synergism among those compounds could also explain the enzyme inhibition values found in the present study. *Lippia chevalieri* can then contain bioactive substances useful in the treatment of Alzheimer's disease and cancer, justifying the popular use of this plant in mental disorders, cardiovascular diseases and inflammation in Burkina Faso folk medicine, but, a detailed examination of polyphenolic content of this plant species is necessary for a comprehensive assessment of the individual compounds enzyme inhibitory ability.

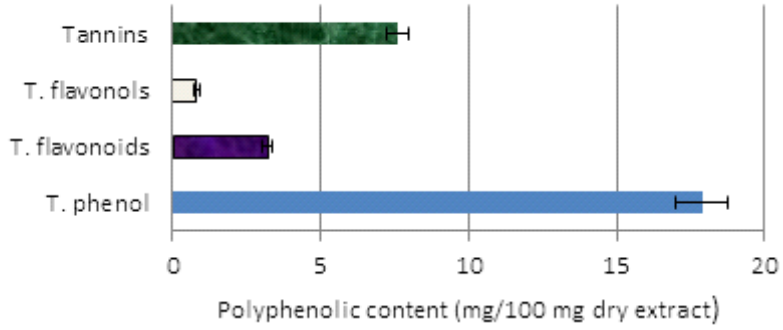

Figure 3. Polyphenolic content of methanolic extracts of the stem-leaves of *Lippia chevalieri*

CONCLUSION

This study has showed that methanolic extract of *Lippia chevalieri* is a potential inhibitor of AChE and GST. The activities seem to be partially correlated to the flavonoid and tannins. *Lippia chevalieri* is indicated for the investigation of new molecules to relieve the diseases in which these enzymes are involved. Future studies should be focused in the screening of others species of Verbenaceae family, in the isolation and identification of active constituents that exhibit significant inhibitory activity through bioassay-guided fractionation, and in the in vivo evaluation of those activities.

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