

The potential therapeutic role of Fenugreek saponin against Alzheimer's disease: Evaluation of apoptotic and acetylcholinesterase inhibitory activities

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ABSTRACT

Alzheimer disease (AD) is known as lacking in the neuro-transmitters within the brain cells due to increase the Acetylcholinesterase (AChE) activity. So, use of AChE inhibitors (AChEI) is believed to be the best way in treatment of Alzheimer. Therefore, the aim of the present work was to evaluate the AChEI and apoptotic activities of fenugreek saponin against AD *in vivo*. Ninety male aged Sprague Dawley rats were allocated in several experimental groups including untreated animals, supplemented animals with 0.05%, 0.1% and 2% of fenugreek saponin (FS), animals treated with $AlCl_3$ to induce AD, AD-induced animals treated with the previous doses of FS or with Rivastigmine. Brain tissues of different groups were used for determine the AChEI and apoptotic activities as well as generation of reactive oxygen species (ROS), DNA damage and expression of apoptotic related genes (Bax; Bcl2 and caspase-3). The results showed that FS increased the AChEI and apoptosis activities as well as elevated the gene expression levels of Bax; Bcl2 and caspase-3 genes in AD-induced rats. However, FS decreased the ROS generation and DNA damage in AD-induced rats compared with control rats. The results suggested that the ability of fenugreek saponin to inhibit AD due to increase AChE inhibition activity might be attributed to increase the antioxidants in this herb. Moreover, enhancement the apoptosis by fenugreek saponin could be attributed mainly to the regulation process of Bax, Bcl-2 and caspase-3 in the apoptotic pathway and not by generation of ROS in the brain cells of the AD-induced rats.

INTRODUCTION

Alzheimer's disease (AD) is considered as a neurological degenerative disorder of the brain tissues. It is known as the most widespread form of insanity among the aged inhabitants (Kim *et al.*, 2009). Alzheimer's society recorded that 13% of the American population beyond age of 65 years and 50% of the Americans higher than 85 years in age have been currently estimated as AD's patients (Kim *et al.*, 2009). Therefore, increasing the number of AD patients which reaching several million in the near future (Kim *et al.*, 2009; Heo *et al.*, 2004; Prasad *et al.*, 2000; Terry and Masliah, 1991) needs a lot of money for the health care scheme of the AD (Kim *et al.*, 2009).

Absence of cholinergic connections in several parts of the brain such as neocortex and hippocampus has been found to be main reason of AD. This fact highlights the requirement to utilize a considerable challenge that regulates the acetylcholinesterase (AChE) role to overcome this failing (Kim *et al.*, 2009).

Several medicines such as rivastigmine and donepezil are used for therapy this disorder as AChE inhibitors which are approved by several global Food and Drug societies (Kim *et al.*, 2009; Candy *et al.*, 1983; Loizzo *et al.*, 2008). In despite of using these inhibitors for regulating the function of the AChE there are increment need to pursuit for new medicaments (Kim *et al.*, 2009). Therefore, several studies for this purpose aimed to find new natural compounds having possible antioxidative properties and with very low side effects have been carried out (Kim *et al.*, 2009, Ak and Gulcin, 2008; Gulcin, 2006; Burda and Oleszek, 2001; Cardenas *et al.*, 2006; Li *et al.*, 2008; Kris-Etherton *et al.*, 2002).

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Consequently, there is rising spotlight on use imitative herbal medicines for AD treatment (Drever *et al.*, 2008). As already known, acetylcholine is main neurotransmitter playing critical role in AD is acetylcholine (Akhondzadeh *et al.*, 2003). For that reason, there have been multiple research to use AChE suppressors. One of the important plants having the antioxidant properties is *Trigonella foenum graecum* L namely, fenugreek. Its seeds and leaves are used for food and also in traditional medicine (Sharma *et al.*, 1996; Warrior and Nambiar, 1995). Some studies reported that trigonelline, a compound isolated from fenugreek, exhibited nerve regeneration and improved memorial activity in AD-induced mice (Tohda *et al.*, 2005). Therefore, in India prepared bread from fenugreek seeds, wheat and maize is used for general population as protection against several diseases including AD. It has been found that fenugreek seeds have many of compounds such as steroidal saponin, vitamins and oils considered as very important materials for medical applications (Jayaweera, 1981). These compounds and materials have been used against ulcer (Almeshal *et al.*, 1985), CNS degeneration (Natrajan *et al.*, 2007), immune diseases (Bilal *et al.*, 2003), oxidation (Kaviarasan *et al.*, 2007). In addition, fenugreek saponin has been reported to have apoptosis activity (Moalic *et al.*, 2001). It has been found that fenugreek saponin is able to inhibit cell proliferation in the tumor cell line by stimulation of apoptosis and arrest of the cell cycle (Moalic *et al.*, 2001). It has been suggested that fenugreek saponin caused apoptotic stimulation and cell cycle arrest mostly by enhancing the gene expression of the onco-protein p53 (Corbiere *et al.*, 2003). However, for our knowledge there are no published data available concerning the possible therapeutic effect of fenugreek saponin on AD. Therefore, the idea of the present study is to evaluate the apoptotic and acetylcholinesterase inhibitory activities of fenugreek saponin against AD *in vivo*.

MATERIAL AND METHODS

Drugs and Reagents

Aluminium Chloride (AlCl_3 , M.W. 133.34): was purchased from Sigma. Rivastigmine: Exelon 1.5 mg, was purchased from Novartis Co. Trizol was bought from Invitrogen (Carlsbad, CA, USA). The reverse transcription and PCR kits were obtained from Fermentas (Glen Burnie, MD, USA). SYBR Green Mix was purchased from Stratagene (La Jolla, CA, USA).

Fenugreek saponin

An 80% methanol extract of fenugreek seeds was produced by grinding seeds to a fine powder and mixing with 80% methanol over night. Saponins were extracted from fenugreek seeds (*T. foenum-graecum* L.) seeds generally according to Marston and Oleszek (2000).

Experimental Animals

Ninety male aged Sprague Dawley rats (14-16 months) weighing 250-300 g (purchased from the Animal House Colony, Giza, Egypt) were maintained on standard laboratory diet and

water *ad libitum* at the Animal House Laboratory, National Research Center, Dokki, Giza, Egypt. After an acclimation period of 1 week, animals were divided into groups (10 rats/ group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled ($23 \pm 1^\circ\text{C}$) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.

Experimental Design

Rats were randomly assigned into nine groups, ten rats each. The first group served as normal control. The second to fourth groups were fed experimental diets containing 0.05%, 0.1% and 2% of fenugreek saponin (FS) forty five days, respectively. The fifth group was provided with AlCl_3 in drinking water in a dose of 0.3% for forty five days (Erazi *et al.*, 2010), and served as AD intoxicated group. The sixth to eighth groups rats were given AlCl_3 in drinking water daily for forty five days then were fed experimental diets containing 0.05%, 0.1% and 2% of fenugreek saponin, respectively, for another forty five days (Hussein, 2008). The ninth group rats were given AlCl_3 in drinking water daily for forty five days then they were orally treated with Rivastigmine in a dose of 0.3 mg/kg b. wt. (Carageorgious *et al.*, 2008) as a reference drug daily for another forty five days.

Tissue Collection

At the end of experimental period the animals were sacrificed under anesthesia and the brains were rapidly dissected, thoroughly washed with isotonic saline. The each brain was mid-sagittally divided into two portions. The first portion was stored in liquid nitrogen for gene expression analysis and the second portion was homogenized, centrifuged under cooling at 3000 rpm for 10 min for biochemical analyses.

Apoptosis assay

The brain tissues (100 mg per sample) were made into single-cell suspensions according to method of Villalba *et al.* (1992). Cells apoptosis was determined by flow cytometry (FCM) assay using Annexin V/PI apoptosis detection kit. The single-cell suspension (1×10^6 cells/mL) was suspended in 200 μL ice-cold binding buffer and then 10 μL horseradish peroxidase FITC labeled Annexin V and 5 μL propidium iodide (PI) were added. The cell suspension was incubated in darkness at room temperature for 15 min. Apoptosis rate was determined by flow cytometer. In this study, both FITC and PI negative cells were considered as normal cells. FITC-single positive and PI negative cells were defined as early apoptotic cells, while both FITC and PI positive cells were considered as late apoptotic or necrosis cells.

Determination of ROS formation

Intracellular ROS generation was measured in brain tissues by a flow cytometer with an oxidation-sensitive DCFH-DA

fluorescent probe, after single-cell suspensions were made. DCFH-DA is a non-fluorescent compound that is freely taken up into cells. DCFH is oxidized to fluorescent dichlorofluorescein (DCF) by the action of cellular oxidants. The suspension was loaded by DCFH-DA solution with a final concentration of 50 μM and was incubated for 30 min at 37°C. Then samples were centrifuged at 1000 rpm for 5 min (4°C), and cells were resuspended with phosphate buffered saline (PBS, pH 7.2–7.4). The fluorescence was detected by flow cytometer (with excitation 488nm and emission 525 nm). For each treatment, 1×10^5 cells were counted, and the experiment was performed in triplicate.

Determination of AChE activity

AChE activity was measured in brain tissues using a 96-well microplatereader (Ellman *et al.*, 1961; Mukherjee *et al.*, 2007). The enzyme hydrolyzes the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 405nm. In the 96-well plates, 125 μl of 3 mM DTNB, 25 μl of 15 mM ATCI, 50 μl of buffer and 25 μl of sample dissolved in phosphate buffer were added. The absorbance was measured at 405nm every 13s for 65s. 25 μl of 0.22U/ml of AChE enzyme was then added and the absorbance was again read every 13s for 104s. The absorbance was read using a BioRad micro-plate reader at 405nm. Absorbance was plotted against time and enzyme activity was calculated from the slope of the line so obtained and expressed as a percentage compared to an assay using a buffer without any inhibitor.

Gene Expression Analysis

Isolation of Total RNA

TRIzol[®] Reagent (Invitrogen, Germany) was used to extract total RNA from brain tissues of male rats according to the manufacturer's instructions with minor modifications. Isolated total RNA was treated with one unit of RQ1 RNase-free DNase (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis (data not shown). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

Reverse Transcription (RT) Reaction

Complete Poly(A)⁺ RNA isolated from brain tissues was reverse transcribed into cDNA in a total volume of 20 μl using RevertAid[™] First Strand cDNA Synthesis Kit (Fermentas, Germany). An amount of total RNA (5 μg) was used with a master mix consisting of 50 mM MgCl₂, 10x RT buffer, 10 mM of each dNTP, 50 μM oligo-dT primer, 20 IU ribonuclease inhibitor and 50 IU MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min,

followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real Time-polymerase chain reaction (qRT-PCR).

Real Time-PCR (qPCR)

QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the cortex cDNA copy number. PCR reactions were set up in 25 μL reaction mixtures containing 12.5 μL $1 \times$ SYBR[®] Premix Ex Taq[™] (TaKaRa, Biotech. Co. Ltd.), 0.5 μL 0.2 μM sense primer, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water, and 5 μL of cDNA template. The reaction program was used as follows: a number of 40 cycles was used in which each cycle divided to: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. afterwards, a last step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. The sequences of specific primer of the genes used are listed in Table 1. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Table 1: Primer sequences used for qPCR

Gene	Primer sequence (5'-3')	References/NCBI
Bax	F: CGA GCT GAT CAG AAC CAT CA R: CTC AGC CCA TCT TCT TCC AG	NM-017059.2
Bcl2	F: CTC AGT CAT CCA CAG GGC GA R:AGA GGG GCT ACG AGT GGG AT	Khalil and Booles [32]
Caspase 3	F: GGA CCT GTG GAC CTG AAA AA R: GCA TGC CAT ATC ATC GTC AG	NM-012922.2
β -actin	F: CAC GTG GGC CGC TCT AGG CAC CAA R: CTC TTT GAT GTC ACG CAC GAT TTC	Khalil and Booles [32]

To calculate the gene expression levels the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet: $Ef = 10^{-1/\text{slope}}$, Efficiency(%) = (Ef - 1) x 100. The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method if Ef for the target (Bax; Bcl2 and caspase 3) and the reference primers (β -Actin) as follows:

$$\Delta C_{T(\text{test})} = C_{T(\text{target, test})} - C_{T(\text{reference, test})}$$

$$\Delta CT (\text{calibrator}) = C_{T(\text{target, calibrator})} - C_{T(\text{reference, calibrator})}$$

$$\Delta\Delta CT = \Delta C_{T(\text{Test})} - \Delta C_{T(\text{calibrator})}$$

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

Comet assay

Isolated brain tissues of all groups of rats were subjected to the modified single-cell gel electrophoresis or comet assay (Fairbairn *et al.*, 1995). In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under

alkaline conditions (30 min) at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a fluorescence microscope with a green filter at $\times 40$ magnification. For each animal about 100 cells were examined to determine the percentage of cells with DNA damage that appear like comets. The non-overlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between $1\times$ and $2\times$ the nuclear diameter; and class 3 = tail longer than $2\times$ the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus (Collins *et al.*, 1997).

RESULTS

Effect of FS on rate of the apoptosis

Determination the effect of different doses of FS on apoptosis induction in brain tissues of male rats was carried out (Fig. 1). The results indicated that FS increased the rates of apoptosis in healthy rats by 259.8, 312.2 and 522% when they treated with low, medium and high doses of FS, respectively, compared with control rats (Fig. 1).

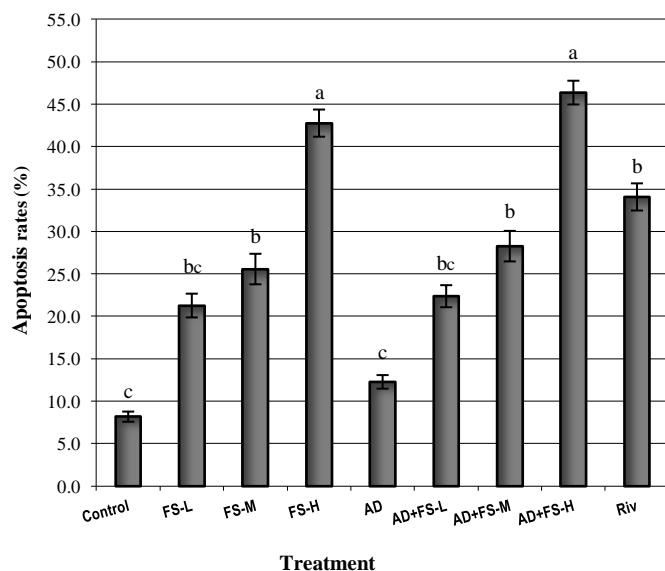


Fig. 1: Alterations in apoptosis rate in brain tissues of healthy or AD-induced rats treated with different doses of fenugreek saponin (FS). Data are presented as mean \pm SEM. ^{a,b,c} Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

Moreover, similar rates of apoptosis were found in brain tissues of AD-induced rats when they treated with FS compared with control rats. The rates of apoptosis were equal to 273.2, 345.1 and 565.9% in brain tissues of AD-induced rats treated with low, medium and high doses of FS, respectively compared with control rats. Furthermore, treatment of AD-induced rats with rivastigmine induced rate of apoptosis similar to those induced by medium dose

of FS. However, less apoptosis rate was found in brain tissues of AD-induced rats (Fig. 1).

Effect of FS on ROS generation

Measurement of the intracellular ROS generation in brain tissues of male rats is summarized in Fig. 2. The results showed that high level of intracellular ROS generation with a significant difference ($P < 0.01$) was found in AD-induced rats compared with control rats. However, low intracellular ROS generation similar to those in control rats was found brain tissues of male rats treated with the all doses of FS. Moreover, treatment of AD-induced rats with FS decreased the intracellular ROS generation compared with those in untreated AD-induced rats. The decrease in the intracellular ROS generation in AD-induced rats was found in the treatment with all doses of FS, where the lowest intracellular ROS generation was found with the high dose of FS. On the other hand, treatment of AD-induced rats with rivastigmine decreased the intracellular ROS generation similar to those induced by high dose of FS (Fig. 2).

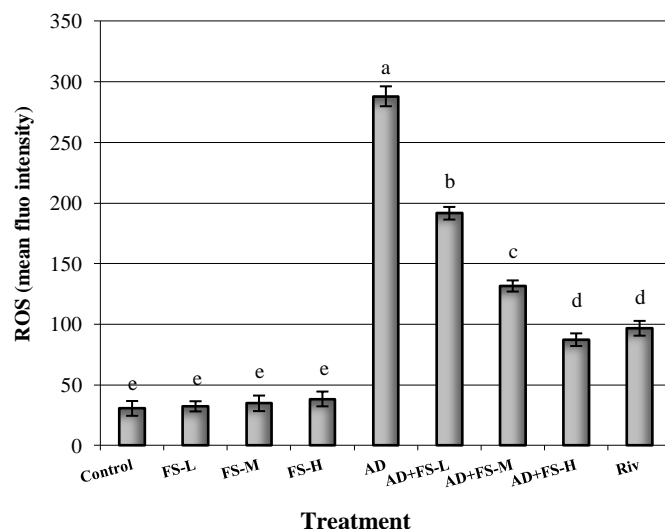


Fig. 2: Intracellular ROS levels in brain tissues of healthy or AD-induced rats treated with different doses of fenugreek saponin (FS). Data are presented as mean \pm SEM. ^{a,b,c,d} Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

Effect of FS on the AChE inhibitory activity

Table 2 shows the effect of different doses of FS on the levels of AChE inhibitory activity. The results found that FS increased significantly the levels of AChE inhibitory activity in male rats by 4, 7 and 9-fold increase when they treated with low, medium and high doses of FS, respectively, compared with control rats (Table 2). In addition, relatively similar mean levels of AChE inhibitory activity were found in brain tissues of AD-induced rats when they treated with FS compared with control rats. The mean values of AChE inhibitory activity were increased by 4.5, 8 and 10.6-fold increase in brain tissues of AD-induced rats treated with low, medium and high doses of FS, respectively compared with control rats. Moreover, treatment of AD-induced rats with

rivastigmine increased the levels of AChE inhibitory activity similar to those induced by medium dose of FS (Table 2).

Table 2: Acetylcholinesterase Inhibitory activity in different treatments by fenugreek saponin.

Treatment	IC ₅₀ values of AChE inhibitory activity (µg/ml)
Control	8.2±2.3 ^c
Fenugreek saponin-low	32.1±4.2 ^b
Fenugreek saponin-medium	58.7±6.1 ^a
Fenugreek saponin-high	78.4±7.3 ^a
Alzheimer	13.6±3.2 ^c
Alzheimer + Fenugreek saponin-low	36.2±7.6 ^b
Alzheimer + Fenugreek saponin-medium	64.1±9.2
Alzheimer + Fenugreek saponin-high	85.6±8.4
Rivastigmine	62.3±7.7

IC₅₀= half maximal inhibitory concentration (IC₅₀). Data are presented as mean ± SEM. ^{a,b,c} Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$)

Effect of FS on the expression alteration of apoptotic related genes

Expression of Bax, Bcl2 and caspase-3 genes quantified by real-time RT-PCR is summarized in Figures 3-5. The results found that low expression levels of Bax, Bcl2 and caspase-3 genes in control rats was in same line with those in AD-induced rats.

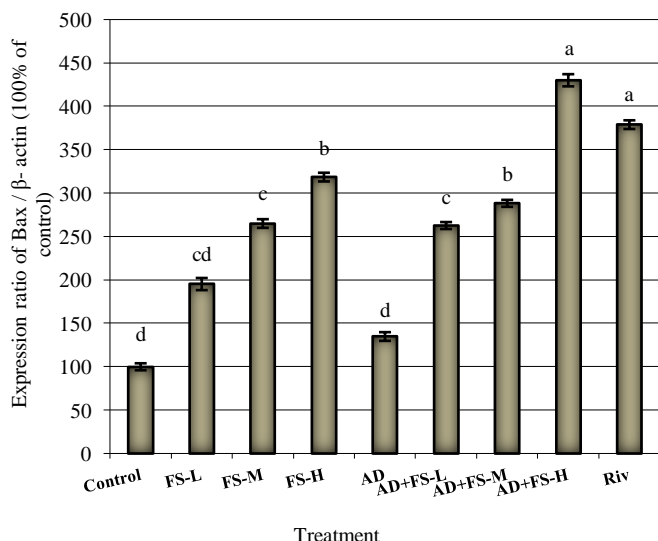


Fig. 3: Expression of Bax gene in the brain tissues of healthy or AD-induced rats treated with different doses of fenugreek saponin (FS) determined by quantitative RT-PCR. Data are presented as mean ± SEM. ^{a,b,c,d} Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

In contrary, FS treatment increased significantly the mRNA expression levels of Bax, Bcl2 and caspase-3 genes compared with control rats. The expression of Bax gene in male rats treated with low, medium and high doses of FS was increased to 195.3, 265.1 and 318.6% of control, respectively (Figure 3-5). The expression of Bcl2 mRNA in male rats treated with low, medium and high doses of FS was increased to 256.7, 321.6 and 345.9% of control, respectively. Also, the expression of caspase-3 mRNA in male rats treated with low, medium and high doses of

FS was increased to 264.3, 350.0 and 392.8% of control, respectively. Moreover, the expression of Bax, Bcl2 and caspase-3 genes was highly over-expressed in AD-induced rats treated with the all doses of the FS. The expression of Bax, Bcl2 and caspase-3 genes in AD-induced rats treated with low, medium and high doses of FS compared to control was increased to 262.8, 288.3 and 430.2% for Bax; 329.7, 354.1 and 470.3 for Bcl2; and 328.6, 450.1 and 567.8 for caspase-3 gene, respectively (Figure 3-5). Moreover, treatment of AD-induced rats with rivastigmine increased the expression levels of Bax, Bcl2 and caspase-3 genes similar to those of high dose of FS (Figure 3-5).

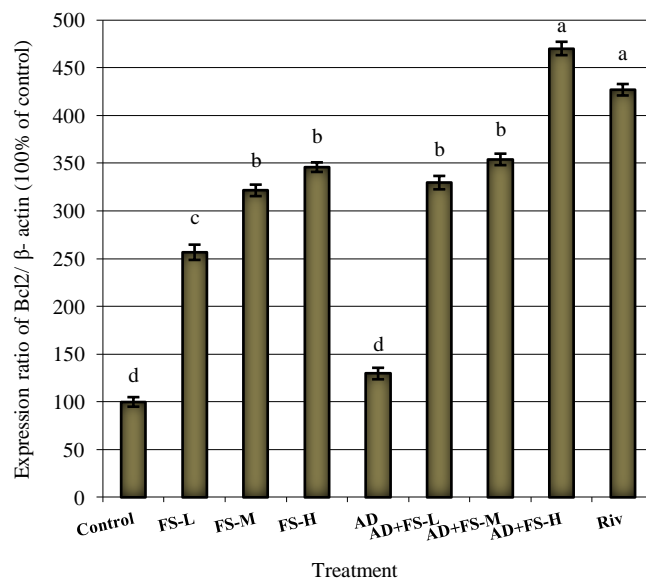


Fig. 4: Expression of Bcl2 gene in the brain tissues of healthy or AD-induced rats treated with different doses of fenugreek saponin (FS) determined by quantitative RT-PCR. Data are presented as mean ± SEM. ^{a,b,c,d} Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

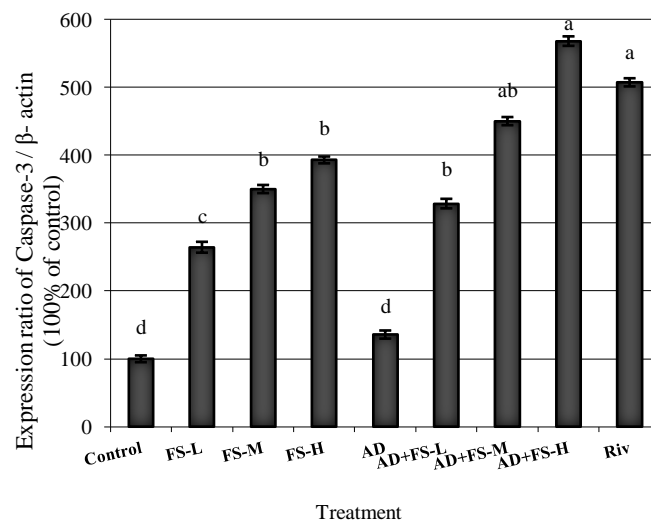


Fig. 5: Expression of Caspase-3 gene in the brain tissues of healthy or AD-induced rats treated with different doses of fenugreek saponin (FS) determined by quantitative RT-PCR. Data are presented as mean ± SEM. ^{a,b,c,d} Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

Effect of FS on DNA damage

Table 3 shows the rates of DNA damage determined by comet assay. The results observed that significantly high rate of DNA damage was found in AD-induced rats compared with control rats. However, low rates of DNA damage similar to those in control rats was found brain tissues of rats treated with the all doses of FS. Additionally, treatment of AD-induced rats with FS decreased the rate of DNA damage compared with those in untreated AD-induced rats. The decrease in the rate of DNA damage in AD-induced rats was found in the treatment with all doses of FS, where the lowest rate of DNA damage was found with the high dose of FS. On the other hand, treatment of AD-induced rats with rivastigmine decreased the rate of DNA damage similar to those induced by high dose of FS (Fig. 2).

Table 3: Visual score of DNA damage in brain tissues of different treatments by fenugreek saponin.

Treatment	No. of cells		0	Class**			DNA damaged cells %
	Analyzed	Comets		1	2	3	
Control	500	37	463	24	13	0	7.4
FS-L	500	36	464	23	12	1	7.2
FS-M	500	38	462	21	15	2	7.6
FS-H	500	41	459	22	13	5	8.2
AD	500	113	387	31	43	39	22.6
AD+FS-L	500	101	399	32	35	34	20.2
AD+FS-M	500	89	411	29	32	28	17.8
AD+FS-H	500	71	429	24	26	21	14.2
Riv	500	79	421	24	29	26	15.8

*: Number of cells examined per a group (n=5), **: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus.

DISCUSSION

This study aimed to evaluate the apoptotic and acetylcholinesterase inhibitory activities of fenugreek saponin against AD *in vivo* as novel therapeutic tool for Alzheimer disorder. The results of the present work found that fenugreek saponin increased significantly the AChE inhibitory activity in AD-induced rats at the all tested doses. In deep agreement with our findings Satheesh Kumar *et al.* (2010) reported that fenugreek fractions and saponin namely trigonelline extracts exhibited high AChE inhibitory activity. Additionally, they found that fenugreek saponin, trigonelline, exhibited higher AChE inhibitory activity than other fenugreek fractions. Moreover, Sharififar *et al.* (2012) indicated that between 3 different plants, fenugreek fraction extract showed high AChE inhibitory activity.

One of the novel strategies of AD therapy is to enhance the cholinergic activity during the pathway of AChE inhibition through using several natural products. Whereas, the major function of AChE is to transfer the impulse of the nervous cells through the cholinergic connection by the acetylcholine decomposition (Rahman and Choudhary, 2001). Therefore, the main point of the AChE inhibition pathway is cutting the nerve signal through the transmission role of AChE. For that reason, one of the important tools is to find natural products or extracts having the properties to improve the cognitive deficiency. This point was verified in the present study, where fenugreek saponin exhibited

the AChE inhibition activity. It has been suggested that the AChE inhibition activity increased with increase the presence of antioxidants in the extracted plant (Sharififar *et al.*, 2012). Depending on this fact, the ability of fenugreek saponin to inhibit AD due to increase AChE inhibition activity might be attributed to increase the antioxidants in this herb (Naidu *et al.*, 2011).

The present study reported that fenugreek saponin increased the rates of apoptosis rates in AD-induced rats. The rates of apoptosis were equal to 273.2, 345.1 and 565.9% in brain tissues of AD-induced rats treated with low, medium and high doses of FS, respectively compared with control rats. In addition, fenugreek saponin increased significantly the mRNA expression levels of apoptotic related genes (Bax, Bcl2 and caspase-3) in AD-induced rats compared with control rats.

In the same line with our findings, Alshatwi *et al.* (2013) demonstrated that methanol fenugreek extract was able to increase the expression levels of apoptotic and pro-apoptotic genes (*such as Caspase 3, Caspase 8, Caspase 9 and Bax*) in human breast cancer cell line. They suggested that the activity of fenugreek extract in inducing apoptosis might be attributed to its effect on the two pathways including apoptotic and death receptor pathways.

The main starting place of inducing apoptosis is taking place in the mitochondria. It considered as the central function in *caspase* cascade regulation and induction of apoptosis (Shafi *et al.*, 2009). It has been found that *caspases* are the most important molecules in the apoptotic process which they enhance the pathway of the apoptotic cascade (Shah *et al.*, 2003). This pathway is starting with releasing the cytochrome-c from the mitochondrial cells which stimulate the procaspase-9 and *caspase-3* expression (Shafi *et al.*, 2009). It has been demonstrated that *caspase-3* stimulation is found to be the significant step in the apoptotic process (Alshatwi *et al.*, 2013) and play an important role with caspase-8 and 9 in initiation of the apoptotic pathway (Riedl and Shi, 2004).

Pommier *et al.* (2004) suggested that caspase-8 is the main molecule in the death receptor pathway, while caspase-9 is the critical molecule in the mitochondrial apoptotic pathway, in which the caspase-3 is associating with these molecules in regulation the both pathways. In addition, they also reported that the role of caspase-8 in activation the link between the both pathways is due to enhancement the cleavage of the pro-apoptotic member of the Bcl-2 family.

Furthermore, the apoptotic member Bax plays an important role with Bak molecule in the apoptotic mitochondrial pathway which activating indirectly the p53 molecule causing cytochrome-c induction followed by apoptosis. This chain of activation steps in the apoptotic pathway enhances the function of caspase-3 indirectly by Caspase-8 and 9 or directly by caspase-9 (Alshatwi *et al.*, 2013). Although of the fact that generation of ROS in the cells triggers the apoptosis pathway by enhancement the DNA strand breaks (Armstrong *et al.*, 2002; Ding *et al.*, 2016; Alan Mitteer *et al.*, 2015) which was found in the AD-induced rats, our study found that treatment of AD-induced rats with fenugreek saponin decreased the intracellular ROS generation and

DNA damage compared with those in untreated AD-induced rats. Therefore, our results indicated that enhancement the apoptosis by fenugreek saponin could be attributed mainly to the regulation process of caspase-3, Bax and Bcl-2 in the apoptotic pathway and not by generation of ROS in the brain cells of the AD-induced rats.

CONCLUSION

The results indicated that FS increased the AChEI and apoptosis activities as well as elevated the gene expression levels of Bax, Bcl2 and caspase-3 genes in AD-induced rats. However, FS decreased the ROS generation and DNA damage in AD-induced rats compared with control rats. The results suggested that the ability of fenugreek saponin to inhibit AD due to increase AChE inhibition activity might be attributed to increase the antioxidants in this herb. Moreover, enhancement the apoptosis by fenugreek saponin could be attributed mainly to the regulation process of Bax, Bcl-2 and caspase-3 in the apoptotic pathway and not by generation of ROS in the brain cells of the AD-induced rats.

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