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BIOASSAY-GUIDED FRACTIONATION AND ISOLATION OF 6-GINGEROL FOR ACETYL CHOLINESTERASE INHIBITORY ACTIVITY FROM *ZINGIBER OFFICINALE* (ROSCOE) RHIZOME

Pharmacology

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ABSTRACT

Background and Objective: *Zingiber officinale* (ginger) is one of the most widely used species in our day-to-day life and is a common condiment in various foods and beverages is being used for various diseases; cardio- protective, anti-inflammatory, anti-cancer and memory impairment.
Materials and Methods: The bioassay guided fractionation and isolation was carried out for 6-gingerol from *Zingiber officinale*. The extract was quantified by LC-MS/MS using 6-gingerol. The extract and 6-gingerol were analyzed for the AChE activity by Ellman's method.
Results: The isolated compound (SF4) was confirmed by ^{1}H NMR and ^{13}C NMR and FTIR. Significant inhibition was found with ginger on AChE enzyme with an IC^{50} of SF4 (42.5±74), ginger methanol extract (140±036) and fraction 1:6 (86.7±118), respectively, which was compared with standard galanthamine hydrobromide (4.73±0.71).
Conclusions: Our investigations indicated that ginger could be a promising anti- Alzheimer agent, thus we propose that Z. *officinale* a promising drug for the treatment of Alzheimer's disease.

KEYWORDS

Zingiber officinale, Bioassay guided fractionation, Isolation, Acetyl cholinesterase, Cholinergic and Alzheimer's disease.

1. INTRODUCTION:

Alzheimer's disease (AD) is an age-associated, irreversible, progressive neurodegenerative disease characterized by severe memory loss, unusual behavior, personality changes and a decline in cognitive function, It is the most common cause for dementia in the elderly and is regarded as the pandemic of the 21st century, imposing enormous social and economic burdens on patients and their families. [1] These plaques can induce neuroinflammatory processes, which trigger the production of reactive oxygen species (ROS) [2] and (i) cholinergic deficit resulting due to enhanced activity of acetyl cholinesterase (AChE), contributing to the cognitive impairments seen in AD (cholinergic hypothesis). [3] Based on these hypotheses, several cholinesterase inhibitors [4] are being investigated for their disease modifying strategies. In the recent years, herbal therapy for AD is gaining momentum. [5] Several ayurvedic medicinal plants and their phyto-constituents have shown promising pharmacological activities for the treatment of AD. [6-8]

The gingerols are the major phenolic pungent principles in the fresh rhizome, with 6-gingerol being the most abundant. [9] Due to its low toxicity and high medicinal potential, [6]- gingerol has become increasingly in demand day to day. [10] Ginger has its particular important medicinal properties, which are antioxidant [11-12] and anti-inflammatory [13] activities, which are the relevant for the treatment of AD. The results reveal that methanolic extract of dry ginger (MEDG) functions through multiple routes by exhibiting antioxidant property, inhibition of cholinesterase activity, prevention of Aβ oligomerisation and conferring protection against Aβ induced toxicity.

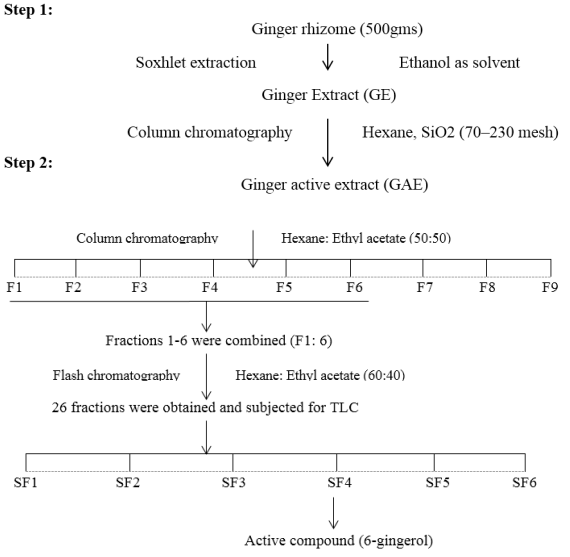
2. MATERIALS AND METHODS:

2.1. Preparation of Plant Extract:

The *Rhizomes of Zingiber* officinale (voucher specimen: JSSPG-277) were thoroughly washed, cut into small pieces. The rhizome (500gm's) was packed in the soxhlet apparatus using solvent system Methanol: Water (70: 30) mixture as a solvent placed in the round bottom flask and heated at 45-550C. The extract obtained was filtered and concentrated using Rota-vapor (IKA RV-10). The obtained semi-solid extract was then lyophilized to remove the residual moisture and the percentage yield was found to be 1.20 %w/w. [14]

2.2. Isolation of 6-Gingerol:

In order to isolate 6-gingerol, methanolic extract of ginger rhizome was chromatographed on SiO2 (70–230 mesh) column with bed height 20 cm (20 cm × 8 cm i.d.), using initially n-hexane (1L), to eliminate the lipids. After, n- hexane/ethyl acetate 50:50 (v/v, 2.5 L) was used as eluent resulting in 12 fractions (F1-12; 53.6%, 8.14g), rich in [6] - gingerol, were joined resulting in the fraction F1–6. Fraction 1-6 (2.4g) was further purified by Flash chromatography (isolera one) using n-hexane/ethyl acetate 60:40 (v/v, 2.5L). Twenty six fractions (15 mL each) were collected and combined after monitoring by TLC resulting in six sub-fractions with the following volumes: 50 mL (SF1), 50 mL (SF2), 100 mL (SF3), 150 mL (SF4), 100 mL (SF5) and 50 mL (SF6). The 6 - gingerol were the main constituent on the fraction SF4 (150 mL, 36 mg, yellow oil). [15]



2.3. Quantification of 6-gingerol in the extract of Z. officinale by HPTLC analysis:

HPTLC analysis of Zingiber officinale with marker 6-gingerol was performed using isocratic technique by external methods. Mobile phase was optimized with Tolune, ethyl acetate, methanol and glacial

acetic acid in a ratio of 5:4:1:1 v/v. The temperature was kept at 25°C and mobile phase was developed in a twin trough glass chamber. Standard stock solution was applied consequently in the range of 2-10 µl with 2 µl gradual increments. All total 8 tracks in HPTLC plate were used for standardization including standard and sample solution respectively in a band wise fashion. After development the plates were dried by hand dryer. Colored bands were observed at 254 to 366 nm and also visualized using vanillin sulphuric acid spraying reagent. After chromatography, the amount of 6-gingerol was determined by means of the calibration plot and the percentage of 6-gingerol was quantified. [20]

2.4. LC/ESI-MS/MS analysis of ginger extracts:

The Mobile phase was optimized with (A) buffer (5mM ammonium formate, 0.1% formic acid, in ddH2O) and (B) acetonitrile. The acquisition parameters for positive and negative modes were: drying N2 temperature, 350°C; Flow rate: 1ml/min; temperature, 400C; injection volume, 5 ml was delivered at a flow rate of 0.5 mL/min with splitter; the total run time was 5 min. The parameters of mass spectrometer were optimized using 100µg/mL of test solution in methanol and the concentration of the isolated compound was 10µg/mL. Good intensity of response was obtained in the positive mode. [16]

2.5. Cholinesterase inhibitory activity by 96 well microtiter plate method:

Ache inhibitory activity was determined by using Ellman's method. [17] Galantamine hydrobromide was used as the standard cholinesterase inhibitor. [18] 20µL of 3mM DTNB, 20µL of 15mM ATCI, 120 µL of buffer and sample was dissolved in tris-HCl buffer and added in increasing order to each well of 96 well plate and the absorbance was read at 405 nm every 13 s for 65 s. 20 µL of 0.22 U/mL of Ache was added to each well and the absorbance was measured at every 13 s for 104 s. The % inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated. Inhibition curves were obtained for each compound by plotting the relative activity versus concentration in the assay solution. The Kinetic analysis of acetylcholinestarse was performed for the Un-competitive inhibition, which can be described by the Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \left(1 + \frac{[I]}{K_i}\right) \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

From Eq. (1), 'V' is the enzyme reaction rate in the absence and presence of ginger extract, F 1:6 & 6-gingerol. Ki and Km are the inhibition constant and Michaelis-Menten constant, respectively. Inhibition constant (Ki) was calculated by plots of Km/ Vmax or 1/ Vmax versus inhibitory concentration. [19]

3. RESULTS:

3.1. 6-Gingerol Isolation:

Ginger MeOH extract (14.26 g) was subjected to column chromatography on SiO2 (70–230 mesh) in a slurry with the increase in polarity of mobile phase using n- hexane/ethyl acetate 50:50 (v/v, 2.5 L), to obtain 12 fractions (8.14g). The fractions 1-6 (2.4g) were combined and were subjected to flash chromatography using n-hexane/ethyl acetate 60:40 (v/v, 2.5L) to obtain 26 fractions, which contains six sub fractions SF1-6.

The sub fraction SF4 was evaporated under vacuum to give compound 6-Gingerol (150 mL, 36 mg, yellow oil), with Rf 0.49 by TLC in toluene: ethyl acetate: MeOH: formic acid (3:4:3:1 v/v). IR Vmax cm-1: 2954.95, 2858.81, 1707.0, 1516.05, 1271.09; 1H NMR (CDCl3): δ _0.870 (3H, t, J = 7.2 Hz, Me), 1.296-1.478 (8H, m, H-9, H-8, H-7, H-6), 2.446 (1H, dd, J =17.4 Hz, 8.0 Hz, H-4), 2.710-2.821 (4H, m, H-1, H-2), 2.873 (1H, broad, s, OH), 3.876 (3H, s, OMe), 4.020 (1H, m, H-5), 4.056 (1H, broad, s, Ar-OH), 6.647 (1H, dd, J =8.1 Hz, 2.0 Hz, H-6'), 6.654 (1H, d, J =2.2 Hz, H-2'), 6.819 (1H, d, J =8.2 Hz, H-5'); 13C NMR (CDCl3): δ _13.997, 22.575, 25.116, 29.287, 31.720, 36.441, 45.428, 49.364, 55.879, 68.00, 111.010, 114.402, 120.740, 132.643, 143.997, 146.461, 211.414.

The IR spectrum showed broad hydroxyl absorption at 2954.95 cm-1, strong carbonyl absorption at 1707.0 cm-1, aromatic stretching at 1610.56 and 1516.05 cm-1 and also C-O stretching at 1271.09 cm-1. Table1. Gives the IR parameter of compound 6-Gingerol.

1H NMR spectrum of this compound indicated that compound 6-Gingerol had a similar structure to compound Shogaol due to the presence of a methoxyl group at 3.876 (3H, s), methyl group at δ _0.870 (3H, t, J=7.2 Hz) and three aromatic protons (H-6', H-5' and H-2') observed at δ _6.647, 6.654 and 6.819 respectively. However, the spectrum for compound 6-gingerol showed a broad peak at δ _2.873 was attributed to the hydroxyl proton. A broad single peak integrating for two protons was assigned to two hydroxyl protons at δ _4.056. A multiplet peak at δ _2.710-2.821 was assigned for four protons (H-1 and H-2). Four methylene groups (H-9, H-8, H-7 and H-6) were observed at δ _1.296-1.478 as multiplet.

The 13C NMR spectrum supported that compound 6-Gingerol based on the 17 peaks, which represents 17 carbons in the molecule. The presence of carbon methyl was observed at δ _13.997 and δ _55.879, seven methylene carbon, C-1 (29.287), C-2 (45.428), C-4 (49.364), C-6 (36.441), C-7 (31.720), C-8 (25.116), C-9 (22.575) and methylene C-5 (67.678), carbonyl carbon C-3 (211.414), quaternary carbon C-1' (132.643), C-3' (146.461), C-4' (143.997) and carbon aryl C-2' (111.010), C-5' (114.402) and C-6' (120.740). That compound has been identified as compound 6-Gingerol based on the physical properties and the spectroscopic data of compound isolated from Zingiber officinale Roscoe and compared with the standard 6-Gingerol. Both of the 1H and 13C NMR data are summarized in Table 2.

3.2. Quantification of 6-gingerol using HPTLC technique:

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for the analysis of 6-gingerol. The Rf of 6-gingerol was found to be 0.53 in which the UV spectra were measured for the bands, which showed a maximum absorbance at 533 nm. The calibration plot (Fig. 1c) was linear in the range of 200–800µg of 6-gingerol with a correlation coefficient of 0.9926 was indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation Y = -3788.573 + 26.061 × X, where Y is the response as peak area and X is the concentration. The densitometric chromatograms obtained from 6-gingerol and the methanol extract are shown in Fig. 1a and 1b respectively. The TLC plates at 254, 366nm and along with a visualizing agent (vanillin sulphuric acid) are shown in figure 2a, 2b and 2c. The amount of 6-gingerol present in the extract was found to be 1.23%w/w.

3.3. LC-MS/MS analysis to determine the 6-gingerol in Zingiber officinale:

The mobile phase were well optimized to obtain chromatograms with good resolution within an acceptable time of analysis. (A) Buffer (5mM ammonium formate, 0.1% formic acid, in ddH2O) and (B) acetonitrile were used as a mobile phase. The mass spectra of fraction SF4 and the ginger extract are shown in figure 3a and 3b. As shown in Figure 3 a and b peaks exhibited the expected mass at m/z 293.10 (SF4) and 290.15 (ginger extract), indicating that the compound was found to be 6-gingerol.

3.4. Acetylcholinesterase inhibitory assay:

AChE inhibitory potentials of the test and standard compounds have been shown in (Table 1). The result shown that the test sample along with standard compound has significant AChE inhibitory activity. SF4 has showed high inhibition of AChE (IC50: 42.5 ± 74), when compared to AChE inhibition value of ginger methanol extract (IC50: 140 ± 036) and fraction 1:6 (IC50: 86.7 ± 118) were compared with a reference standard galanthamine hydrobromide which have possessed the highest AChE inhibition activity with an IC50 value of 4.73 ± 0.71 shown in (Figure 4).

Enzyme kinetics of AChE inhibitory activities of methanol extract of Z. offcinale, F 1:6, 6-gingerol and galanthamine is shown in [(Figure 5a, 5b and 5c)]. V max, Km and Ki values were determined through Line-weaver Burk plot the values are shown in Table 4. The Line-weaver Burk plot for ginger extract, F 1:6 and 6-gingerol showed un-competitive inhibition where as galanthamine showed competitive type of inhibition. Moreover, 6-gingerol and galanthamine was shown significant ($P < 0.05$) kinetic value, when it compared with the control group.

4. DISCUSSION:

It has been assumed that cognition behaviors of AD patients are closely

associated with a decrease in concentration of brain ACh level. The cholinesterase inhibitors used such as galantamine is obtained from plant sources. This provides an opportunity to explore new ChIs from plant sources. AChE inhibition potential of Zingiber officinale were confirmed by the inhibitory potential of ginger extract, F 1:6, 6-gingerol and galantamine in 96 well plate microtitre assay were applied. Enzyme inhibition efficiency of 6-gingerol showed a better inhibition than the ginger extract. 6-gingerol demonstrated the highest AChE inhibitory among the extract and fraction, but less active than that of the reference compound galantamine.

Enzyme kinetics of AChE inhibitory activities of methanol extract of Zingiber officinale extract, F 1:6, 6-gingerol and galantamine were screened. The V max value of ginger extract, F 1:6 and 6-gingerol were decreased by comparing with the control group, while the K m values of the above were different, in which both the km and Vmax values are different which indicated a Un-competitive and competitive type of inhibition. However, V max value of reference compound galantamine did not showed any significant change with respect to control group, but K m value decreases, which suggested a competitive type of inhibition. The phyto-constituents of the Zingiber officinale extract and its oil may be explored as potential lead for the treatment of AD.

5. CONCLUSION:

The present study suggested that Zingiber officinale extract and its oil has shown a potential inhibition against anticholinesterase. Therefore, ginger may be explored further for its use in the management of AD and related cognitive disorders.

ACKNOWLEDGEMENT:

I am very thankful to Dr. S. Rajan, Centre of Medicinal Plants Research in Homeopathy, Ministry of AYUSH, Indra nagar, Emerald for his encouragement and helpful in the identification and authentication of the plants. I am also very much thankful to my beloved guide Dr. S.P. Dhanabal who has supported me for doing this work.

"Table 1: IR Parameter for Compound 6-Gingerol."

Frequency (cm-1)	Intensity	Type of Bond
2954.95	M	O-H
2931.60 and 2858.51	S	C-H
1707.0	S	C=O
1610.56 and 1516.05	M	C=C aromatic
1271.09	S	C-O stretch

m = medium, s = strong

"Table 2: *^{1}H NMR and ^{13}C NMR Parameters for Compound 6-Gingerol."*

Carbon	13C, δ (ppm)	^{1}H, δ (ppm)
1	29.287	2.710-2.821
2	45.428	2.710-2.821
3	211.414	
4	49.364	2.446, 2.504
5	67.678	4.020
6	2.873 (-OH)	
7	36.441	1.296-1.478
8	31.720	1.296-1.478
9	25.116	1.296-1.478
10	22.575	1.296-1.478
1'	13.997	0.870
2'	132.643	
3'	111.010	6.647
4'	146.461	
5'	143.997	4.056
6' Ome	114.402	6.819
	120.740	6.647
	55.879	3.876

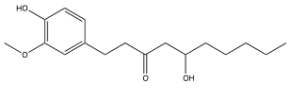


Figure: 6- gingerol structure
(5S)-5-hydroxy-1- (4-hydroxy-3-methoxyphenyl) decan-3-one

"Figure 1 and 2: *Densitometric chromatogram and TLC plate visualization"*

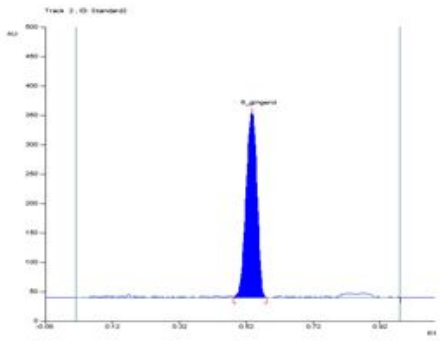


Fig. 1a. Densitometric chromatogram of standard 6- gingerol

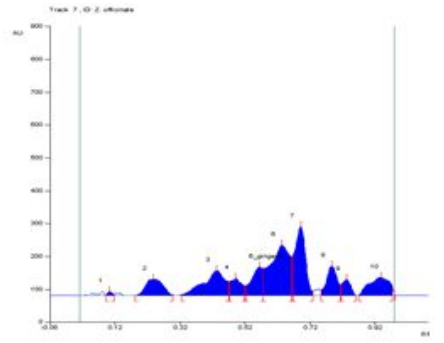


Fig. 1b. Densitometric chromatogram of Zingiber officinale MeOH extract

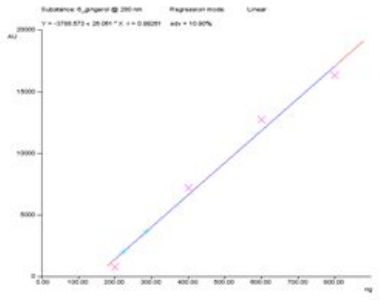


Fig. 1c. Calibration curve of Zingiber officinale MeOH extract

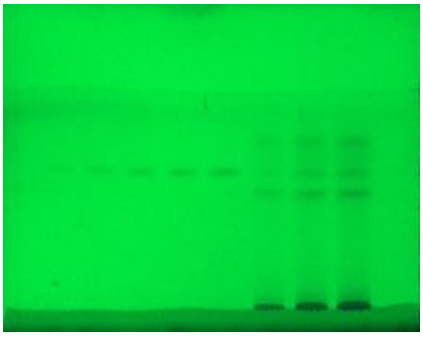


Fig. 2a. TLC plate at 254nm

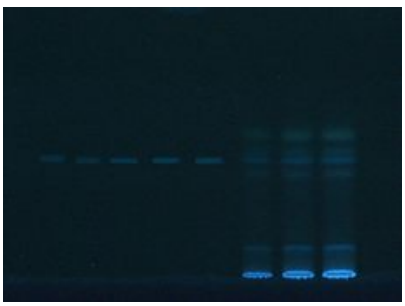


Fig. 2b. TLC plate at 366nm

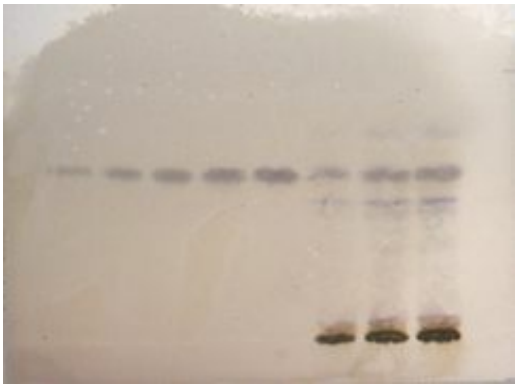
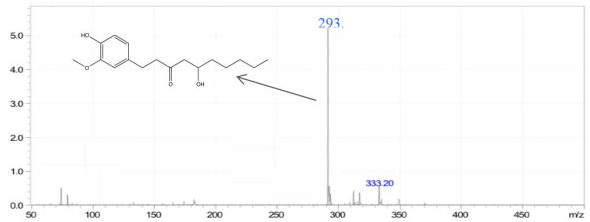
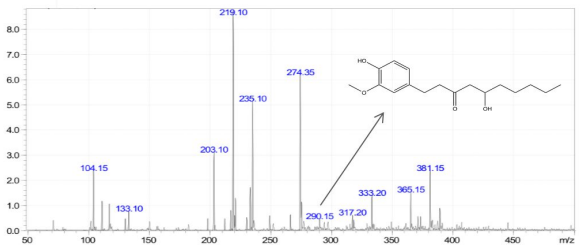


Fig. 2c. TLC plate using vanillin sulphuric acid spraying reagent

***"Figure 3a & 3b:** Mass spectra of the SF4 fraction & ginger extract."*

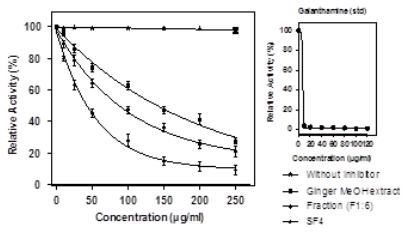


3a



3b

"Figure 4: *Inhibition pattern of Ginger methanol extract and 6-gingerol of Z. officinale on Acetylcholinestarse."*



[(★) Without inhibitor; (■) Ginger methanol extract; ( ) Fraction F1: 6 and (●) SF4 (6-gingerol); Data are presented as Mean ± SD (n = 3)].

"Figure 5: *Lineweaver-Burk plot of acetylcholinesterase inhibitory activity at different substrate concentration."*

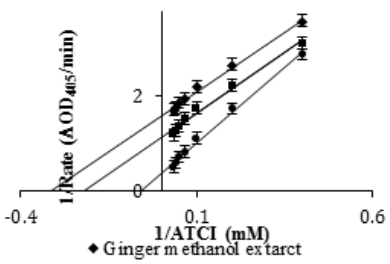


Fig. 5a. Lineweaver-Burk plot of acetylcholinesterase inhibitory activity at substrate concentration 10mM

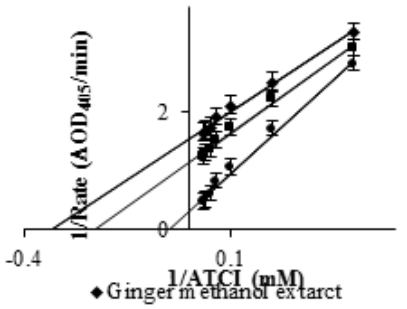


Fig. 5b. Lineweaver-Burk plot of acetylcholinesterase inhibitory activity at substrate concentration 15mM

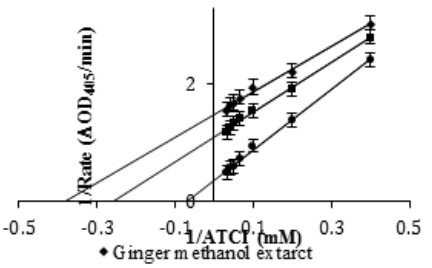


Fig. 5c. Lineweaver-Burk plot of acetylcholinesterase inhibitory activity at substrate concentration 20mM

***"Table 4:** Lineweaver-Burk plot of acetylcholinesterase inhibitory activity."* *"Table 4: Lineweaver-Burk plot of acetylcholinesterase inhibitory activity."*

Enzyme	Substrate	Inhibitor	Vmax (n mole/min/mg of protein)	Km (mM)	Ki (mg/ml)	Type of Inhibition
AChE	ATCI	- Control - Galanthamine - Ginger ME - Fraction 1:6 - SF4	91.54±3.23 91.54±3.23 1.5554±1.38 1.144±0.59 0.3258±1.63	41.67±1.27 42.23±4.0 2.97±1.54 4.36±2.79 19.62±0.76	- 2.63±0.28 149.27±1.7 85.7±2.38 41.5±0.57	- Competitive Un-competitive Un-competitive Un-competitive

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