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EVALUATION OF ACETYLCHOLINESTERASE INHIBITION BY *BAUHINIA ALBA AERIAL PARTS* METHANOL EXTRACT AND BIO-ACTIVE CONSTITUENTS

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ABSTRACT

Objective: This study was carried out to evaluate acetylcholinesterase activity of methanol 80% extract of *Bauhinia alba* aerial parts and to identify the bio-cative phytoconstituents present in the plant extract. **Methods**: The acetylcholinesterase inhibition was detected using Ellman's method and the methanol extract was subjected for phytochemical analysis to identify phytochemical constituents present in the extract. **Results**: The methanol extract of *B. alba* has shown (IC_{50} = 0.222 mg/mL), assuming that the extract has compounds with a similar activity to neostigmine (IC_{50} = 1.87 µg/mL) and galanthamine (IC_{50} = 0.37 x10^{-3} mg/mL) which are considered to be the most effective compounds in the treatment of Alzheimer's disease. Phytochemical investigation of the extract revealed the presence of triterpenes, flavonoids, tannins, alkaloids and carbohydrates. Chromatographic separation and fractionation of the methanol extract of *B. alba* resulted in the isolation of lupeol, luteolin, kaempferol, vitexin, isovitexin, kaempferol 3-*O*-*β*-glucoside and rutin. **Conclusion**: The results suggest that methanol extract of *B. alba* is very interesting as acetylcholinesterase inhibitor which can be widely used in the treatment of Alzheimer's disease.

Key Words: *Bauhinia alba*, aerial parts, Anticholinesterase activity, Alzheimer's disease, bio-active phytoconstitutents.

INTRODUCTION

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body and these plants are being used against various infections and diseases in the world since past history. Alzheimer's disease (AD) is one of the most common forms of dementia affecting approximately 10% of the population over the age of 65 years[1]. It is a neuropsychiatric condition

with progressive neurodegeneration, dementia and decline of cognitive function, usually accompanied by behavioral disturbances[2]. Several acethylcholinestrase (AChE) inhibitors such as tacrine, donepzil, rivastigmine and galanthamine, are available for the treatment of mild to moderate AD[3]. Although the use of these drugs are beneficial in the treatment of AD symptoms, they may also cause some adverse side effects[4]. The most common side effects of these drugs include: anorexia, diarrhea, fatigue, nausea, muscle cramps as well as gastrointestinal, cardiorespiratory, genitourinary and sleep disturbances[4]. *Bauhinia alba* is a tree from *Caesalpiniaceae* family and it is also known white orchid semi-tropical tree with smooth or slightly ridged grey bark grows in moist rich soil in mild climates. The large fragrant blooms ranges in color from snowy to creamy white. In traditional medicine, *B. alba* was used for treating skin diseases, asthma, diarrhea and it was used as a blood purifier and tonic[5]. Few reports about *B.alba* biological activities and bio-active phytoconstituents where antioxidant activity of different extracts from the flowers of *B. alba* was determined by (Ghias et al., 2012)[6].In the current study, we evaluated acetylcholinesterase inhibitory activity of *B. alba* aerial parts methanol extract and also investigated the bio-active phytoconstituents from the plant extract.

MATERIALS AND METHODS

General experimental procedures

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). ^{1}H-NMR and ^{13}C-NMR (Varian Unity Inova). MS (Finnigan MAT SSQ 7000, 70 ev). (Silica gel (0.063-0.200 mm for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Thin layer chromatography (TLC) F_{254} plates. Solvent mixtures, BAW (*n*-butanol: acetic acid: water 4:1:5 upper phase, 15% acetic acid: water: glacial acetic acid: 85:15). Paper Chromatography (PC) Whatman No.1 (Whatman Led. Maid Stone, Kent, England) sheets for qualitative detection of flavonoids and sugars were used in this study.

Plant identification and collection

Bauhinia alba aerial parts were collected from Al-Zohiriya garden, Giza, Egypt in May 2012. The plant was identified by Dr. Mohammed El-Gebaly, department of botany, National research centre (NRC) and by Mrs. Tereeza Labib consultant of plant taxonomy at the ministry of agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen was deposited in the herbarium of Al-Zohiriya garden, Giza, Egypt.

Plant extract preparation

Air dried aerial parts of *B. alba* (650g) were extracted with methanol: distilled water 80:20 (v/v) several times at room temperature by maceration method. The extract was concentrated under reduced pressure to give 32g of methanol extract. The extract was phytochemically screened according to the methods described by Yadav and Agarwala (2011)[7].

Isolation of the bioactive components of methanol extract of *Bauhinia alba*

Aerial parts of *Bauhinia alba* methanol extract (30g) was subjected to silica gel column chromatography eluting with n-hexane, dichloromethane, ethyl acetate and methanol gradually. One hundred and thirty fractions of 100 ml conical flask were collected. The fractions that showed similar Paper Chromatography (PC) in two solvent systems, butanol–acetic acid–water (BAW) 4:1:5 and 15% acetic acid were combined to give 4 fractions (I, II, III and IV).

Fraction I (1.9 g) was subjected to sub–column of silica gel eluted with n-hexane: dichloromethane (50:50) gave compound 1. Fraction II (2.4 mg) was subjected to sub–column of silica gel eluted with ethyl acetate :dichloromethane (50:50) yielded compound 2 and further elution with ethyl acetate :dichloromethane (80:20) gave compound 3. Fraction III (2.1 g) was subjected to sub–column of silica gel eluted with ethyl acetate : methanol (95: 5) to give compound 4 while compound 5 was

obtained from further elution with ethyl acetate: methanol (90:10). Fraction IV (1.7 g) was subjected to sub–column of silica gel eluted with ethyl acetate: methanol (70:30) gave compound 6 while compound 7 was obtained by elution with further elution of methanol: ethyl acetate (80:20). All the isolated compounds were purified on sephadex LH–20 column using different systems of methanol and distilled water.

General method for acid hydrolysis of flavonoid glycosides

5 mg of each flavonoid glycoside 4, 5, 6 and 7 in 5 ml 10% HCl was heated for 5 h. The aglycones were extracted with ethyl acetate and identified by co-TLC with authentic standards. The sugars in the aqueous layer were identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (*n*-BuOH-AcOH-H_2O 4:1:5 upper layer).

Acetylcholinesterase inhibition assay

The methanol extract of *B. alba* was dissolved in methanol to prepare solution of 10 mg/mL. Then, 1.5 µL of the extract was spotted on silica gel TLC plate and developed with chloroform: methanol 9:1 after which the enzyme inhibitory activity was detected using Ellman's method "in situ" on the plate[8,9]. The developed plate was sprayed with 1 mM Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB) and 1 mM acetylthiocholine iodide (ATCI) in buffer A. It dried for 3-5 minutes, then an enzyme solution of AChE from an electric eel (type VI-s lyophilized, 261 U/mg solid, 386 U/mg protein) dissolved in buffer A (500 U/mL stock solution) was diluted with buffer A to obtain 5 U/mL enzyme and was then sprayed on the plate[9]. Yellow background with white spot for inhibiting extract was visible after about 5 minutes. The observation must be recorded within 15 minutes because they fade after 20-30 minutes. To observe whether the positive results of the extract in TLC or the microplate assay are due to enzyme inhibition or to the inhibition of the chemical reaction between DTNB and thiocholine, (the product of the enzyme reaction), 5 units/mL of AChE was premixed with 1 mM ATCI in buffer A and incubated for 15 minutes at 37°C. This enzyme-substrate mixture was used as thiocholine spray[9]. The extract was spotted on the silica gel TLC plate developed as described above and sprayed with 1 mM solution DTNB followed by the thiocholine spray. White spot on a yellow background was observed for false positive extract. The inhibitory effect quantitative of methanol extract of *B. alba* on acetylcholinesterase activity is evaluated using and adaptation of the spectrophometric method of Ellman et al. (1961)[8] modified by Rhee et al. (2001)[9]. Five different concentrations were prepared in triplicate, starting from the methanol extract of *B. alba* (1 mg/mL; 0.5 mg/ml; 0.25 mg/mL; 0.125 mg/mL and 0.0625 mg/mL). The reaction was monitored at 412 nm for 5 min in spectrophotometer.

In test tube is placed 100 µL of the extract (concentration 0.1% solution in 50 mM Tris-HCl pH 8, and methanol 10%) was mixed with 100 µL of AChE 0.22 U / ml (22 U of enzyme diluted in 100 mL of 50 mM Tris-HCl pH 8, 0.1% BSA) and 200 µL of buffer (50 mM Tris-HCl, pH 8, BSA 0.1%). Incubating the mixture for 5 min at 30°C. Subsequently add, 500 µL of DTNB (concentration of the 3 mM in Tris-HCl pH 8, 0.1 M NaCl, 0.02 M $MgCl_2$) and 100 µL of ATCI (4 mM in water). A blank should also be prepared by substituting AChE with 100 µL of buffer (50 mM Tris-HCl buffer pH 8, 0.1% BSA). The reaction is monitored for 5 min at 412 nm and initial velocity (V0) recorded. Anticholinesterase activity (%) was calculated:

$$I\ (\%) = (1 - \frac{Vo\ sample}{Vo\ white}) \times 100$$

Sample Vo and Vo represents the initial rates blank samples and white.

Inhibition concentration 50% (IC_{50}) values so obtained by plotting Log-Probit. Neostigmine (or other commercial acetylcholinesterase inhibitor) is used as positive control at the same concentration of the extract.

RESULTS

This present study was focused on the evaluation of acetylcholinesterase activity of *B. alba* aerial parts methanol extract where the extract showed a significant inhibition for acetylcholinesterase. enzyme with (IC_{50} = 0.222 mg/mL). We investigated the presence of phytochemicals and bioactive constituents in *B. alba* methanol extract. Phytoconstituents of *B. alba* methanol extract are shown in table 1. The major bioactive components of *B. alba* aerial parts methanol extract are lupeol, luteolin, kaempferol, vitexin, isovitexin, kaempferol 3-*O*-*β*-glucoside and rutin. The chemical structures of the bio-active components were elucidated by different spectroscopic analyses (UV, ^{1}H-NMR, ^{13}C-NMR and MS) and their chemical structures are shown in Figure 1.

Structure elucidation of the isolated compounds

Compound 1 (Lupeol): 14 mg, white powder, ^{1}H-NMR ($CDCl_3$, 400 M Hz): δ 0.75, 0.8, 0.85, 0.96, 0.98, 1.08, 1.75 (each 3H, s), 3.25 (1H, dd, *J* = 5.6, 10.8 Hz, H-3), 4.58 (1H, s, H-29a), 4.68 (1H, s, H-29b). ^{13}C-NMR($CDCl_3$, 100MHz): δ 151.4 (C-20), 108.7 (C-29), 78.6(C-3), 55.8 (C-5), 50.7 (C-9), 48.7 (C-18), 48.4 (C-19), 43.2 (C-17), 43.2 (C-14), 40.8 (C-8), 39.7 (C-22), 38.7 (C-4), 38.5 (C-1), 38.7 (C-13), 37.6 (C- 10), 35.7 (C-16), 34.5 (C-7), 29.4 (C-21), 28.4 (C-23), 27.6 (C-2), 27.6 (C-15), 25.4 (C-12), 21.4 (C-11), 19.4 (C-30), 18.7 (C-6), 18.4 (C-28), 16.5(C-25), 16.2 (C-26), 15.7(C-24), 15.2 (C-27).

Compound 2 (Luteolin): 12 mg, yellow powder. ^{1}H-NMR: δ ppm 12.9 (1H, s, 5-OH), 7.4 (1H, d, *J* = 8 Hz, , H-6´), 7.38 (1H, d, *J* = 2 Hz, H-2´), 6.85 (1H, d, *J* = 8 Hz, H-5´), 6.6 (1H s, H-3), 6.4 (1H, d, *J* = 2 Hz, H-8), 6.15 (1H, d, *J* = 2 Hz, H-6). EI-MS: m/z 286.

Compound 3 (Kaempferol): 10 mg, yellow powder, ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 8.12 (2H, d, *J* = 8 Hz, H-2', 6'), 6.96 (2H, d, *J* = 8 Hz, H-3',5'), 6.47 (1H, d, *J* = 2 Hz, H-8), 6.19 (1H, d, *J*= 2 Hz, H-6). (+) ESI-MS: m/z 287$[M+H]^+$.

Compound 4 (Apigenin 8-*C*-*β*-glucoside), (Vitexin): 18 mg, yellow power. UV λmax (MeOH): 271, 339; (NaOMe): 278, 392; ($AlCl_3$): 275, 303 sh, 349; ($AlCl_3$/HCl): 269, 350; (NaOAc): 279, 372; (NaOAc/H_3BO_3): 269, 345. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 13.12 (1H, s, 5-OH), 7.92 (2H, *d*, *J* = 8.9Hz, H-2′,6′), 6.85 (2H, *d*, *J* = 8.9 Hz, H-3′,5′), 6.72 (1H, s, H-6), 6.24 (1H, s, H-3), 4.64 (1H, *d*, *J* =10 Hz, H-1′′), 3.2-3.9 (rest of sugar protons, H-2′′-6′′). (-) ESI-MS: m/z 431 $[M-H]^{-}$.

Compound 5 (Apigenin 6-*C*-*β*-glucoside) (Isovitexin): 16 mg, yellow amorphous power. UV λmax (MeOH):272, 334; (NaOMe): 275, 331sh, 399; ($AlCl_3$): 271, 304, 353, 383; ($AlCl_3$/HCl): 271, 304, 345, 381; (NaOAc): 278, 395; (NaOAc/H_3BO_3):275, 336. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 7.94 (2H, *d*, *J* = 8.5 Hz, H-2′,6′), 6.89 (2H, *d*, *J* = 8.5 Hz, H-3′,5′), 6.75 (1H, s, H-6), 6.54 (1H, s, H-3), 4.62 (1H, *d*, *J* =10 Hz, H-1′′), 3.2-3.9 (rest of sugar protons, H-2′′-6′′). (-) ESI-MS: m/z 431 $[M-H]^{-}$.

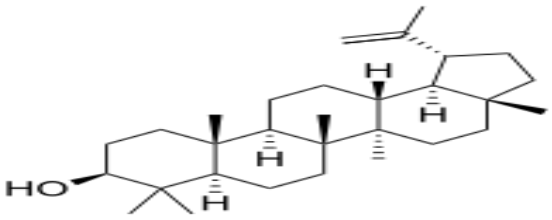
Compound 6 (Kaempferol 3-*O*-*β*-glucoside): 12 mg, yellow amorphous powder. UV λmax (MeOH): 268, 348, (NaOMe): 272, 324 sh, 398, ($AlCl_3$): 273, 302 sh, 348, 398, ($AlCl_3$/HCl): 273, 302 sh, 348, 398 (NaOAc): 274, 312 sh, 367, (NaOAc/H_3BO_3): 268, 344. ^{1}H-NMR (500 MHz, CD_3OD) : δ 8.15 (2H, d, *J* = 9 Hz, H-2', H-6'), 6.95 (2H, d, *J* = 9 Hz, H-3', H-5'), 6.42 (1H, d, *J* = 1.9 Hz, H-8), 6.25 (1H, d, *J* = 1.9 Hz, H-6), 5.32 (1H, d, *J* = 7.8 Hz, H-1''), 3.1-3.9 (5H, m, H-2'', 3'', 4'', 5'', 6'').

Compound 7 (Quercetin 3-O-rutinoside), (Rutin): 20 mg, yellow powder: UV λmax (MeOH): 258, 269, 361; (NaOMe): 276, 322, 416; ($AlCl_3$): 232, 276, 302, 366; ($AlCl_3$/HCl): 232, 276, 302, 366; (NaOAc): 284, 306, 381; (NaOAc/H_3BO_3): 261, 312, 376. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 7.54 (2H, m, H-2'/6'), 6.85 (1H, d, *J* = 9 Hz, H-5'), 6.38 (1H, d, J = 2.5Hz, H-8), 6.19 (1H, J = 2.5 Hz, H-6), 5.35 (1H, d, *J* = 7.5 Hz, H-1''), 4.39 (1H, s, H-1'''), 3.90-3.20 (m, remaining sugar protons), 0.99 (3H, d, *J* = 6 Hz, H-6''''). ^{13}C NMR (DMSO-d_6, 100 MHz): δ ppm 177.85 (C-4), 164.70 (C-7), 161.68 (C-5), 157.14 (C-2), 156.95 (C-9), 148.92 (C-4'), 145.25 (C-3'), 133.76 (C-3), 122.12 (C-

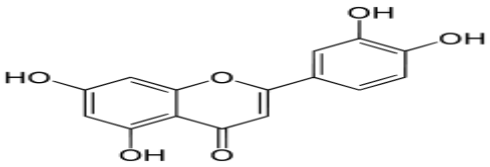
6'), 121.66 (C-1'), 116.73 (C-2'), 115.72 (C-5'), 104.41 (C-10), 101.66 (C-1'''), 101.23 (C-1''), 99.24 (C-6), 94.16 (C-8), 74.58 (C-3''), 72.33 (C-5''), 72.2 (C-4'''), 71.05 (C-2''), 70.8 (C-2'''), 70.87 (C-3''), 70.49 (C-4''), 63.74 (C-6''), 18.19 (C-6''').

Table.1. Phytochemical analysis of the methanol extract of *B. alba* aerial parts

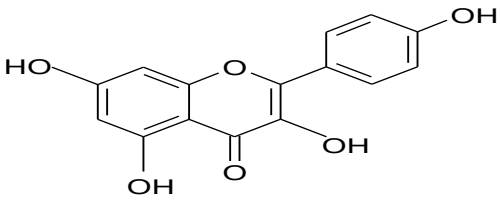
Chemical Constituents	Methanol extract
Carbohydrates and/or glycosides	+
Tannins a. Condensed tannins b. Hydrolysable tannins	 + +
Alkaloids and/or nitrogenous bases	+
Flavonoids	+
Sterols and/or triterpenes	+
Saponins	-
Coumarins	-
(+) indicate the presence of constituents, (-) indicate the absence of constituents	



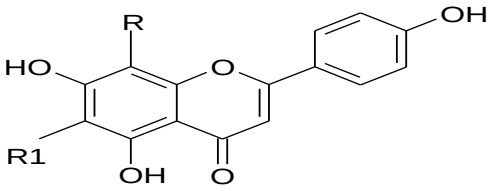
Lupeol (**1**)



Luteolin **(2)**

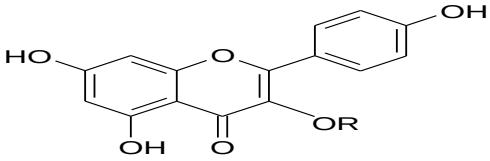


Kaempferol (**3**)

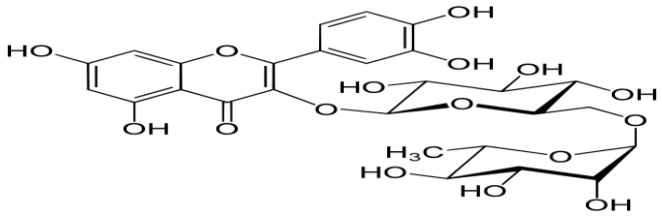


Vitexin **(4),** (R=glucose, R1=H)

Isovitexin **(5),** (R1= glucose, R=H)



Kaempferol 3-*O*-*β*-glucoside (**6**), (R= glucose)



Rutin **(7)**

Fig.1. Chemical structures of the compounds isolated of *B. alba* methanol extrac

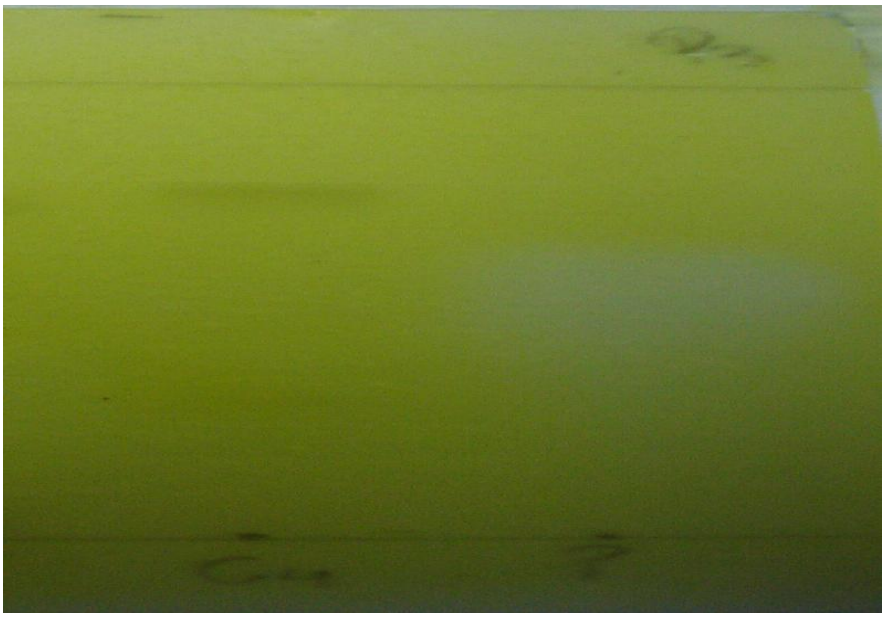


Fig.2.Acetylcholinesterase inhibition of *B. alba* methanol extract (C-4) in TLC and caffeine is used as positive control for acetylcholinesterase inhibitor.

DISCUSSION

Identification of the bio-active phytoconstituents of methanol extract of *B. alba* aerial parts

Compound 1 (lupeol) gave a dark spot under short UV light and changed to pink to violet upon spraying with vanillin-sulphuric acid and heating in an oven at 110°C for 5 min. NMR spectral data showed signals very similar to lupeol.[10] Compound 2 (luteolin) showed a deep purple spot under UV light which changed to yellow with ammonia vapor indicating that a flavone with free 5-OH and 4'-OH and spectral data of compound 2 is very close to that described by Owen *et al.* 2003 [11]. Compound 3 (kaempferol) yielded yellow colour under UV light and after exposure to ammonia or spraying with $AlCl_3$ reagent, it gave florescent yellowish green colour. ^{1}H-NMR and MS spectral data were in agreement with kaempferol.[12] Compound 4 (vitexin) and compound 5 (isoviotexin), each compound gave deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$. With complete acid hydrolysis, there is no change for compounds 4 and 5 and thus, both compounds were subjected to ferric chloride degradation, the products being chromatographed with authentic flavonoid aglycone and sugar samples, where apigenin as an aglycone and glucose moiety were detected and all spectral data of both compounds were very close to that described by Yun-Lian et al., 2000.[13] Compound 6 (kaempferol 3-*O*-*β*-glucoside) yielded a deep purple spot under UV light which changed to yellow with ammonia vapor and spraying with $AlCl_3$. With complete acid hydrolysis, it yielded kaempferol as an aglycone and glucose as sugar moiety. The spectral data of compound 6 is very similar to that described by Amal et al. 2009 [14]. Compound 7 (rutin) gave a deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$ and complete acid hydrolysis gave quercetin as an aglycone and glucose and rhamnose as sugar moieties and its spectral data was very similar to that described by Biruk *et al.*, 2012.[15]

Acetylcholinesterase inhibition by methanol extract of *B. alba* aerial parts

The qualitative results of inhibition of enzyme acetylcholinesterase in Thin Layer Chromatography (TLC) showed that the methanol extract the *B. alba* significantly inhibited the enzyme by the appearance yellow backgrounds with white spots for inhibiting compounds were visible after about 5 minutes. This are the results of the first tests, yellow backgrounds with white spots for inhibiting compounds were visible after about 5 minutes for methanol extract of *B. alba* apparently tested positive enzyme inhibition in concentration of 10 mg/mL (Figure 2).

The results of acetylcholinesterase inhibition quantitative for methanol extract of *B. alba* that presented strong activity in both tests, the IC_{50} values were determined (IC_{50} = 0.222 mg/mL). The concentration of inhibition 50% (CI_{50}) was tested starting at five different concentrations (1 mg/mL; 0.5 mg/ml; 0.25 mg/mL; 0.125 mg/mL; 0.0625 mg/mL) tested in triplicate, showed that methanol extract of *B. alba* has higher inhibition activity (*B. alba*, IC_{50} = 0.222 mg/mL), in comparison to commonly used drugs neostigmine (IC_{50} = 1.87 µg/mL) and galanthamine (IC_{50} = 0.37 x10^{-3} mg/mL). Galanthamine which is alkaloid considered to be the most effective compound in the treatment of Alzheimer's disease[16]. *B. alba* aerial parts methanol extract seems of interest for further study. Plants that have shown favorable effects in relation to cognitive disorders, including anticholinesterase, anti-inflammatory and antioxidant activities or other relevant pharmacological activities are potentially of interest to clinical use for Alzheimer's disease[17]. Eighteen medicinal plants of Brazil were screened for inhibitory activity on AChE, the results show that various plants are very interesting for further isolation of acetylcholinesterase inhibitors, which are widely used in the treatment of Alzheimer's disease, galanthamine, an alkaloid from plants of the Amaryllidaceae family, is a selective reversible long-acting and competitive acetylcholinesterase inhibitor (AChEI). The extract is considered to be more effective in the treatment of Alzheimer's disease (AD) and to have fewer limitations than physostigmine and tacrine are relevant in terms of searching for novel formulations or compounds for AD treatment[16]. This is the result of the first tests, yellow backgrounds with white spots for inhibiting extract was visible after about 5 minutes and so *B. alba* aerial parts methanol extract apparently tested positive enzyme inhibition in concentration of 10 mg/mL. The activity of the methanol extract of *B. alba* may be explained by the presence of triterpenes, flavonoids, tannins, alkaloids and carbohydrates and also for the isolated bioactive compounds where some of the isolated compounds (kaempferol sugars) showed a significant acetylcholinesterase inhibiton[18]. Also many plants as *Sophora flavescens* showed a significant acetylcholinesterase inhibition and this activity is due to prenylated flavonoid, 8-lavandulylkaempferol which exhibited significant inhibitory effects with IC_{50} values of 7.10 and 8.11μM for butyrylcholinesterase and acetylcholinesterase[19], also Inhibition of acetyl cholinesterase by *Indigofera* species extracts was due to the potential contribution of tannins and flavonols present in the extracts [20].

CONCLUSION

In the present article, we evaluated acetylcholinesterase inhibition by methanol extract of *B. alba* aerial parts, also we determined the main phytoconstituents and identified the bio-active phytoconstituents of the plant extract. The methanol extract of *B. alba* apparently tested positive enzyme inhibition, it has shown (IC_{50}= 0.222 mg/mL) and this activity is due to the bioactive compounds (lupeol, luteolin,

kaempferol, vitexin, isovitexin, kaempferol 3-*O*-*β*-glucoside and rutin) isolated from the extract and these active compounds are with a similar activity to neostigmine, which should contain about 1% of an active compound, or if present at lower levels even more active compounds than neostigmine (IC_{50} = 1.87 µg/mL) and galanthamine (IC_{50} = 0.37 x10^{-3} mg/mL) should be present. The results show that the extract is very interesting as acetylcholinesterase inhibitor which can be widely used in the treatment of Alzheimer's disease.

CONFLICT OF INTEREST

There is no conflict of interest associated with the authors of this paper.

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