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ARTICLES

Full Length Research Paper

Phytochemical Analysis and Evaluation of Analgesic and Anti-inflammatory Properties of *Xanthoxylum fraxineum*

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Purpose of this study was to standardize the drug *Xanthoxylum fraxineum* by performing chemical analysis including FTIR and to evaluate its analgesic and anti-inflammatory properties as the drug has folkloric uses as an anti- inflammatory, antispasmodic and analgesic agent. Chemical analysis revealed presence of flavonoids, alkaloids, tannins and phytosterols. Mice were employed to determine the analgesic activity by inducing writhes with acetic acid and licking by formalin injection. Both methods revealed good analgesic activity. Drug was evaluated at 300 and 500 mg/kg doses and the analgesic effects were compared with that of the standard that is, aspirin 300 mg/kg. Both doses produced significant analgesic activity at $P<0.05$. Analgesia caused by the drug was higher than that caused by the standard. Moderate anti-inflammatory activity was also observed during formalin test. The results were in consistence with the folkloric claims made for this drug suggesting that the drug can be used for the claimed purposes. However, identification, safety and mechanism of action of its active constituents should be established to compare it with already available analgesics.

Key words: Standardize, analgesic, anti-inflammatory, Fourier transform infra-red.

INTRODUCTION

Xanthoxylum fraxineum Mill is native to northern and eastern America and Canada. Genus *Xanthoxylum* consists of two hundred and fifty species either shrubs or trees belonging to family Rutaceae. These species are indigenous to temperate, warm and subtropical areas. Their generic name is due to their yellow heart heartwood (Grant et al., 2001). The native North Americans used *X.*

fraxineum for the treatment of fever, cough, rheumatism and gonorrhoea (Erichsen-Brown, 1979). The plant is commonly known as Toothache Tree because its bark is chewed to relieve toothache (Felter and Lloyd, 1983). Bark is also used in the form of a tincture or infusion for problems like renal calculi, heart troubles, dyspepsia, dysentery, neuralgia and rheumatic conditions (Foster

and Duke, 2000; Grieve, 1931). Fruit which is a berry is thought to be a good stimulant and tonic. It is used for various chest and throat problems (Erichsen-Brown, 1979). Fruits also have powerful anti-spasmodic, anti-rheumatic and diuretic properties (Foster and Duke, 2000; Grieve, 1931). Significant anti-fungal activity by extracts of different parts of the plant has also been reported. Extracts of leaves and fruits showed greater antifungal activity than that of stem and roots (Bafi-Yeboa et al., 2005). Decoction made by roots of the plant is used to cure throat inflammation and to increase sweating (Erichsen-Brown, 1979).

Present study was carried out to standardize the drug *X. fraxineum* by performing chemical analysis and also by evaluating its anti-inflammatory and analgesic properties as the drug has a good reputation as an anti-inflammatory, antispasmodic and analgesic agent (Foster and Duke, 2000; Grieve, 1931). In homoeopathy, it is used for the treatment of paralysis, rheumatic affections, painful hemorrhages, neurasthenia, neuralgia etc. (Boericke, 1906).

MATERIALS AND METHODS

The drug *X. fraxineum (*mother tincture), Lot No.1010509, manufactured by Willmar Schwabe, Germany was procured from the local market in Karachi, Pakistan. The drug was dried by rotary evaporator to obtain a dark solid residue.

Experimental animals

The experiments were conducted on Swiss albino mice (25 to 30 g) of either sex. Animals were kept on standard diet and water *ad libitum*. Animals were allowed to get used to the environment before carrying out experiments. Four groups each consisting of 6 mice, were formed. First group, used as control was administered only vehicle. Second and third groups were given the drug (300 and 500 mg/kg body weight respectively). The fourth group was treated with the standard drug that is, aspirin 300 mg/kg body weight. Prior to experiments, permission was sought from the Ethical Committee Research Institute of Pharmaceutical Sciences, (Reference number: FAM/13/XF University of Karachi and the animals were disposed of after experiments in accordance to the standard procedure.

Chemical screening of crude extract of *X. fraxineum*

The phytochemical screening of the extract was performed by using different chemical tests. Fourier transform infra-red (FT-IR) spectrophotometric analysis was performed by using FT-IR Spectrophotometer: Thermo Electron Corporation, Nicolet Avatar 330 FT-IR, USA. Following chemical tests were performed for the identification of main constituents present in the alcoholic extracts of crude drugs. The precipitates / colour produced in these reactions were noted.

Test for alkaloids with Mayer's reagent

Two milliliter of Mayer's reagent was added to the extract and colour of the product was recorded after comparison with blank (Brain and Turner, 1975; Purohit, 2007). Presence of alkaloids is indicated by formation of yellow cream precipitates.

Test for alkaloids, Dragendorff's reagent

2.5 ml of the extract was shaken with 2 ml of Dragendorff's reagent in a test tube. The test tubes were agitated and colour of the product was recorded after comparison with blank (Brain and Turner, 1975; Purohit, 2007). Formation of orange red precipitates indicates alkaloids.

Test for alkaloids with Wagner's reagent

The extract, approximately 10 ml was taken in a test tube and 5 ml of Wagner's reagent was added. Colour of product was recorded (Gutal, 2011; Purohit, 2007). Formation of red to reddish brown precipitates is indicative of the presence of alkaloids.

Test for alkaloids with Hager's reagent

2.5 ml of the extract was taken in a test tube and 2 ml of Hager's reagent test solution was added in it. The test tubes were shaken and colour of the product was recorded after comparison with blank (Gutal, 2011; Purohit, 2007). Yellow precipitates indicate the presence of alkaloids.

Test for reducing sugar with Fehling's reagent

2.5 ml of extract was shaken and heated in a test tube with Fehling's reagent. Colour of the product was recorded after comparison with blank (Gutal, 2011; Sharma et al., 2013). Red to brown ppt. shows the presence of reducing sugars.

Test for carbohydrates with Molisch's reagent

Take 2.5 ml of extract in a test tube and add few drops of Molisch's reagent and then few drops of concentrated H_2SO_4 along the sides of test tube (Gutal, 2011; Sharma et al., 2013). Change of colour was noted. Formation of brown ring indicates carbohydrates.

Test for amino acids/protein with Ninhydrin reagent

2.5 ml of the extract was taken in a test tube and 2 ml of Ninhydrin reagent was added in it. The mixture was then heated on a water bath (Gutal, 2011; Koster et al., 1959; Purohit, 2007). The colour of the product was noted. Blue colouration indicates presence of amino acids or proteins.

Test for lignins with Phloroglucinol reagent

5 ml of the extract was taken in a test tube and concentrated by heating it on a water bath. Few drops of concentrated hydrochloric acid were added in it. The mixture was then cooled and 5 ml of phloroglucinol test solution was added to it. Colour of the resultant product was recorded (Gutal, 2011). Red violet colour is a positive indication of lignins.

Test for lignins with Safranin reagent

2.5 ml of the extract was taken in a test tube and concentrated by

heating it on a water bath. The concentrated extract was cooled and 2 ml of safranin test solution was added to it. Colour of the resultant product was recorded (Gutal, 2011). Red colour indicates lignins.

Test for flavonoid with Lead acetate

Test solution (2 ml) was taken in a test tube and few drops of lead acetate solution were added to it and observed for yellow colored precipitate (Koster et al., 1959; Sharma et al., 2013).

Froth test for Saponins

A small quantity of the extract was shaken with water. Formation of foam indicates the presence of saponins (Shah and Seth, 2010).

Test for tannins with ferric chloride test solution

5 ml of the extract was taken in a test tube and concentrated it to about 2.5 ml by heating it on a water bath. The concentrated extract was cooled and 2 ml of ferric chloride test solution was then added to it (Gutal, 2011; Koster et al., 1959). Green or bluish black colour indicates the presence of tannins.

Test for tannins with gelatin test solution

5 ml of the extract was taken in a test tube and concentrated it to about 2.5 ml by heating it on a water bath. The concentrated extract was cooled and 2 ml of 1% gelatin test solution was then added to it (Gutal, 2011; Koster et al., 1959; Sharma et al., 2013). White precipitates are formed in case of tannins.

Libermann Burchard's test for phytosterols

Alcoholic extract was dried and extracted with chloroform. The filtrate was treated with few drops of acetic anhydride followed by concentrated H_2SO_4 from side walls of the test tube. Formation of brown ring at the junction indicates the presence of phytosterols (Brain and Turner, 1975).

Analgesic activity

By writing method

The tests were performed according to the modified method of Hunskaar and Hole (1987). Mice were used as the test animals in this method. According to this method writhes were induced by intra-peritoneal administration of 1% acetic acid solution (10 ml/kg body weight). Thirty minutes prior to administration of acetic acid, the animals were treated orally with the test substance. Number of writhes was counted for 30 min immediately after acetic acid administration. A reduction in the number of writhes as compared to the control animals was considered as evidence for the presence of analgesia and expressed as percent inhibition of writhing. Mice were divided into 4 groups of 6 animals each (Group-A for control, Group-B and Group-C for 300 and 500 mg/kg oral doses of crude extract respectively, and Group-D for standard). Each group comprised 6 animals, weighing 25 to 30 g. Acetyl salicylic acid (aspirin) as 300 mg/kg orally was used as the reference compound. The crude drug and the acetyl salicylic acid were diluted in distilled water and administered orally. The control animals were treated orally with the same volume of saline as the crude extract.

Formalin test

Swiss albino mice (25 to 30 g) were divided into 4 groups of 6 animals each. 20 µl of 2% formalin was injected in the right hind paw and the left hind paw was injected with an equal volume of normal saline. Two distinct phases of intensive licking and biting of right hind paw were observed during 0 to 5 min (early phase) and 15 to 30 min (late phase) after formalin injection. These phases were scored separately for studying drug effect. Vehicle and the drug were administered orally 30 minutes before formalin injection (Hunskaar and Hole, 1987; Rathi, 2003).

Anti-inflammatory activity

Formalin test in mice (Vernier caliper method)

Mice were divided into four groups each consisting of 6 mice. First group served as control and received only vehicle. Second and third groups were given crude extracts (300 and 500 mg/kg body weight). The fourth group was treated with the standard drug that is, aspirin 300 mg/kg body weight. Inflammation was induced in the left hind paw by injecting 20 µl of 2% formalin into the left hind paw, 30 to 40 min after administering the oral doses of crude extracts and aspirin. The induced edema due to inflammation in the plantar tissue was measured as increase in the size of the paw after 30 min of formalin injection by using a Vernier Caliper in millimeters. This increase in the paw volume was noted in the control, treated and the standard groups up to 4 h. Percentage inhibition in the edema was calculated as:

% inhibition of edema= (Vc-Vt)/Vc X 100

Where Vc and Vt are the mean paw volumes of the control and the treated mice respectively (Rana, 2008). The experimental data were calculated as ± S.E.M., evaluated by student t-Test. Values of P≤0.05 were considered statistically significant (Posten 1978).

RESULTS

The drug was standardized using FTIR and performing phytochemical analysis. The results obtained are shown in Table 1 and Figure 1. The standardization data can be used in the future for the identification of this plant. Phytochemical screening using different reagents revealed presence of different compounds present in the drug as mentioned in Table 2.

The drug produced very significant dose dependent analgesic activity during acetic acid induced writhing method. Drug was evaluated at 300 and 500 mg/kg doses and the analgesic effects were compared with that of standard (aspirin 300 mg). Both doses produced very significant activity in comparison to the standard. In the 1st phase (0 to15 min), the lower dose (300 mg/kg) produced 50% analgesia in comparison to 36.6% analgesia produced by the standard. In the 2nd phase (15-30 minutes), it caused even greater inhibition of pain that is, 72.7% in comparison to 38.6% by the standard (Table 3). The higher dose (500 mg/kg) produced 64 and 80% analgesia in the 1st and 2nd phase respectively.

Almost similar analgesic activity was observed in the formalin test (Table 4). Reduction in licking time indicated dose dependent anti-nociceptive effects in both

Table 1. FTIR Peak cm^{-1}, *X. fraxineum.*

No. of peak	cm^{-1}	Functional group
1	2921	CH of aldehyde
2	2843	C-H stretch
3	1695	C=O
4	1638	C=C (aromatic)
5	1437	C=C (aromatic)
6	1245	C=O stretch
7	972, 910, 804, 702	C-H (aromatic) out of plane bend.
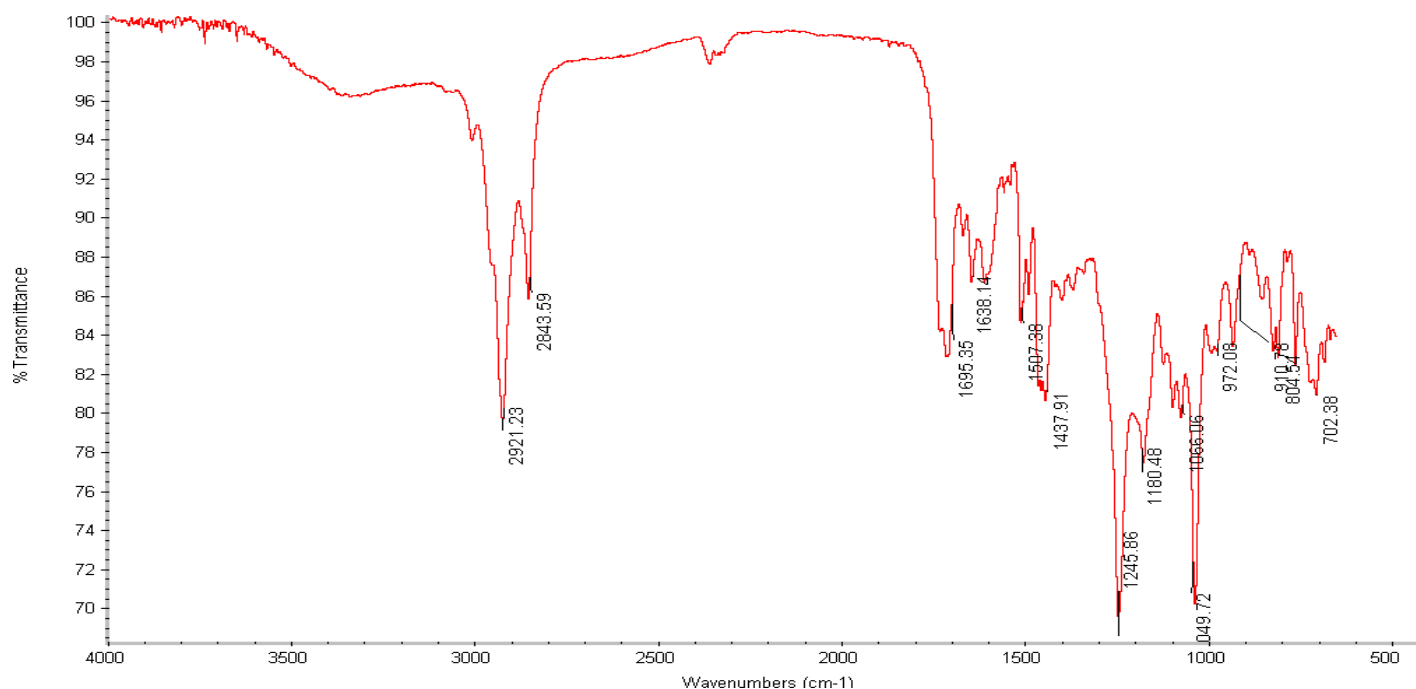
Figure 1. FTIR of *X. fraxineum.*

Table 2. Phytochemical Analysis of *X. fraxineum.*

Phyto-constituents	Result
Alkaloids	+
Carbohydrates	-
Amino acids	-
Protein	-
Flavonoids	+
Tannins	+
Phenolic compounds	+
Phytosterols	+

neurogenic and inflammatory phases. Anti -nociceptive effects were more pronounced in the 2nd phase (15 to 30 min). The drug also showed moderate dose-dependent anti-inflammatory effects during formalin induced edema test on the hind paw of mice. Results are shown in Table 5.

Table 3. Analgesic activity of *X. fraxineum* in mice by acetic acid induced writhing method.

Treatment	Dose mg/kg orally	Mean no. of writhes ± SEM 0 to 15 min	% Inhibition	Mean no. of writhes ± SEM 15 to 30 min	% Inhibition
Control	0.5 ml saline	30±0.77	-	36.66±0.71	-
Xanthoxylum fraxineum	300 mg/kg orally	15±1.2	50*	10±1	72.7**
	500 mg/kg orally	10.83±1.8	64**	7.33±1.3	80**
Aspirin	300 mg/kg orally	19±2.03	36.66	22.5±0.76	38.6

Results are shown as Mean±SEM, *Significant at $P<0.05$; **Highly significant at $p<0.01$.

Table 4. Analgesic activity of *X. fraxineum,* formalin test.

Treatment	Dose mg/kg orally	Licking time seconds ±SEM 1st Phase (0 to 5 min)	% Inhibition	Licking time seconds ±SEM 2nd Phase (15 to 30 min)	% Inhibition
Control	0.5 ml saline.	47±0.427	_	30±1.8	-
Xanthoxylum fraxineum	300 mg/kg orally	26±1.32	44.5*	5±1.4	83.3**
	500 mg/kg orally	11±1.75	76.5**	2±0.41	93.3**
Aspirin	300 mg/kg orally	35±0.76	25.5	18±0.8	40*

Results are shown as Mean ± SEM, *Significant at $p<0.05$; **Highly significant at $p<0.01$.

Table 5. Anti-inflammatory activity of *X. fraxineum* by formalin edema test.

Treatment	Dose mg/kg	Mean paw volume increase after 1 h ±S.E.M (mm)	% Inhibition	Mean Paw volume increase after 2 h ±S.E.M (mm)	% Inhibition	Mean Paw volume increase after 3 h ±S.E.M.(mm)	% Inhibition	Mean Paw volume increase after 4 h ±S.E.M (mm)	% inhibition
Control	0.5 ml saline	4.04±0.05	-	4±0.05		3.89±0.05	-	3.82±0.05	-
Xanthoxylum fraxineum	300 mg/kg orally	3.23±0.1	20	3±0.04	25	2.84±0.19	27	2.87±0.19	25
	500 mg/kg orally	3.11±0.07	23	2.6±0.1	35	2.72±0.09	30	2.79±0.1	27
Aspirin	300 mg/kg orally	2.83±0.03	30	2.6±0.17	35	2.41±0.3	38	2.3±0.03	40*

Results are shown as Mean±SEM, *Significant at $p<0.05$; **Highly significant at $p<0.01$.

DISCUSSION

Inflammation causes redness, swelling and pain. Although inflammation is an important part of body's defense against infective organisms but excessive and prolonged inflammation may damage different tissues and organs resulting in great pain and discomfort. During inflammation, activated macrophages and monocytes produce large quantities of cytokines like TNF-α, IL-6, IL-1β, PGs and reactive oxygen species (Janero, 1990). Different plant drugs have been used traditionally to treat pain and inflammatory conditions. Based on folkloric uses, we investigated the analgesic and anti-inflammatory properties of *X. fraxineum* (Mother Tincture) in animal model by using two methods, acetic acid induced writhing method and formalin method.

Acetic acid causes release of inflammatory substances like PGs, serotonin and cytokines which results in painful sensation (Manjavachi et al., 2010). NSAIDs and centrally acting analgesics like morphine can block this nociceptive effect. In the present study we studied and compared the analgesic and anti-inflammatory effects of *X. fraxineum* (300 and 500 mg/kg) with control and the standard, aspirin (300 mg/kg). Both doses produced very significant analgesic effects during acetic acid induced writhing test and formalin test.

Formalin test consists of two phases, neurogenic nociceptive phase and inflammatory nociceptive phase. Centrally acting drugs inhibit both these phases while NSAIDs block only the second phase which occurs from 15 to 30 min after injecting formalin (Reeve and Dickenson, 1995). According to results, *X. fraxineum* inhibited both phases, predominantly the second phase. Aspirin, being a non-steroidal anti-inflammatory drug inhibited the second phase mainly. Good peripheral analgesic activity was noted during acetic acid induced writhing method also.

The drug showed moderate anti-inflammatory effects in formalin induced edema which could be attributed to the flavonoid content of the drug detected during phytochemical analysis. Flavonoids present in plants have been found to have anti-inflammatory activity by decreasing reactive oxygen species and inflammatory cytokines (Jin et al., 2010; Serafini, Peluso and Raguzzini, 2010).

Conclusion

During this study the preliminary phytochemical screening indicated the presence of alkaloids, flavonoids, tannins and phytosterols. Results of the present study validated the folkloric use of *X. fraxineum* as an analgesic to treat various types of pain like toothache, nerve pain, rheumatic conditions, renal colic etc. Moderate anti-inflammatory activity was also noted during the experiments.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vivo hypoglycemic effect of ethanol extract and its fractions of *Rhaphidophora glauca* (Wall.) Schott leaves with area under curve (AUC) during oral glucose tolerance test (OGTT)

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This study was carried out to investigate the hypoglycemic effects of ethanol extract and its chloroform and ethyl acetate fractions of *Rhaphidophora glauca* (Wall.) Schottleaves in normal and glucose induced hyperglycemic mice (*in vivo*). Area under curve (AUC) was also calculated during oral glucose tolerance test (OGTT). Ethanol extract and its fractions of *R. glauca* leaves at 400 and 800 mg/kg doses significantly (P < 0.05-0.001) reduced fasting glucose level in normal mice as compared to standard drug glibenclamide (5 mg/kg). Ethanol extract of *R. glauca* (EERG) at 800 mg/kg dose showed the highest hypoglycemic effect among the extract and fractions and it decreased 25.13% of the blood glucose level after 2 h of administration in normal mice, where glibenclamide decreased to 49.30%. In oral glucose tolerance test, at 400 and 800 mg/kg dose of extract and fractions significantly reduced blood glucose level (P < 0.05) at 30 min, but at 60 and 90 min, blood glucose level reduction is not all properly significant as compared to the control. At 120 min, both doses of extract and fractions significantly (P < 0.01) reduced blood glucose level. Whereas glibenclamide (5 mg/kg) significantly reduced glucose level at every hour after administration. EERG at 800 mg/kg dose showed the highest hypoglycemic effect among the extract and fractions and it decreased to 13.28% of blood glucose level after 2 h of administration in glucose induced mice, where glibenclamide decreased to 41.18%. AUC during OGTT of extract and fractions are at the range of 12.713 to 13.188 h.mmol/L., and 14.573 and 9.835 h.mmol/L for control and glibenclamide, respectively. These findings suggest that the plant may be a potential source for the development of new oral hypoglycemic agent.

Key words: *Rhaphidophora glauca*, hypoglycemic activity, area under curve (AUC), ethanol extract, fractions, glucose induced.

INTRODUCTION

Several medicinal plants have been reported to be useful in treating diabetes globally and have been used empirically in antidiabetic and antihyperglycemic cures.

Antihyperglycemic activity of the plants is principally due to their ability to reinstate the function of pancreatic tissues by causing an increase in insulin production or

inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin reliant processes. More than 400 plant species having hypoglycemic activity have been accessible in literature, though, searching for new antihyperglycemic drugs from natural plants is still striking because they contain substances which make obvious alternative and safe property on diabetes mellitus (Haddad et al., 2012; Patel et al., 2012a, b). Most of the plants contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids, etc., that are habitually implicated as having hypoglycemic effect (Salihu et al., 2015).

Diabetes is a metabolic disorder of sugar, fat and protein, influencing a substantial number of populaces on the planet (Matsumoto et al., 2015). Diabetes mellitus is not a solitary disorder, rather it is a gathering of metabolic disorder described by ceaseless hyperglycemia, coming about because of deformities in insulin discharge, insulin activity, or both. Expanded thirst increased urinary yield, ketonemia and ketonuriaare, the basic side effects of diabetes mellitus which occur due to the abnormalities in carbohydrate, fat, and protein metabolism. At the point when ketones body is available in the blood and urine, it is called ketoacidosis; hence, legitimate treatment ought to be taken quickly, else it can prompts other diabetic complications (Low et al., 2015). Diabetes mellitus has brought about critical grimness and mortality because of microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (heart assault, stroke and fringe vascular sickness) complexities (Singh et al., 2015).

Diabetes is basically ascribed to the fast ascent in undesirable way of life, urbanization and maturing. Hyperglycemia which is the primary side effect of diabetes mellitus produces reactive oxygen species (ROS) which cause lipid peroxidation and layer harm. ROS assumes an imperative part in the improvement of auxiliary complications in diabetes mellitus such as cataract, neuropathy and nephropathy. Antioxidants protect β-cells from oxidation by inhibiting the peroxidation chain reaction and along these lines they assume an important part in the diabetes. Plants containing regular cancer prevention agents, for example, tannins, flavonoids, vitamin C and E can safeguard β-cell work and anticipate diabetes prompted ROS development. Polyphenols, which are ordered into numerous gatherings, for example, flavonoids, tannins and stilbenes, have been known as health-beneficial properties, which incorporate free radical searching, restraint of hydrolytic and oxidative proteins, antiinflammatory activity and hypoglycemic potentiality (Patel et al., 2011; Roy et al., 2015). Aldose reductase as

a key catalyst, catalyze the diminishment of glucose to sorbitol and is related in the perpetual complications of diabetes, such as peripheral neuropathy and retinopathy. Utilization of aldose reductase inhibitors and α-glucosidase inhibitors has been reported for the treatment of diabetic complications (Jung et al., 2011).

Oral hypoglycemic medications can bring about different adverse reactions, including hypoglycemia, weight gain, fluid retention, cardiac failure, and gastrointestinal side effects (El-Refaei et al., 2014; Chiniwala and Jabbour, 2011; Kar et al., 2015). Specifically, hypoglycemia is connected with cardiovascular incidents and cognitive dysfunction, and it is known that hypoglycemic episodes can lead to falls and cracks (Geier et al., 2014). Elderly patients are more inclined to create hypoglycemia than more youthful patients when treated with different medications, and in addition quickly after release from healing center, if they have renal failure, and if their diet is poor and they are also less likely to detect the onset of hypoglycemia (Kamei et al., 2015; Penfornis et al., 2015). These distinctions make treatment of diabetes more troublesome in elderly patients, so that cautious training and drug selection are needed.

Rhaphidophora glauca (Wall.) Schott (family: Araceae), is an aroid liane native to the subtropical and warm temperate regions of the eastern Himalaya, which is also distributed in Nepal through North East India to Bangladesh and Myanmar and North Thailand to North Laos and Vietnam. Leaves of *R. glauca* have activities like antiarthritic, membrane stabilizing, α-amylase inhibitory and anthelmintic (Hossain et al., 2015; Kabir et al., 2015).

This study intends to explore the ethanol extract and its chloroform and ethyl acetate fractions of *R. glauca* for its hypoglycemic activity in normal and glucose induced hyperglycemic mice.

MATERIALS AND METHODS

Plant collection and identification

Leaves of *R. glauca* were collected from Alutila, khagrachari, Chittagong, Bangladesh in the month of September 2014 at the last time of its flowering. It was authenticated by reputed plant taxonomist, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh. A specimen of the plant has been preserved in the national herbarium with the Accession No.30145.

Extraction and fractionations

Leaves were cleaned with fresh distilled water and dried for a

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period of 10 days under shade and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The powdered of leaves (900 g) of *R. glauca* was soaked in 1.3 L ethanol for 7 days with occasional shaking and stirring and filtered through a cotton plug followed by Whatman filter paper number-1. The extract was then concentrated by using a rotary evaporator at reduced temperature and pressure. A portion (50 g) of the concentrated ethanol extract (EERG) was fractioned by the modified Kupchan partitioning method (Islam et al., 2010; Bulbul et al., 2011) into chloroform (CHFRG, 13 g) and ethyl acetate (EAFRG, 12 g).

Chemicals and reagents

All the chemicals and reagents were of analytical grade. Ethanol, chloroform and ethyl acetate were purchased from Merck, Germany. Normal saline solution was purchased from Beximco Infusion Ltd. Rapid View™ (Blood glucose monitoring system, Model: BIO-M1, BIOUSA Inc, California, USA) with strips were purchased from Andorkilla, Chittagong. Glucose was purchased from local scientific market, Chowkbazar, Chittagong. Glibenclamide was obtained from Square Pharmaceutical Ltd., Bangladesh.

Animals and experimental set-up

Swiss albino mice, weighing about 28 to 35 g, were collected from Jahangir Nagar University, Savar, Bangladesh. The animals were furnished with standard lab nourishment and refined water *ad libitum* and maintained at natural regular day-night cycle having proper ventilation in the room. All the experiments were conducted in an isolated and noiseless condition. Then, the study protocol was approved by the P&D Committee, Department of Pharmacy, International Islamic University Chittagong, Bangladesh. The animals were acclimatized to laboratory condition for 7 days prior to experimentation.

Acute toxicity study

For acute toxicity study, forty Swiss albino female mice were used. According to the method of Walum (1998), mice were divided into four groups of five animals each. Different doses (1000, 2000, 3000 and 4000 mg/kg) of ethanol extract and its chloroform and ethyl acetate fraction of *R. glauca* leaves were administered by stomach tube. Then, the animals were observed for general toxicity signs.

Experimental protocol for *in vivo* hypoglycemic activity

Hypoglycemic effect in normal mice

Mice were kept fasting overnight with free access to water. Group I was treated as control group, Group II was treated with glibenclamide (5 mg/kg body weight), Groups III to VIII were treated with ethanol extract and its chloroform and ethyl acetate fraction of *R. glauca* leaves at 400 and 800 mg/kg body weight, respectively. Before administration of the drug, extract and fractions solutions fasting blood glucose levels were estimated by glucose oxidase method (Barham and Trinder, 1972). Then blood glucose levels were again estimated after 2 h of administration of drug and extract solutions. Glucose levels were measured by Rapid View™ (Blood glucose monitoring system, Model: BIO-M1, BIOUSA Inc, California, USA). The maximum hypoglycemic effect of glibenclamide was found after 2 h of its administration. Percent decrease of blood glucose level after 2 h was measured using the

following equation:

$$\text{Decrease (\%)} = \frac{GL_{before} - GL_{after}}{GL_{before}} \times 100$$

GL_{before}= Blood glucose level before drug or extract and fractions administration, GL_{after}=Blood glucose level after drug or extract and fractions administration.

Hypoglycemic effect in glucose induced hyperglycemic mice (OGTT)

Oral glucose tolerance test (OGTT) was performed according to the standard method (Xia et al., 2013) with minor modification. Group I was treated as normal control group, Group II was treated with glibenclamide (5 mg/kg body weight), Groups III to VIII were treated with ethanol extract and its chloroform and ethyl acetate fraction of *R. glauca* leaves at 400 and 800 mg/kg body weight, respectively. Glucose solution (1 g/kg body weight) was administered at first. Then, drug and extract solutions were administered to the glucose fed. Serum glucose level of blood sample from tail vein was estimated using glucometer at 0, 30, 60, 90 and 120 min. Areas under the curves (AUC) for OGTT were calculated to evaluate glucose tolerance (Purves, 1992). Percent decrease of blood glucose level after 120 min was measured using the following equation:

$$\text{Decrease (\%)} = \frac{GL_{0\ min} - GL_{120\ min}}{GL_{0\ min}} \times 100$$

$GL_{0\ min}$= Blood glucose level at 0 min, $GL_{120\ min}$=Blood glucose level at 120 min

Statistical analysis

The results were expressed as the mean±standard error of mean (SEM). The results were statistically analyzed using repeated measures analysis of variance with Dunnett's and Bonferroni multiple comparison when compared with control in OGTT. Paired t test was performed to show significant variation between before and after blood glucose level. $P<0.05$, $P<0.01$ and $P<0.001$ were considered as statistically significant. Statistical programs were used GRAPHPAD PRISM® (version 6.00; GraphPadSoftware Inc., San Diego, CA, USA).

RESULTS

Acute toxicity study

None of the animals showed behavioral, neurological or physical changes characterized by symptoms such as reduced motor activity, restlessness, convulsions, coma, diarrhea and lacrimation at the limit dose of 4000 mg/kg of ethanol extract and its chloroform and ethyl acetate fraction of *R. glauca* during the observation period. In addition, no mortality was observed at the test dose. Thus, the median lethal dose (LD_{50}) of the plant extracts was found to be greater than 4000 mg/kg.

Table 1. Effect of *R. glauca* leaves extract and fractions on fasting blood glucose level (mmol/L) in normal mice.

Group	Dose (oral)	Before administration	After administration	Decrease (%)
Control (1% Tween)	10 ml/kg	5.49±0.134	6.29±0.181	-
Glibenclamide	5 mg/kg	5.74±0.167[a]	2.91±0.164[c,β,z]	49.30±0.75
EERG	400 mg/kg	5.06±0.092[a]	4.32±0.086[a,α,z]	14.62±0.75
EERG	800 mg/kg	5.00±0.164[a]	3.74±0.103[c,β,z]	25.13±0.69
CHFRG	400 mg/kg	5.20±0.226[a]	4.58±0.235[b,z]	12.04±0.77
CHFRG	800 mg/kg	4.98±0.201[a]	3.86±0.186[a,β,z]	22.58±0.90
EAFRG	400 mg/kg	5.22±0.218[a]	4.66±0.227[b,y,z]	10.82±0.98
EAFRG	800 mg/kg	4.88±0.188[a]	3.88±0.137[b,β,z]	20.44±0.72

Values are presented in mean±SEM (n=5). EERG: Ethanol extract of *R. glauca*; CHFRG: Chloroform fraction of ethanol fraction; EAFRG: Ethyl acetate fraction of ethanol extract. (A) Values in same row with different superscripts are significantly different for [a]$P>0.05$, [b]$P<0.01$ and [c]$P<0.001$. Paired t-test was performed to analyze before and after relationship. (B) Values with different superscripts in same column are significantly different from control after the administration of standard and different doses of the extract and fractions for [α]$P>0.05$, [β]$P<0.01$ and [γ]$P<0.001$. One-way ANOVA followed by Dunnett's multiple comparison was performed to analyze this comparison. (C) Values with different superscripts ([x, y, z]) are significantly different from each other in the same column among standard and different doses of the extracts after administration. One-way ANOVA followed by Bonferroni multiple comparison was performed to analyze this inter relationship.
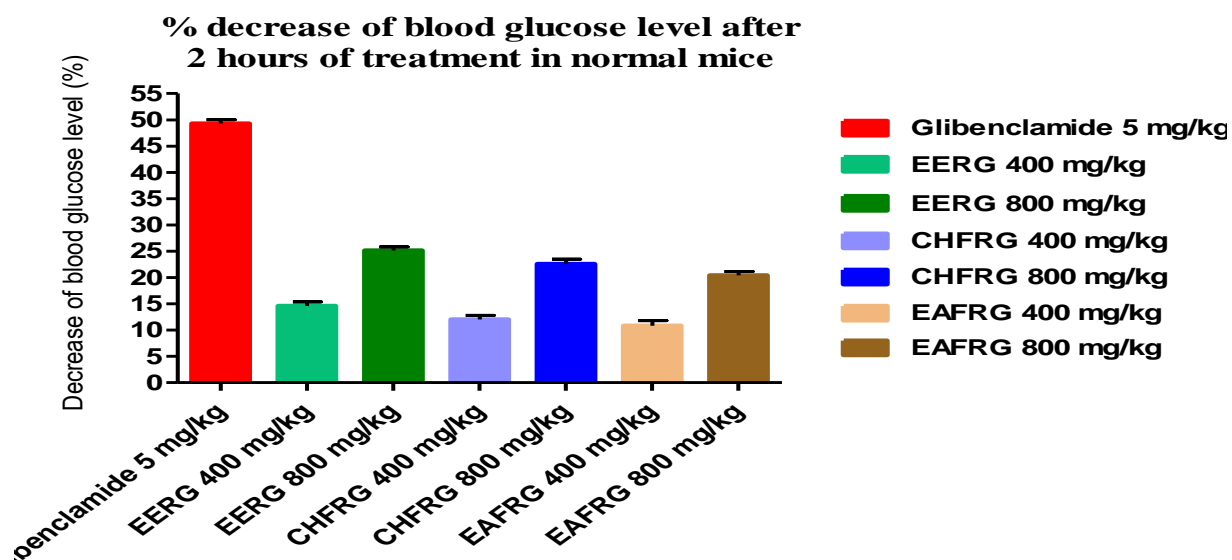
Figure 1. Percentage decrease of blood glucose level after treatment with drug, extract and fractions in normal mice.

Hypoglycemic effect in normal mice

Both doses of ethanol extract, fractions and glibenclamide significantly reduced fasting blood glucose level. Glibenclamide showed significant reduction at level of $P<0.01$. Dose of 400 and 800 mg/kg leaves ethanol extract of *R. glauca* and its fractions showed significant reduction at the level of $P<0.05$ and $P<0.001$, respectively. These results suggest that hypoglycemic activity of 800 mg/kg dose and glibenclamide has similar significant level. All results are presented in Table 1 and percentage of decrease of blood glucose level in normal mice after 2 h with different treatment are shown in Figure 1. EERG at the highest dose of 800 mg/kg decreased blood glucose level (25.13%) than other treatments (accept standard Glibenclamide).

Hypoglycemic effect in glucose induced hyperglycemic mice (OGTT)

Investigational induction of hyperglycemia resulted in increased glucose level in blood (comparing the result of control of 0 and 1 h) (Table 2). Both dose of leaves

Table 2. Effect of *R. glauca* leaves extract and fractions on glucose induced hyperglycemia (mmol/L) in normal mice.

Group	Dose	0 min	30 min	60 min	90 min	120 min	Decrease (%)	AUC
Control (1% Tween)	10 m l/kg	6.52±0.35	7.46±0.37	7.80±0.49	7.28±0.39	6.69±0.21	-	14.573
Glibenclamide	5 mg/kg	6.46±0.24	5.6±0.18[b,γ]	4.7±0.19[c,α]	4.24±0.17[c,γ]	3.8±0.15[c,γ]	41.18±0.89	9.835
EERG	400 mg/kg	5.38±0.22	7.98±0.33[a,β]	6.58±0.22[β]	5.76±0.27[a,β]	4.96±0.20[b,γ]	7.79±0.60	12.745
EERG	800 mg/kg	5.90±0.14	8.49±0.12[a,β]	6.04±0.33[b,α]	5.82±0.31[β]	5.12±0.32[b,γ]	13.28±0.84	12.930
CHFRG	400 mg/kg	5.48±0.21	8.16±0.17[a]	6.64±0.10[β]	5.70±0.16[a,β]	5.10±0.22[b,γ]	6.95±0.47	12.895
CHFRG	800 mg/kg	5.94±0.17	8.32±0.13[a,α]	6.32±0.23[b,β]	5.78±0.28[β]	5.21±0.19[b,γ]	12.35±0.59	12.998
EAFRG	400 mg/kg	5.26±0.18	8.02±0.09[a]	6.36±0.09[α]	5.96±0.15[a,α]	4.91±0.17[b,γ]	6.64±0.19	12.713
EAFRG	800 mg/kg	6.02±0.18	8.22±0.12[a]	6.57±0.17[b,α]	5.93±0.16[α]	5.29±0.19[b,γ]	12.08±0.62	13.188

Values are presented in mean±SEM (n=5). EERG: Ethanol extract of *R. glauca*; CHFRG: Chloroform fraction of ethanol fraction; EAFRG: Ethyl acetate fraction of ethanol extract. (A) Values with different superscripts in same column are significantly different from control at each specific hour after the administration of standard and different doses of the extract and fractions for [a]$P>0.05$, [b]$P<0.01$ and [c]$P<0.001$. One-way ANOVA followed by Dunnett's multiple comparison was performed to analyze this comparison. (B) Values with different superscripts ([α, β,γ]) are significantly different from each other in the same column among standard and different doses of the extract, fractions and standard. Bonferroni multiple comparison was performed.
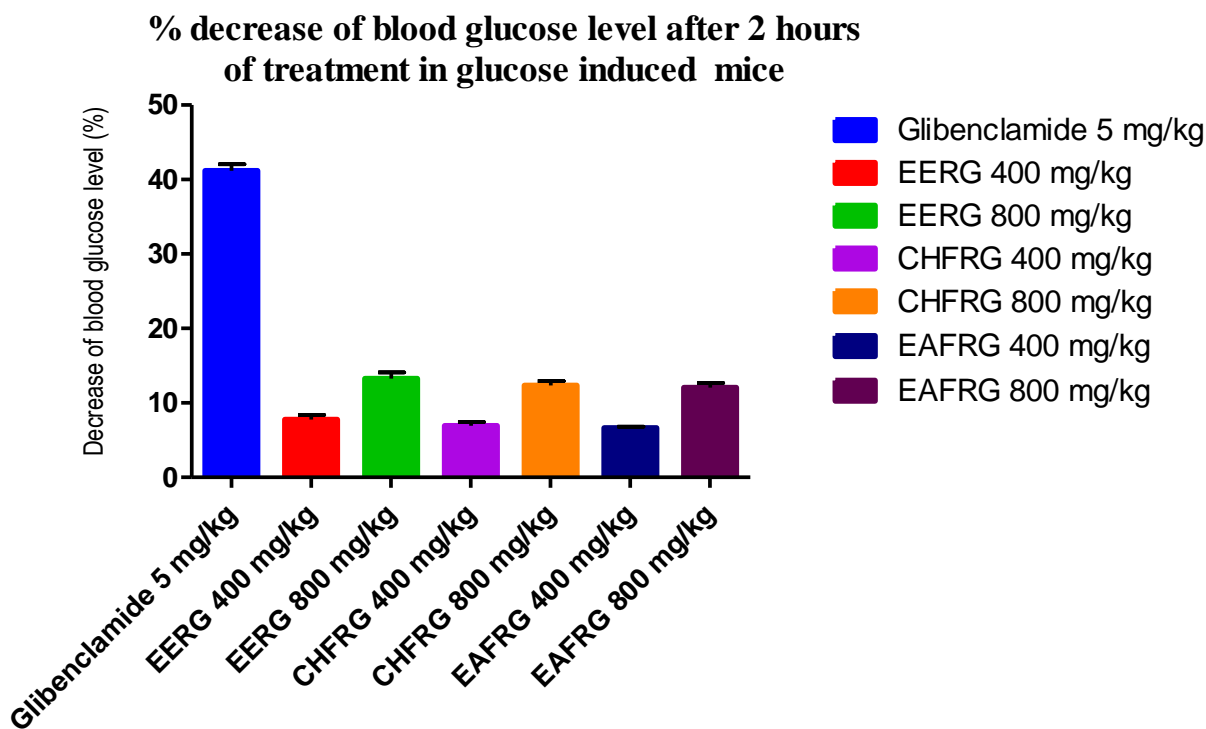
Figure 2. Percentage decrease of blood glucose level after treatment with drug, extract and fractions in glucose induced mice.

extract and its fractions did not manifest any significant reduction in 30 min after administration. Most significant reduction ($P<0.05$) was observed for 800 mg/kg dose of ethanol extract of *R. glauca* and its fractions at 120 min. At 120 min, this dose also showed significant reduction ($P<0.05$). Standard glibenclamide (5 mg/kg) showed significant reduction in 30, 60, 90 and 120 min. These findings suggest that evidently 800 mg/kg dose is more potent than 400 mg/kg dose. Time interaction with each specific hour in this experiment was also found significant ($P<0.05$ and 0.001). All results are presented in Table 2 and results of AUC of OGTT are also presented in Table 2. Percentage of decrease of blood glucose level in glucose induced mice after 2 h with different treatment are shown in Figure 2. EERG at the highest dose of 800 mg/kg decreased blood glucose level (13.28%) than

other treatments (accept standard Glibenclamide).

DISCUSSION

In this study, ethanol extract and its chloroform and ethyl acetate fractions of leaves of *R. glauca* exerted significant hypoglycemic activity in both fasting glucose level reduction in normal mice and oral glucose tolerance test in glucose induced hyperglycemic mice. Decrease of blood glucose level after 2 h of treatment is very well significant when compared with the control.

There are numerous pharmaceutical items which are accessible in current medicinal treatment have a long history of utilization as home grown cures, including ibuprofen, opium, digitalis and quinine. An expansive number of world's populace who live in creating nations cannot take the advantages of the present day pharmaceuticals as those are extremely expensive. More or less 25% of modern drugs used in the United States have been derived from plant origins (WHO, 2008). So, research on phytomedicine has got great momentum in recent years to find out noble pharmaceuticals.

Diabetes is a metabolic disease connected with host of difficulties, for example, intense and endless inconveniences. The intense intricacies may be activated by metabolic disorders including ketoacidosis and non-ketotic coma and infections, but these indications can be moderately very much controlled. Then again, the endless entanglements have a tendency to exacerbate as the diabetes advances. Chronic complications associated with diabetes include macroangiopathies, such as coronary artery disease and cerebrovascular disease, and microangiopathies, such as neuropathy, orthostatic hypotension, retinopathy and nephropathy (Chang et al., 2006; Takayuki et al., 2006; Gogoi et al., 2014; Toma et al., 2015).

In the normal mice, ethanol extract and all fractions reduced fasting blood glucose level in normal mice after the treatment. EERG at 800 mg/kg dose decrease 25.13% glucose level among extract and fractions, where standard drug glibenclamide decreased 49.30%. This is evident that this extract did not supply glucose in the blood due to its administration.

The OGTT is generally considered as more susceptible for the screening of impaired glycemia, because it detects changes in post-prandial glycemia that tend to precede changes in fasting glucose. All the current diagnostic criteria for diabetes depend on a threshold value imposed on a continuous distribution of blood glucose levels. Yet, the correct glycemic threshold that discriminates 'normal' from diabetic is not obvious. Though screening for undiagnosed type 2 diabetes remains a contentious issue, there is clear evidence that once it is diagnosed, complications can be prevented in many patients (Vamos et al., 2012; Hemmingsen et al., 2015). OGTT measures the body's ability to use glucose, the body's main source of energy. OGTT can be used to diagnose pre diabetes and diabetes. The ethanol extract and its fractions of leaves of *R. glauca* showed significant ability to reduce the elevated glucose level in normal mice as compared to the standard drug glibenclamide, where EERG showed the highest hypoglycemic activity in both examined model.

The OGTT determines the shape of the glucose curve based on the measurements at 0, 30, 60, 90 and 120 min. From the glucose curve, AUC was determined using Trapezoidal Rule. AUC during OGTT for screening hypoglycemic effect of extract and fractions are at the range of 12.713 to 13.188 h.mmol/L, and 14.573 and 9.835 h.mmol/L for control and glibenclamide, respectively.

These outcomes suggest that ethanol extract and its chloroform and ethyl acetate fractions of *R. glauca* leaves possess a hypoglycemic principle and can be useful for the treatment of diabetes. Further studies are warranted to isolate the active principle and to find out its accurate mechanism of action.

Conclusions

From the study, it was concluded that *R. glauca* may have hypoglycemic effect, but not sure about how this extracts and fractions can exert potent hypoglycemic activity. It is a logical inference that this plant may recover the metabolism of glucose and increase insulin secretion by stimulating beta cells. It is possible to propose that the bioactive compounds present in the leaves extract and its fractions may be responsible for versatile effects. Based on the literature search, this is the first study about hypoglycemic activity of *R. glauca*. That is why the exact mode of action is not determined yet. However, further co-ordinate and well-structured studies would be required to isolate the bioactive compounds and determine their underlying molecular mechanism of action on diabetes induced mice model. These findings suggest that the plant may be a potential source for the development of new oral hypoglycemic agent.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Efficacy and effectiveness of drug treatments in amyotrophic lateral sclerosis: A systematic review with meta-analysis

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The results of published studies with various neuroprotectors seeking to preserve motor neuron function and improve survival in amyotrophic lateral sclerosis patients have poor evidence in humans, although there are several studies in animal models with positive results. A systematic review and meta-analysis of studies on drug treatment options and survival times in animal models and patients with amyotrophic lateral sclerosis from March, 2009 to March, 2015 was conducted. Four hundred eighty-nine (489) articles were found, and from these, we selected 30 preclinical '*in vivo*' studies, 18 randomized controlled trials, and four systematic reviews. A meta-analysis confirmed the effectiveness of various drugs in improving the life span in preclinical trials, in particular, Resveratrol, which had a mean difference of 10.8 days (95% CI: 9.57 to 12.02), whereas no drug showed efficacy in clinical trials. The positive results of preclinical studies should be interpreted with caution because there is a mismatch between those results and the negative results in clinical trials.

Key words: Amyotrophic lateral sclerosis (ALS), motor neuron disease, drug, treatment.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease. It is more often sporadic and characterized by the progressive degeneration of both upper and lower motor neurons in the brain, brainstem and spinal cord (Gordon, 2013). Its incidence may vary between 1.2 and 4.0 per 100,000 individuals per year (Logroscino et al., 2010; Marin et al., 2009). It is more predominant in males (3.0 per 100,000 individuals per year, 95% CI 2.8 to 3.3) than females (2.4 per 100,000 individuals per year, 95% CI 2.2 to 2.6). Its onset occurs

between 58 and 63 years of age, and its incidence decreases considerably after the age of 80 (Chiò et al., 2013).

The few advances in knowledge about the mechanisms of development of ALS are primarily due to the understanding of familial forms, which correspond to 5 to 10% of cases (Ravits et al., 2013; Strong, 2010). The pathophysiology of the disease is still poorly understood, but it is believed that the disease's injury mechanisms involve both glial cells and neurons (Strong, 2010). The main known mechanisms are oxidative stress with damage to RNA species, mitochondrial dysfunction, impairment of axonal transport, glutamate excitotoxicity as a mechanism contributing to motor neuron injury, protein aggregation, endoplasmic reticulum stress, abnormal RNA processing, neuroinflammation, and excitability of peripheral axons (Mancuso, 2015).

Death occurs on average between 2 and 4 years after onset due to respiratory complications, but patients given multidisciplinary care and undergoing enteral nutrition and noninvasive ventilation have extended survival (Miller et al., 2009). A single drug, Riluzole, approved in 1996 by the Food and Drug Administration (FDA), slows the progression of the disease by approximately 2 to 4 months, but it does not prevent the disease's fatal outcome (Miller et al., 2009).

Studies on the use of various neuroprotectors seeking to preserve motor neuron function and reduce the toxic levels of glutamate have been giving any evidence of efficacy in humans, although their use has been fairly efficacious in experimental animal models (Orrell, 2010). Considering the need to identify new alternatives to treat ALS, the aim of this study was to investigate the efficacy and effectiveness of drug treatments in clinical and preclinical trials through a systematic review of the literature in the field.

MATERIAL AND METHODS

Strategies to search for and select studies

In May, 2015, we investigated primary preclinical *in vivo* studies, clinical trials and systematic reviews with subsequent meta-analyses published between March, 2009 and March, 2015 in the following electronic databases: Medline, Embase, Cochrane Library and Lilacs. The following Medical Subject Headings (MeSH) and Health Science Descriptors (HScDe) were used: 'Amyotrophic Lateral Sclerosis' OR 'Motor Neuron Disease' AND 'Treatment' AND 'drug' AND 'survival'. Two authors independently evaluated the titles and abstracts of all studies identified in the search in the aforementioned electronic databases based on the descriptors. The following inclusion criteria were adopted:

i) In clinical studies, including prospective randomized trials and meta-analytical systematic reviews, patients diagnosed with a motor neuron disease by means of anamnesis and electromyography according to the El Escorial and Awaji criteria (Costa et al, 2012);
ii) In preclinical studies, *'in vivo'* studies with assessment of survival compared to control group and studies of treatment after the onset

of weakness; and
iii) Studies based on the use of any drug to increase survival time compared to placebo or other treatments used by the control group.

The exclusion criteria were studies in which participants presented with respiratory failure or spinal muscular atrophy; studies in which the treatment was administered only prior to disease; or narrative reviews, letters, editorials, case reports, duplicate publications or those without objective data to be evaluated. Articles published in all languages were included. The studies that met the inclusion criteria were obtained in full. References were also considered, and communication with the authors was established in cases of doubt. Disagreements were resolved by consensus, and when this was impossible, there was subsequent analysis by two additional reviewers.

Data extraction

Data were obtained from each study using a review form with the following content: author, place where the work was conducted, year of publication, intervention, study design, number of participants, age, analysis by intention to treat, declaration of conflict of interest, evaluation by a research ethics committee, and animal species used if the study was preclinical. The following outcomes were assessed:

i) Comparison between two drugs and/or placebo;
ii) Analysis of mean survival and absolute days of survival. In preclinical studies, the authors converted the survival in days, when it was clearly reported as animals alive;
iii) Mean duration of the disease until the start of intervention;
iv) Alteration of the Revised ALS Functional Rating Scale - ALSFRS-R (The Amyotrophic Lateral Sclerosis Functional Rating Scale, 1996) between the start and end of the study;
v) Incidence of reactions and adverse effects of proposed treatments.

Assessing the quality of the studies

Quality was assessed by two independent authors, and in cases of disagreement, the situation was resolved by consensus among all authors. The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) model (Guyatt et al., 2008) was used for primary studies and/or the Assessment of Multiple Systematic Reviews (AMSTAR) criteria were used for systematic reviews (Kung et al., 2010). The following data were observed in the studies:

Methods: Research question, treatment sequence, allocation confidentiality, post-intervention follow-up, blinded outcome assessment, primary clinical outcome measures, location of study, protection against contamination, calculation of statistical power, sample representativeness, conflict of interest, and ethical aspects.
Participants: Inclusion criteria, exclusion criteria, age, gender, disease severity, and disease variants.
Interventions: Medications and doses or procedures, follow-up time, and method for monitoring disease progression.
Outcomes assessed in the review: Disease duration before intervention, survival time, and/or alteration of the ALSFRS-R.

The results of the primary outcomes were obtained based on the intention-to-treat principle: for each dichotomous outcome, the total number of participants in each group divided the number of events; for continuous outcomes, the following variables were calculated:
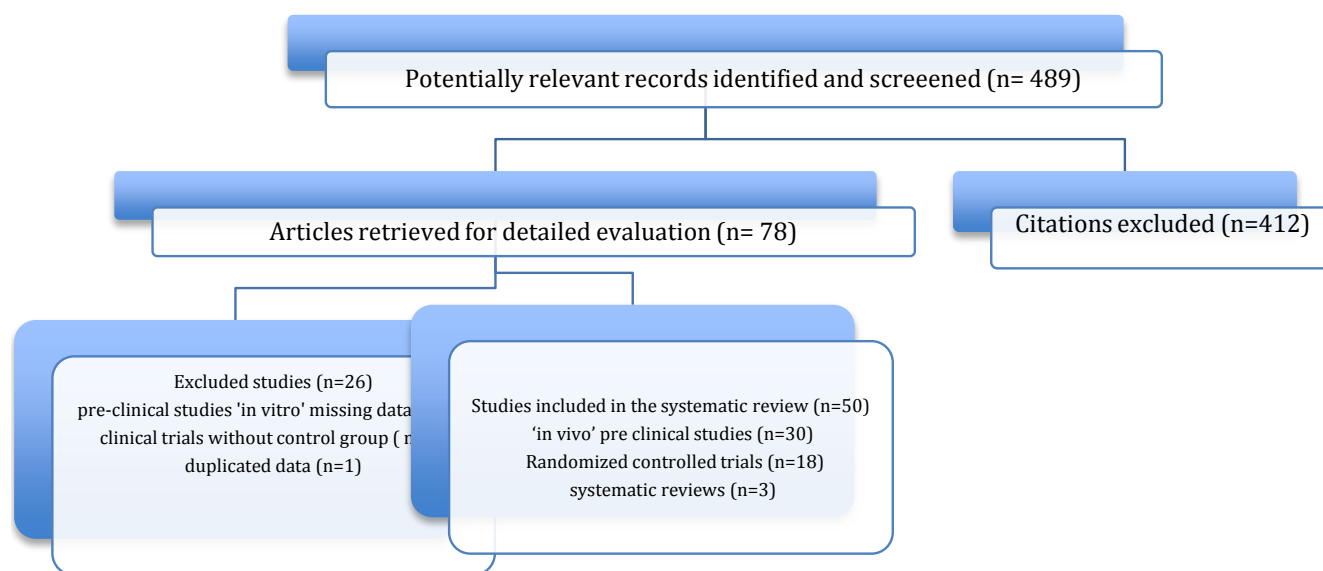
Figure 1. Systematic review flowchart.

mean, standard deviation and number of participants in each group. Data from work published more than once were obtained from the more thorough study. The reviewers rated each primary study according to the overall quality of evidence: A-high; B-moderate; C-low and D-very low, assigning scores of 1 to 5 according to the number of biases. The total AMSTAR score was used for systematic reviews. For analytical purposes, the studies were grouped as i) interventions in animal models and ii) clinical studies.

Statistical analysis

Statistical analysis was performed in preclinical trials using RevMan software, version 5.3. All p values < 0.05 were considered to be statistically significant. For dichotomous variables, such as patients who were alive at the end of the period analyzed, the absolute risk reduction method with a confidence interval of 95% (random effects model) was used.

For continuous variables, such as animal survival in days, the weighted mean difference (random effects model) was calculated based on the DerSimonian and Laird method, with a corresponding confidence interval of 95%. To evaluate heterogeneity among studies, a heterogeneity test was performed by calculating both the Q-test of heterogeneity and the I^2 test of inconsistency. Heterogeneity was considered significant when p < 0.10. In addition, a sensitivity analysis was conducted using the funnel plot to quantify the presence of publication bias.

RESULTS

Initially, 489 articles were obtained. Based on their abstracts, the following were selected: 23 prospective clinical trials, 48 preclinical trials, and 3 systematic reviews. After each original document was reviewed and data were obtained, 18 preclinical trials were excluded; of

these, 2 were '*in vitro*' (Calderó et al., 2010; Schuster et al., 2012), 6 did not include a quantitative evaluation of survival (Gu et al., 2010; Tovar-y-Romo et al., 2012; Cappello et al., 2012; Sunyach et al., 2012; Zhao et al., 2012, Yazhou et al., 2012), one had duplicate data (Jablonka et al., 2011), 7 only reported survival ratios and proportions (Seo et al., 2011; Ferrucci et al., 2010; Katsumata et al., 2012; Fidler et al., 2011; Gianforcaro et al., 2012, Gianforcaro et al., 2013; Schuster et al., 2010), and 2 involved interventions that occurred only prior to disease onset (Couglan et al., 2015; Goursaud et al., 2015). The authors of 16 studies were contacted via email for completion of missing data, without any success. Five clinical trials were excluded. One contained duplicate data (Rudnicki et al., 2013), and 4 were not controlled (Chiò et al., 2011; Atassi et al., 2010; Fondell et al., 2012; Grassinger et al., 2014). Finally, 30 preclinical '*in vivo*' studies, 18 randomized and controlled clinical trials, and 3 systematic reviews of the Cochrane Collaboration were included. A flowchart illustrates the selection process adopted in the systematic review (Figure 1).

Clinical studies

Table 1 lists the primary randomized clinical trials and systematic reviews of the Cochrane Collaboration, classifying them according to year, intervention, mean disease duration at baseline, follow-up time, number of participants, completion and quality. Only quality A and B primary studies were selected. Great heterogeneity

Table 1. Selected clinical trials including ALS patients with random allocation, according to intervention, mean time of disease, follow-up time, outcomes and quality (GRADE/AMSTAR) 2009 - 2015. E: experimental group; C: control group; TG: Therapeutic group; STG: sub therapeutic group; IV: intravenous.

Intervention (Ref))	N	Disease (Months)	Follow-up (Months)	Survival	ALSFRS-R Slope	Grade
Talampanel 50 mg (Pascuzzi)	59	12	9	Not reported	E=(-7,1);C=(-10,2)(P=0,081)	1B
Ursodeoxycholic acid (min)	64	12.5	8	Not reported	E=1.04±0.28; C= 1.61±0.28 (P= 0.16)	1B
Dexpramipexole 300 mg (Cudkowicz)	102 -	15.4 -	9 -	68% HR: 0,32 (95%CI 0.086 to 1.18) log rank p=0.07	31% Reduction:0,40 (95%: CI 0,38 to 1,18)	1A -
Dexpramipexole 300 mg (Cudkowicz)	943	12	12	HR 1·03 (95%CI 0·75 to 1·43) p=0·84	E=13·34;C=-13·42(p=0·90)	1A
COQ10 1.800 OU 2.700 mg (Kaufmann)	185	27	18	Not reported	E=8.4± 7.3;C= 9.0±8.2(P=)	1A
BCAA/Threonin (Parton)	476/86 -	Not reported -	6 -	BCAA HR 1.57 P=0.209 (95%CI 0.78 to 3.19); L-TR HR=2.76 P=0.151 (95%CI :0.71 to 9.27)	Not reported	31* -
Ciliary neurotrophic factor –CTNF(Bongioanni)	1300	Not reported	6	RR 1.07(95% CI 0.81 to 1.41)	RR 1.07(95% CI 0.81 to 1.41)(P= 0.85)	31*
Creatine (Pastula)	386	Not reported	18	Dosing Escalation: 83%, 96% and 96%	Difference in slope 0.09(P= 0.76)	30*
Growth hormone- 2UI(Saccà)	40	15.6	18	RR: 1.03± 0.15	E=-45.2±6.1 C= 33.1±7.8(P=0.61)	1B
Acetyl-l-carnitine(Beghi)	82	Not reported	12	HR 0.72(95%CI 0.45 to 1.16) P=0.1804	Monthly E=-0.97±1.09; C=1.60±1.39	1A
Ceftriaxone 2G 4G IV/DAY(Berry)	66	18	3	Not reported	E=36.8±6.0; C=35.2±5.7	1A
Lithium TG × STG (Chiò)	117	24	15	NR; did not differ P= 0.94	TG=1.26±1.43; STG=1.15±1.03(P= 0.60)	1B
Lithium + Riluzole (Aggarwal)	84	20.3	5.4	HR 1.13 (95%CI=0.61 to 2.07)	0.15(95%CI -2.58 to 0.13) p=0.08	1A
Lithium + Riluzole (UKMND)	214	20.5	18	HR 1.35(95%CI 0.90 A 2.02)	9.50 (95% CI -10.31 to-8.70) slope 0.19(95% CI -1.28 to 0.90)	1A
Lithium × Placebo (Verstraete)	133	13	16	HR 1.03 (95%CI 0.66 A 1.63)	E= 40- 22; C= 40- 24 (P =0,74)	1A
G-CSF × Placebo (Duning)	39	22.4	12	Not reported	E=-4.66±3.37; C= -6.56±5.3 (P=0.289)	1B
G-CSF(Nefussy)	10	13.2	0.9	Not reported	E=35.3±9.4); C=34.4± 8.2	1B
Olesoxime (Lenglet)	512	17.5	18	E= 69.4%(95%CI 63.0 to 74.9);	HR 0.997 (95%CI 0.958 to 1.04)(p= 0.87)	1A
Pioglitazone (Dupuis)	219	18.9	15	HR:1.21 (95% CI: 0.71-2.07, p=0.48). C=67.5 %(95%CI 61.0 to 73.1)(P=0.71)	Not reported	1A
Lithium + Valproic acid (Boll)	49	46.5	17	HR 0.72± 0.6(12 months); 0.59± 0.07(16 months)(P= 0.016)	Better in experimental group (p<0.05)	2B
Ceftriaxone 4 G IV × Placebo (Cudkowicz)	340	18	72	No difference (P=0.5972)	HR 0.9 (95%CI 0.71 to 1.15) P= 0.4146	1A

among the studies was observed. Heterogeneity in relation to the method, time of patient follow-up and intervention prevented us from performing a meta-analysis with respect to the proposed outcomes with the exception of three studies using Lithium, in which it was possible to conduct

Study or Subgroup	Lithium Events	Total	Placebo or Riluzole Events	Total	Weight	Risk Ratio M–H, Fixed, 95% CI
Aggarwal 2010	18	40	24	44	18.3%	0.82 [0.53, 1.28]
Al–Chalabi 2013	54	107	63	107	50.6%	0.86 [0.67, 1.10]
Verstraete 2012	33	66	39	67	31.1%	0.86 [0.63, 1.18]
Total (95% CI)		213		218	100.0%	0.85 [0.71, 1.02]
Total events	105		126			

Heterogeneity: Chi^2 = 0.03, df = 2 (P = 0.99); I^2 = 0%
Test for overall effect: Z = 1.77 (P = 0.08)
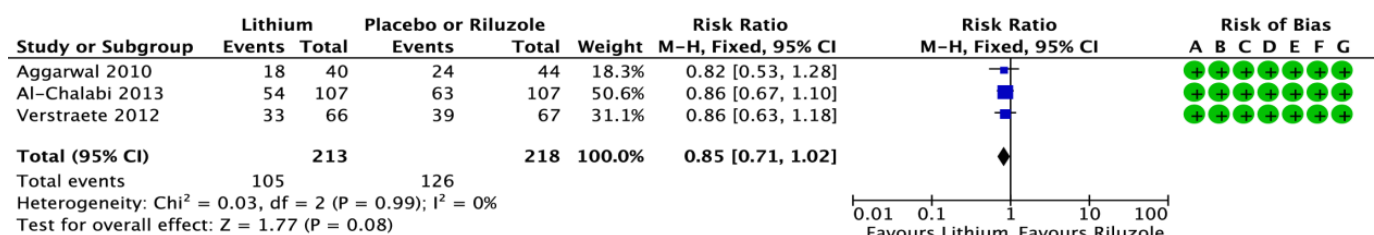
Risk of bias legend
(A) Random sequence generation (selection bias)
(B) Allocation concealment (selection bias)
(C) Blinding of participants and personnel (performance bias)
(D) Blinding of outcome assessment (detection bias)
(E) Incomplete outcome data (attrition bias)
(F) Selective reporting (reporting bias)
(G) Other bias

Figure 2. Forest plot - use of lithium versus Placebo in ALS patients. Risk ratio analysis related to the endpoint in 15 to 16 months of follow-up. Period: 2009 to 2015.

a meta-analysis based on the number of survivors at the end of 15 to 16 months (Figure 2).

The mean disease duration at the time of randomization ranged from 12 to 72 months, with a weighted average of 16.2 months and a median of 15.6 months. The follow-up period after intervention ranged from 25 days to 18 months and was performed in a variable manner relative to the ALSFRS-R, survival, forced vital capacity (FVC) and occurrence of adverse events.

Lithium, dexpramipexole and Granulocyte-colony stimulating factor (G-CSF) had greater consideration in the review because they were the most studied and a larger number of patients were enrolled in the trials. A phase II clinical trial using Dexpramipexole (Cudkowicz et al., 2011) in 102 patients conducted in two phases with duration of nine months and dose escalation moderately slowed disease progression and increased survival time (HR: 0.32 95% CI; 0.086 to 1.18). With the dosage increase to 300 mg/day, in relation to the ALSFRS-R, a reduced functional decline of 31% was observed in the first stage compared to placebo, particularly in the subdomain of the scale relative to fine motor control, where the difference was greater (-1.4 SD 0.30 versus -0.60 SD 0.24), favoring larger studies with the drug. However, a phase III, multicenter, placebo-controlled clinical trial of 943 patients involving a 12-month follow-up was conducted and found no changes in survival time or ALSFRS-R scores (Cudkowicz et al., 2013).

The use of Lithium associated with Riluzole in ALS patients was evaluated in four randomized clinical trials (RCTs) (Aggarwal et al., 2010; Chiò et al., 2010; Verstraete et al., 2012; UKMND-LiCALS Study Group, 2013). In 2010, a multicenter study was conducted on the use of Riluzole and Lithium combined (Aggarwal et al., 2010) at a dose of 150 to 1050 mg per day in 84 patients

while maintaining serum concentration between 0.4 to 0.8 meq/L. The hazard ratio for final outcome (a drop of 6 points in the ALSFRS-R or death) was 1.13 (95% CI; 0.61 to 2.07). Patients were monitored for 5.4 months, and the study was interrupted because most patients in the experimental group presented with the final outcome. The mean difference in decline in the ALSFRS-R between the group that used lithium and the placebo group was 0.15 (95% CI; -0.43 to 0.73, p = 0.61).

In that same year, in the USA and Canada (Chio et al., 2010), the drug was administered in two dosages-subtherapeutic (serum concentration of 0.2-0.4 mEq/L) and therapeutic (serum concentration of 0.4 to 0.8 mEq/L) to a group of 171 patients with a 15-month follow-up, but 85% had discontinued the drug by the end of this period due to adverse effects or lack of efficacy. No statistically significant difference was observed in the decline of the ALSFRS-R and FVC among the groups.

The results of two randomized trials using a similar method were published in the Netherlands in 2012 (Verstraete et al., 2012) and in Great Britain in 2013 (UKMND-LiCALS Study Group, 2013). Both studies evaluated Lithium and Riluzole versus placebo. The studies included 133 and 214 patients, respectively, with a 16- to 18-month follow-up. Hazard ratios (HR) of 1.13 (95% CI; 0.61 to 2.07) and 1.35 (95% CI; 0.90 to 2.02), respectively, were observed for survival, with no evidence of better performance of treated patients in relation to the ALSFRS-R or FVC. A meta-analysis was conducted to assess survival, including the number of patients who survived 15 to 16 months after treatment with Lithium at a variable dose with a serum level of 0.4 to 0.8 mEq/L, in the three studies that used Riluzole or a placebo as control (44, 46, 47). The study that used subtherapeutic Lithium concentrations as a control was excluded. Two hundred thirty-one events were observed in 431 patients

Table 2. Adverse events in clinical trials with ALS patients. 2009-2015. NR: Not Reported

Author (REF)	Drug	Adverse Events (AE)	N (EXPOSED)	N (AE)	N (DISCONTINUED)
Nefussy	G-CSF	Bone and muscle pain after the injections	19	2	0
Pascuzzi	TALAMPANEL	Dizziness, drowsiness, asthenia, depression,	40	24	2
Min	Oral solubilized ursodeoxycholic acid 500 mg	Abdominal pain, anorexia, dysphagia, dyspepsia, nausea, vomiting	40	25	5
Cudkowicz	Dexpramipexole 50 mg, 150 mg and 300 mg	Dizziness, headache, adbominal pain, anorexia, dysphagia, diarrhea, neutropenia	123 + 474	NR + 459	5 +35
Kaufmann	COQ10 1.800 OU 2.700 MG	Respiratory and gastrointestinal events, fall, pain, nausea	110	63	0
Parton	Aminoacid - BCAA or threonin	Headache and gastrointestinal upset, gout	90	0.58 × control	1.35 × control
Bongioanni	Ciliary neurotrophic factor (cntf) -0,5 a 30 mcg/kg	Weight loss, anorexia, asthenia, cough	914	RR 1.55	NR
Pastula	Creatine - 5 a 10 g dia vo ou placebo	No significant	173	NR	NR
Saccà	Growth hormone	Increase of hepatic enzymes, joint swelling, hypertension, weakness , glucose INCR and INCR local reactions	20	15	NR
Beghi	Acetyl-l-carnitine 500 mg	Mai, pneumonia, urinary, dizziness retinal haemorrhage, gastric intolerance	42	27	4
Berry	Ceftriaxone 2 g and 4 g iv	Pseudomembranous colitis, cholelithiasis, catheter related	21	5	4
Aggarwal	Lithium conc: 0.4-0.8 MEQ/L	Fatigue, sedation, raised tsh, anorexia , nausea, muscle weakness	40	20	3
Ukmnd – alchalabi	Lithium 295 mg conc 0.4-0.8 mmol/l	Nausea, vomiting	107	61 HR=1.14	2
Verstraete	Lithium conc 0.4-0.8 mmol/L	Nausea, vomiting, polydipsia	66	47	16
Dupuis	Pioglitazone - 45 mg vo	Dysphagia, dyspnea, depression, oedema, weight loss,	109	35	NR
Duning	G-CSF	Leukocytosis, nervous system disorders, skin, musculoskeletal and connective tissue	5	11	0
Chiò	Lithium conc 0,4-0,8 mmol/l or 0,2-0,4 mmol/l	Cardiac, cystitis, deep vein thrombosis, edema, retinal, dehydration	171	38	117 (68.4%)

(Figure 2). The risk ratio obtained was 0.85 (95% CI; 0.71 to 1.02). The funnel graph showed that the studies presented similar findings.

A clinical trial conducted in Germany (Duning et al., 2011) used recombinant human Granulocyte-colony Stimulating Factor (G-CSF) to slow the progression of ALS symptoms in 10 patients at a dose of 10 mcg/kg/day versus placebo. Patients were monitored for 100 days and showed no differences in the percentage of decline in ALSFRS-R scores. The results of a previous pilot study conducted in Israel, in which half the suggested dose of the same drug was administered to 39 patients, suggested smaller declines in the ALSFRS-R variation (p: 0.289) and FVC (p: 0.854) in the experimental group (46). The studies did not assess survival. The remaining interventions evaluated in single studies (Pascuzzi et al., 2010; Min et al., 2012; Kaufmann et al., 2009; Pastula, 2012; Sacca, 2012; Beghi et al., 2013, Berry et al., 2013; Dupuis et al., 2012; Lenglet et al., 2014; Boll et al., 2014; Cudkowitz et al., 2014) were not significantly successful in slowing disease progression or reducing ALSFRS-R variation compared to the control group.

Table 2 lists the adverse events (AEs) related to the use of drugs in clinical trials. It was observed that most of the studies reported weakness, gastrointestinal intolerance, and dizziness as adverse drug events, but they rarely reported severe events. Adverse events caused by Lithium are controversial because more adverse effects are known - 126 total, but with little discontinuation except for a clinical trial that was interrupted due to an excessive number of adverse events (Verstraete et al, 2012). In summary, none of the drugs demonstrated unequivocal effectiveness in controlling the progression of the disease in humans.

Preclinical studies

Preclinical 'in vivo' trials were conducted in transgenic mice expressing human mutated superoxide dismutase 1 (SOD1[G93A]) and showed experimental ALS treatments using neuroprotective therapies. The studies were considered homogeneous with respect to the method and the evaluation of outcomes in
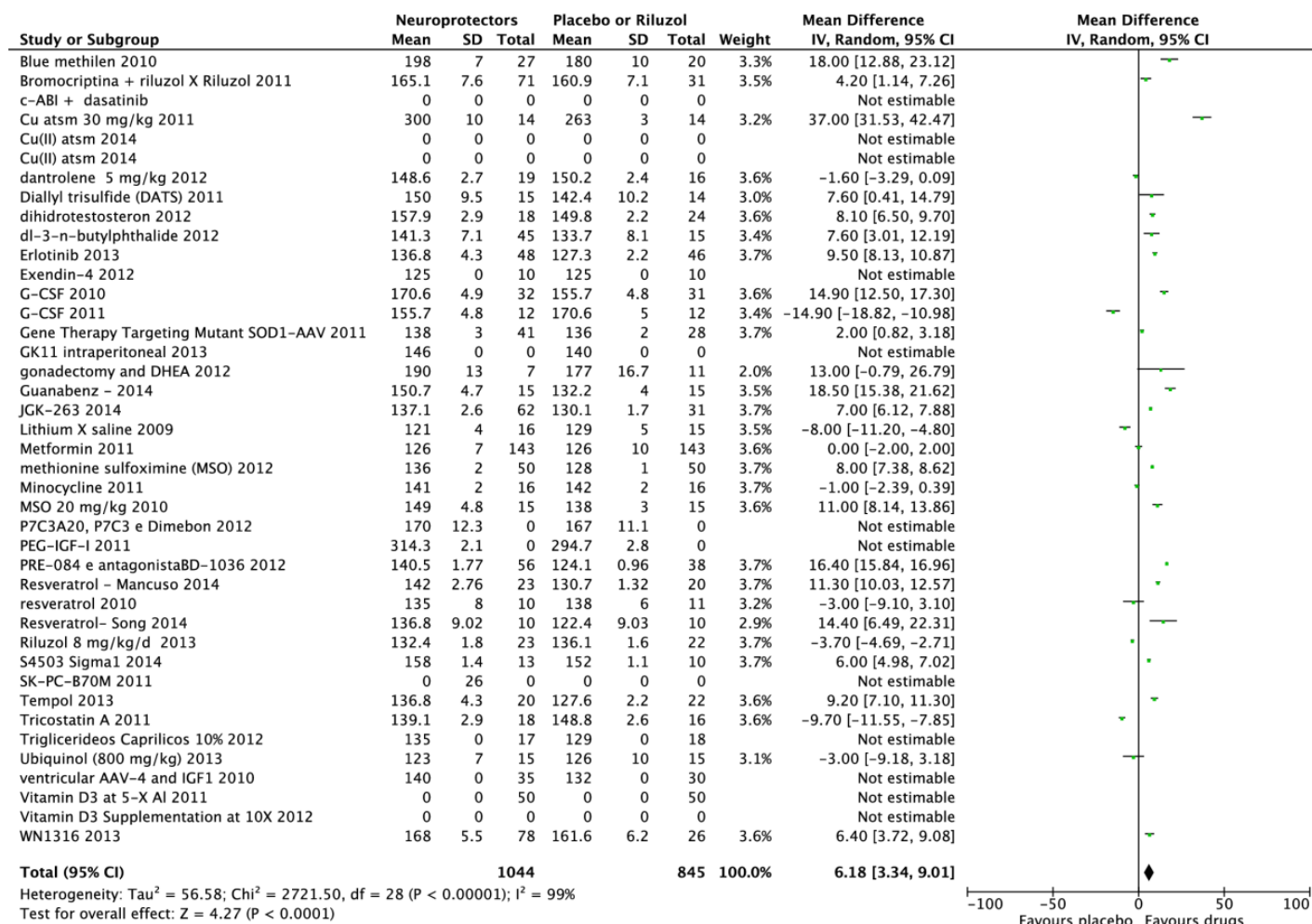
Study or Subgroup	Neuroprotectors			Placebo or Riluzol			Weight	Mean Difference IV, Random, 95% CI	Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Blue methilen 2010	198	7	27	180	10	20	3.3%	18.00 [12.88, 23.12]	
Bromocriptina + riluzol X Riluzol 2011	165.1	7.6	71	160.9	7.1	31	3.5%	4.20 [1.14, 7.26]	
c–ABl + dasatinib	0	0	0	0	0	0		Not estimable	
Cu atsm 30 mg/kg 2011	300	10	14	263	3	14	3.2%	37.00 [31.53, 42.47]	
Cu(II) atsm 2014	0	0	0	0	0	0		Not estimable	
Cu(II) atsm 2014	0	0	0	0	0	0		Not estimable	
dantrolene 5 mg/kg 2012	148.6	2.7	19	150.2	2.4	16	3.6%	−1.60 [−3.29, 0.09]	
Diallyl trisulfide (DATS) 2011	150	9.5	15	142.4	10.2	14	3.0%	7.60 [0.41, 14.79]	
dihidrotestosteron 2012	157.9	2.9	18	149.8	2.2	24	3.6%	8.10 [6.50, 9.70]	
dl–3–n–butylphthalide 2012	141.3	7.1	45	133.7	8.1	15	3.4%	7.60 [3.01, 12.19]	
Erlotinib 2013	136.8	4.3	48	127.3	2.2	46	3.7%	9.50 [8.13, 10.87]	
Exendin–4 2012	125	0	10	125	0	10		Not estimable	
G–CSF 2010	170.6	4.9	32	155.7	4.8	31	3.6%	14.90 [12.50, 17.30]	
G–CSF 2011	155.7	4.8	12	170.6	5	12	3.4%	−14.90 [−18.82, −10.98]	
Gene Therapy Targeting Mutant SOD1–AAV 2011	138	3	41	136	2	28	3.7%	2.00 [0.82, 3.18]	
GK11 intraperitoneal 2013	146	0	0	140	0	0		Not estimable	
gonadectomy and DHEA 2012	190	13	7	177	16.7	11	2.0%	13.00 [−0.79, 26.79]	
Guanabenz – 2014	150.7	4.7	15	132.2	4	15	3.5%	18.50 [15.38, 21.62]	
JGK–263 2014	137.1	2.6	62	130.1	1.7	31	3.7%	7.00 [6.12, 7.88]	
Lithium X saline 2009	121	4	16	129	5	15	3.5%	−8.00 [−11.20, −4.80]	
Metformin 2011	126	7	143	126	10	143	3.6%	0.00 [−2.00, 2.00]	
methionine sulfoximine (MSO) 2012	136	2	50	128	1	50	3.7%	8.00 [7.38, 8.62]	
Minocycline 2011	141	2	16	142	2	16	3.7%	−1.00 [−2.39, 0.39]	
MSO 20 mg/kg 2010	149	4.8	15	138	3	15	3.6%	11.00 [8.14, 13.86]	
P7C3A20, P7C3 e Dimebon 2012	170	12.3	0	167	11.1	0		Not estimable	
PEG–IGF–I 2011	314.3	2.1	0	294.7	2.8	0		Not estimable	
PRE–084 e antagonistaBD–1036 2012	140.5	1.77	56	124.1	0.96	38	3.7%	16.40 [15.84, 16.96]	
Resveratrol – Mancuso 2014	142	2.76	23	130.7	1.32	20	3.7%	11.30 [10.03, 12.57]	
resveratrol 2010	135	8	10	138	6	11	3.2%	−3.00 [−9.10, 3.10]	
Resveratrol– Song 2014	136.8	9.02	10	122.4	9.03	10	2.9%	14.40 [6.49, 22.31]	
Riluzol 8 mg/kg/d 2013	132.4	1.8	23	136.1	1.6	22	3.7%	−3.70 [−4.69, −2.71]	
S4503 Sigma1 2014	158	1.4	13	152	1.1	10	3.7%	6.00 [4.98, 7.02]	
SK–PC–B70M 2011	0	26	0	0	0	0		Not estimable	
Tempol 2013	136.8	4.3	20	127.6	2.2	22	3.6%	9.20 [7.10, 11.30]	
Tricostatin A 2011	139.1	2.9	18	148.8	2.6	16	3.6%	−9.70 [−11.55, −7.85]	
Triglicerideos Caprilicos 10% 2012	135	0	17	129	0	18		Not estimable	
Ubiquinol (800 mg/kg) 2013	123	7	15	126	10	15	3.1%	−3.00 [−9.18, 3.18]	
ventricular AAV–4 and IGF1 2010	140	0	35	132	0	30		Not estimable	
Vitamin D3 at 5–X Al 2011	0	0	50	0	0	50		Not estimable	
Vitamin D3 Supplementation at 10X 2012	0	0	0	0	0	0		Not estimable	
WN1316 2013	168	5.5	78	161.6	6.2	26	3.6%	6.40 [3.72, 9.08]	
Total (95% CI)			1044			845	100.0%	6.18 [3.34, 9.01]	

Heterogeneity: Tau² = 56.58; Chi² = 2721.50, df = 28 (P < 0.00001); I² = 99%
Test for overall effect: Z = 4.27 (P < 0.0001)

−100 −50 0 50 100
Favours placebo Favours drugs

Figure 3. Forest Plot – network meta-analysis including days of survival (life span) in pre-clinical studies with drugs. Period: 2009 to 2015.

animals, making the comparison by meta-analytical methods possible. All included studies analyzed survival by the Kaplan-Meier method with the log-rank test, but only those studies that included disease prior to treatment and evaluated the survival of animals with a mean in days and standard deviation were included in our meta-analysis. The authors of all the studies that did not include such data were contacted via email.

Figure 3 describes the global effect of various drugs on the survival of animal models with ALS compared to placebo by means of network meta-analysis conducted from 2009 to 2015. It was observed that there are several effective medications. Studies on vascular endothelial growth factor (VEGF) (Tovar-y-Romo et al., 2012), Olesoxime (Sunyach et al., 2012), Exendin-4 (Yazhou e al., 2012), and SK-PC-B70M (Seo et al., 2011) were excluded from the meta-analysis for not including results about survival, although they did report improvements in motor performance and a neuroprotective effect of

motor neurons in the spinal cord.

A study that investigated the effects of Dasatinib at a dose of 25 mg/kg/day (Le Pichon et al., 2013) reported improvement in animal survival associated with weight gain (log rank test, p < 0.01). Studies on PEGylated insulin-like growth factor (Jablonka et al., 2011; Saenger et al., 2012) showed prolonged survival (p < 0.05) associated with its use in the initial stage of the disease (Saenger et al., 2012), while a study of Gacyclidine (Gerber et al., 2013) showed a 4.3% increase in survival (p = 0.034). It was also observed that dietary therapy with caprylic triglyceride (Zhao et al., 2012) and vitamin D3 (Gianforcaro et al., 2012; Gianforcaro et al., 2013) improved motor performance without influencing animal survival.

The most effective drugs in prolonging survival are CuII(atsm) (Soon et al., 2011), dihydrotestosterone (Yoo et al., 2012), Granulocyte Colony-Stimulating Factor (G-CSF) (Henriques et al., 2011), Methionine Sulfoximine
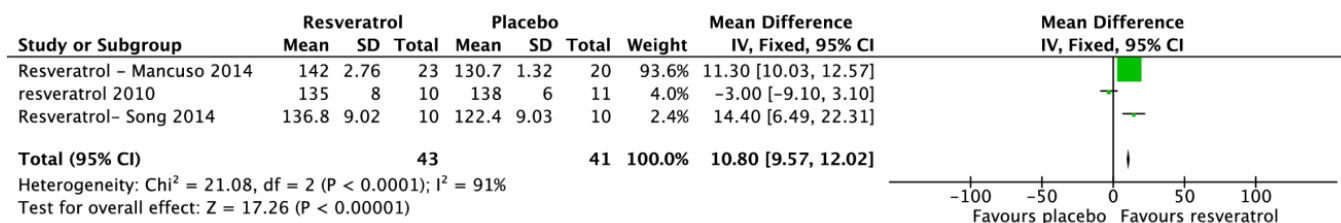
Study or Subgroup	Resveratrol Mean	SD	Total	Placebo Mean	SD	Total	Weight	Mean Difference IV, Fixed, 95% CI
Resveratrol – Mancuso 2014	142	2.76	23	130.7	1.32	20	93.6%	11.30 [10.03, 12.57]
resveratrol 2010	135	8	10	138	6	11	4.0%	−3.00 [−9.10, 3.10]
Resveratrol– Song 2014	136.8	9.02	10	122.4	9.03	10	2.4%	14.40 [6.49, 22.31]
Total (95% CI)			43			41	100.0%	10.80 [9.57, 12.02]

Heterogeneity: Chi^2 = 21.08, df = 2 (P < 0.0001); I^2 = 91%
Test for overall effect: Z = 17.26 (P < 0.00001)

Figure 4. Forest plot - meta-analysis including days of survival (life span) in pre-clinical studies with Resveratrol. Period: 2009 to 2015.
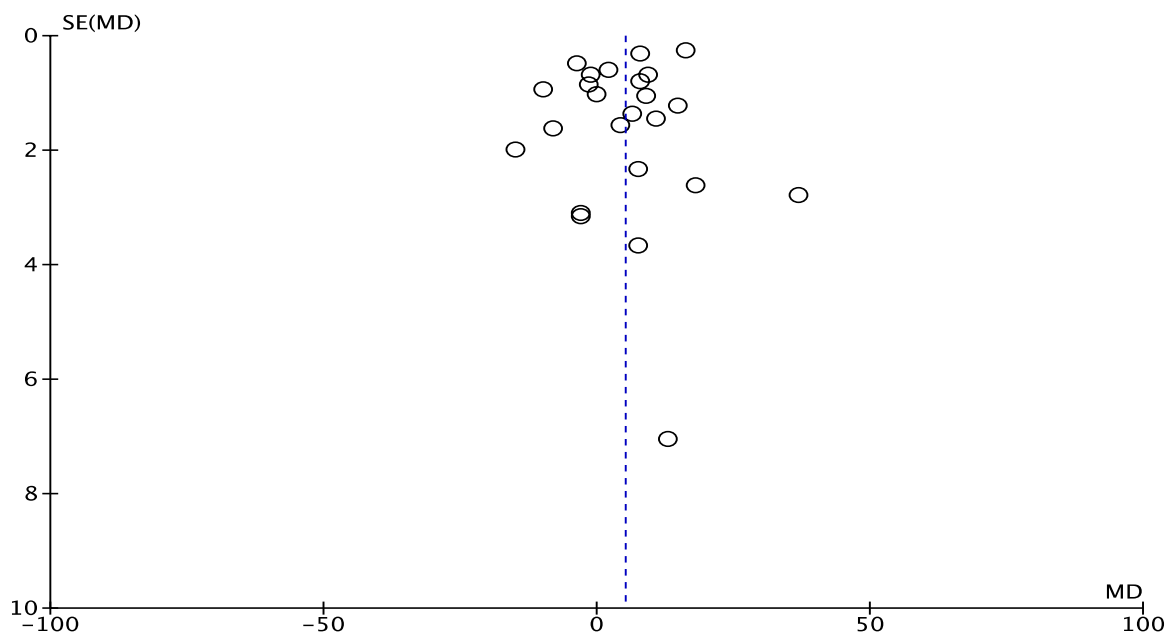

Figure 5. Funnel plot of preclinical studies in SOD1^{G93A} mice. 2009-2015.

(MSO) (Ghoddoussi et al., 2010; Bame et al., 2012), PRE-084 (Mancuso et al., 2012), Tempol (Linares et al., 2013), WN1316 (Tanaka et al., 2014), n-butylphthalide (Feng et al., 2012), Guanabenz (Wang et al., 2014; Jiang et al., 2014) and Resveratrol (Markert et al., 2010; Song et al., 2014; Mancuso et al., 2014), with a total mean meta-analytical difference of 6.18 (95% CI 3.34 to 9.01) days of survival, favoring the group of drugs tested when compared to placebo. It was observed that Resveratrol showed major positive effect in three studies, and a meta-analysis (Figure 4) with this drug showed a mean difference of 10.8 (95% CI 9.57 to 12.02) days of survival, favoring the Resveratrol group. The authors analyzed the positive studies, as a group to demonstrate that more than one drug could potentially be effective in disease control. The funnel plot (Figure 5) showed a symmetrical distribution. The other drugs evaluated, including Riluzole

(Li et al., 2013) and Lithium (Pizzasegola et al., 2009), showed little or no impact on the survival of animal models.

DISCUSSION

Motor neuron disease is a group of progressive neurodegenerative disorders with different etiologies and clinical spectra that have a loss of lower and/or upper motor neurons in common (Mancuso, 2015). Although the heterogeneous and complex nature of ALS has been studied extensively, the absence of early detection biomarkers has not allowed the identification of patients at different stages or those developing the disease. With regard to treatments aimed at slowing the progression of ALS, an analysis of the results of preclinical trials in

animal models and clinical trials shows that there is great disparity between the findings of animal trials, which are often positive, and their replication in humans, which almost always yield negative results. In clinical studies, Riluzole (Miller et al., 1996) remains the single drug that has successfully slowed disease progression in a systematic review with a meta-analysis involving 1,477 patients; a 2- to 3-month increase in survival time was observed with a relative risk of 0.78 (95% CI 0.65 to 0.92) at 18 months.

According to some authors (Mancuso, 2015; Perrin, 2014), there are several possible explanations for the failure of translation from preclinical studies to effective human treatments. In preclinical studies, SOD1 animal models represented familial ALS more than sporadic ALS. In addition, pathophysiology of ALS spectra is poorly understood and it is possible that familial and sporadic ALS differ in some fundamental mechanisms that determine the effectiveness of treatments (Mancuso, 2015). On the other hand, some drugs used in animal models are used prior to symptom onset, which cannot be replicated in patients. It is also difficult to estimate the optimal target dose of an experimental drug in humans with the absence of the biomarkers.

The accessed preclinical trials used were relatively young, mutant SOD1 ^{G93A} mice in homogeneous groups and a controlled environment, in which they showed a similar clinical condition. The drugs were used in the pre-symptomatic and early symptomatic stages, and many studies reported success in slowing the progression of symptoms and prolonging the survival of the animals, as shown in Figure 1. Most animal studies used the G93A SOD1 mice, but others used a low copy number G93A SOD1 strain or different familiar ALS mouse models with different onset and survival times. These studies have suggested that direct injury on the superoxide dismutase (SOD1) protein in neuronal tissues is crucial for the onset of motor neuron disease but not for the clarification of its progression, which is largely determined by microglia and astrocyte responses (Boillée et al., 2006).

The main mechanisms leading to neuronal death after onset of the disease include mitochondrial dysfunction, formation of free radicals and protein aggregates, glutamate excitotoxicity, axonal transport disruption, apoptosis, and inflammatory processes. There is evidence that protein aggregates can actively spread in the cerebral cortex and spinal cord via communication between cells, a process known as prion-like spread (Grad et al., 2015).

The role of autophagy in the injury mechanisms of the disease has also been discussed. Autophagy and the ubiquitin-proteasome (UP) system are two ways in which cells can degrade intracellular components. The UP system degrades short-lived proteins, whereas autophagy is responsible for the degradation of long-lasting proteins and damaged mitochondria, but when present in excess, it can lead to self-digestion and cell death (Pasquali et al., 2009).

Despite the biases present in pre-clinical studies, negative results (95%) obtained in the randomized clinical trials are also influenced by several factors, among which are clinical heterogeneity, little knowledge about the disease prognosis, the highly variable course of the disease, the small number of participants, the inclusion of patients who had had the disease for a long time and not just incidental cases, withdrawal due to the adverse effects or lack of efficacy of drugs, and the use of different outcome measures (Traynor et al., 2006), as shown in Tables 1 and 2. The mean duration of the disease at the beginning of the studies ranged from 12 to 31.8 months, with a weighted average of 16.2 months and a median of 15.6 months. There was great variability among groups, which compromises the assessment of treatment effectiveness because it directly interferes with the scores of functional scales and survival time (Table 1).

Another factor that may be associated with inadequate group set up in clinical trials is the delay in establishing the diagnosis. A recent study conducted in the United States (Paganoni et al., 2014), in which logistic regression was used to assess 103 patients, showed that the total median time for diagnosis is 11.5 months. In this study, it was shown that 52% of patients had previously received another diagnosis; on average, evaluation by three doctors was necessary for a conclusive diagnosis. According to some authors (Paganoni et al., 2014; Gordon, 2011), dose escalation is important in phase II clinical trials before conducting phase III trials, in which efficacy and safety are determined for a greater number of patients. This did not occur in most of the studies selected and could explain why so many phase II trials are positive and phase III trials proved to be negative. Moreover, the ALSFRS-R, the only widely validated clinical scale and survival, are clinical outcomes that should be used to establish the efficacy of the tested compounds.

The effectiveness of various drugs in slowing the progression of motor neuron disease was tested in Cochrane systematic reviews, including Riluzole, Creatine, amino acids, and ciliary neurotrophic factor (CNTF), and only Riluzole yielded positive results. The systematic review of Riluzole included three clinical trials (Riluzole 876, placebo 406) and one of those included patients of advanced age (Mancuso, 2015). Daily treatment with 100 mg of Riluzole increased survival by two to three months in two studies (p = 0.039, hazard ratio (HR) 0.80, 95% CI 0.64 to 0.99), but in patients presenting with advanced disease, the result was not significant (p = 0.056, HR 0.84, 95% CI 0.70 to 1.01). Since then, various drugs have been used in clinical trials in an attempt to slow disease progression without success. Lithium was used in four clinical trials for

this purpose, motivated by positive results in multiple cell culture and animal assays (Fornai et al., 2008), but to date, the same results have not been observed in clinical trials (Aggarwal et al., 2010; Chiò et al., 2010; Verstraete et al., 2012; UKMND-LiCALS Study Group, 2013).

The most significant multicenter clinical trial was conducted in the UK and used Lithium carbonate to treat ALS - LiCALS (UKMND-LiCALS Study Group, 2013) in 214 patients over 18 months. Although the number of adverse events observed was not significant (hazard ratio for serious adverse events 1.14, 95% CI 0.79 to 1.65), the drug was not beneficial (Mantel-Cox log-rank $\chi2$ on 1 df = 1.64, p = 0.20). Three published articles that were included in the review reported negative results in terms of disease progression and many adverse events (Table 2) with the use of Lithium. However, these studies used a non-traditional method, included few patients and did not exclude the possibility of a minor drug effect on survival and disease progression (Aggarwal et al, 2010; Chiò et al, 2010; Verstraete et al, 2012). A meta-analysis of these studies that considered the dichotomous variable survivors in the experimental and control groups at 15 to 16 months yielded a hazard ratio of 0.85 (95% CI: 0.71 to 1.02), without statistical significance.

In preclinical trials, a significant improvement in survival with the use of recombinant human granulocyte-colony stimulating factor (G-CSF) was observed in a selected study (Henriques et al., 2011). The efficacy and safety of the drug in humans with ALS was evaluated based on two articles that had a total of 49 patients and a maximum follow-up time of 12 months (Duning et al., 2011; Nefussy et al., 2010). Subcutaneous injections of G-CSF or saline solution (5 to 10 mg/kg/day of G-CSF for 4 days every three months during 1 year or during 10 days, from day 20th to 25th) were administered. Survival was not assessed, and the primary outcome was disease progression. The ALSFRS-R score was evaluated, and it was observed that the drug was well tolerated, with no significant evidence of efficacy.

In addition, some researchers have found that hypermetabolism is present in ALS patients and that there is a correlation between dyslipidemia, good nutrition status and a better prognosis (Schmitt et al., 2014). However, studies with caprylic triglyceride in animal models (Zhao et al., 2012) and hypercaloric enteral nutrition in patients with ALS with a short-term follow up that assessed safety and tolerability found that survival was not modified (Dorst et al., 2014).

To standardize the results of clinical trials and make them more reliable, approximating the results obtained in animal models, some authors (Gordon, 2011; Beghi et al., 2011) have suggested using samples of a representative number of patients, having a shorter diagnosis time; avoiding prevalent cases; stratifying patients into groups with a homogeneous clinical condition; and having as an endpoint validated functional scales such as the ALSFRS-R, death or survival, or mechanical ventilation use. It is also important to conduct studies of different populations to compare patients with different genetic susceptibility and exposure to various environmental risk factors. However, the implementation of these strategies involves wider samples and higher costs.

Guidelines have been introduced (Ludolph et al., 2010) that should reduce the number of false positives in preclinical studies and therefore prevent unnecessary clinical trials. These recommendations include rigorously assessing animals' physical and biochemical traits in terms of human disease; characterizing when disease symptoms and death occur and being alert to unexpected variations; and creating a mathematical model to aid experimental design, including how many mice must be included in a study. Perrin (2014) also suggested excluding irrelevant animals; balancing for gender; avoiding putting siblings into the same treatment group; and tracking genes that induce non-inherited disease.

Conclusion

Amyotrophic lateral sclerosis (ALS) is a rare, heterogeneous disease that is still poorly understood in its pathophysiology and is difficult to manage from a clinical point of view. Great efficacy of preclinical studies was observed, whereas the clinical ones showed no effectiveness in improving survival. Thus, the positive results of preclinical studies should be interpreted with caution. Translatability of the preclinical findings to clinical studies requires accurate standardization of preclinical research. On the other hand, a better control of the bias of clinical trials is needed to allow a greater potential of generalizing the findings. Additionally, interventions should be tested in patients who have been more recently—diagnosed, and samples should be stratified into more homogeneous groups. The most promising drugs observed in preclinical studies were Resveratrol, CuII(atsm), Dihydrotestosterone, Erlotinib, Granulocyte Colony-Stimulating Factor (G-CSF), Methionine Sulfoximine (MSO), PRE-084, Tempol, WN1316, and n-butylphthalide. The new experimental drugs that demonstrate success in slowing the progression of the disease could be used alone compared to placebos but also in combination.

Conflicts of interest

The authors declare no conflicts of interest and have followed the Sources of Funding Statement.

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Full Length Research Paper

Impact of cooking and conservation for twelve days on total polyphenols content, antioxidant and anticholinesterase activities of red onion

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The objective of this work was to determine the impact of three cooking modes (boiling, steaming and microwaving) and the conservation of onion at 4°C for 12 days on total polyphenol, flavonoid, tannins, phenolic acids contents antioxidant and anticholinesterase activity of red onion: *Allium cepa.* The results showed that the three cooking modes caused an increase of the levels of total polyphenols, flavonoids and tannins, but decreased the levels of phenolic acids. Storage at 4°C for 12 days caused a decrease in levels of total polyphenols, flavonoids, tannins and phenolic acids. A decrease in anti-radical activity during storage was found. Three cooking modes resulted in a decrease in antioxidant activity. However, the use of microwave was more effective as' to higher polyphenol contents. The level of anticholinesterase activity steadily decreased during refrigerated storage and after 12 days it was 1/4 of the value found in the raw material.The raw onion showed a moderate activity which increased after most cooking treatments. The highest level of capacity was observed after microwaving.

Key words: Total polyphenol, flavonoids, tannins, phenolic acid, cooking, conservation, antioxidant activity, anticholinesterase activity, red onion.

INTRODUCTION

Onion (*Allium cepa*) is a versatile vegetable that is consumed fresh as well as in the form of processed products. More recently, there has been renewed attention given to the antioxidant content of onions, because many epidemiological studies suggested that regular consumption of onions in food is associated with a reduced risk of neurodegenerative disorders, many forms of cancer, cataract formation, ulcer development, reduction in symptoms associated with osteoporosis (NOA), prevention of vascular and heart diseases by inhibition of lipid peroxidation (LPO) and lowering of low density lipoprotein (LDL) cholesterol levels (Kaneko and Baba, 1999; Kawaii et al., 1999; Sanderson et al., 1999; Shutenko et al., 1999; Singh et al., 2009). It is an important food because it supplies various activated phytomolecules such as phenolic acid, flavonoids copaenes, thiosulfinate, organosulfur compounds (OSCs), and anthocyanin (Slimestad et al., 2007).

Similarly, protection against neuronal degeneration in Alzheimer's disease can be achieved using natural antioxidants. Acetylcholinesterase inhibitors (AChEI) are used for the treatment of Alzheimer's disease because they enhance neuromediator acetylcholine level (Grossberg, 2003). In contrast, polyphenols and flavonoids have shown certain stability when exposed to high temperatures, a quality that is reflected in the preservation of their antioxidant capacity (Vallejo et al., 2003). Studies performed on different vegetables after cooking or conservation showed that the total polyphenol content and antioxidant capacity could be either higher or lower in comparison to the fresh food (Lombard et al., 2005; Turkmen et al., 2005). The purpose of this study is to analyze differences in the antioxidant, anticholinesterase capacity, and polyphenol retention after preservation and simulated domestic processing (boiling, steaming and microwave).

METHODOLOGY

Samples

Onion samples (*A. cepa* L.) were collected in the region of Djenan el Anab, Beni bechir, 10 km from Skikda, Algeria and they were placed in plastic bags and taken to the laboratory for analysis.

Effect of preserving vegetables on the content of total polyphenols

The effect of preserving vegetables on the content of total polyphenols was studied. Conservation is stopped when a beginning of softening fabrics onion was seen.

Raw vegetables are stored (4°C) in the refrigerator in the laboratory for 12 days. Every two days, an aliquot of these vegetables was washed and cut into small pieces, cooked (in water, steam and microwave), ground and homogenized for analysis.

Sample preparation and cooking

Cooking and pretreatment procedures are reported by Turkmens (2005). Cooking conditions are optimized by preliminary experiments (Miglio et al., 2008). Vegetables (raw or stored) are washed and all inedible parts are removed manually or by using a steel knife. Then, they are cut into small pieces of uniform shapes. 900 g onions are reserved for cooking procedures, using 300 g per method applied. All culinary experiments were performed in triplicate, using 100 g of vegetable each time.

Boiling

Onion (100 g) is placed in a stainless steel pan with 150 ml of distilled water bouillanteà 100°C. Cooking time varies between 16 and 18 min. After this procedure, the vegetables are drained to remove excess water and then cooled in a water bath.

Steaming

Onion (100 g) is placed in a stainless steel steam cooker which was covered with a lid and steamed, over boiling water. The baking time varies between 15 and 20 min. After this step, the vegetables are cooled in a water bath in a steel container.

Microwave

Onion (100 g) is placed in a glass dish in a microwave oven Giant CE137NM (consumption: 220 to 240, operation 245 MHZ frequency, power 1200 W, External Dimensions: 262 mm (H) × 452 mm (W) × 330 mm (D), size of the cavity of the oven: 198 mm (H) × 315 mm (W) × 297 mm (D), oven capacity: 20L Net Weight Env. 10.5 kg). The vegetables are cooked in a microwave oven at full power and then cooled in a water bath, the cooking time is 4 min.

Polyphenol extraction

The samples of raw and cooked vegetables were homogenized using a commercial juice extractor obtaining a fluid vegetable extract (VE). Polyphenols extraction method was adapted from Vinson et al. (2001) and Faller and Fialho (2009).

Briefly, 1 ml of each VE was extracted using 500 µl of 50% methanol (methanol:water, 50:50, v/v) for the soluble polyphenols and 50% methanol (methanol:water, 50:50, v/v) acidified with 1.2 M HCl for the hydrolyzable polyphenols, in screw capped eppendorfs. The samples were then placed in a water bath at 90°C for 180 min with constant shaking. After 3 h, the samples were removed from water bath and cooled at room temperature (approximately 2 min). The volume was adjusted to 1 ml with 95% methanol and the eppendorfs were centrifuged at 12,000 g for 5 min. The supernatants, considered to be polyphenol extracts, were immediately used for the determination of polyphenol content.

Determination of total phenolic content

The total phenolic was determined according to the method of Velioglu et al. (1998) and Ismail et al. (2004) which used Folin-Ciocalteu reagent. Extract was prepared at a concentration of 1 mg/ml. 100 µl of extract was transferred into a test tube and 0.75 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with deionised water) were added and mixed. The mixture was allowed to stand at room temperature for 5 min. 0.75 ml of 6% (w/v) sodium carbonate was added to the mixture and then mixed gently. After standing at room temperature for 90 min, the absorbance was read at 725 nm using a UV–Vis spectrophotometer. The standard calibration (0 to 1000 ppm) curve was plotted using gallic acid. The total phenolic content was expressed as gallic equivalents in microgram per 1 g vegetable extract.

Total flavonoid contents

The total flavonoid content was determined according as the aluminum chloride colorimetric method described by Chang et al. (2002) and Lin and Tang (2007). Briefly, aliquots of 1 g of onion sample were, respectively, dissolved in 1 ml deionized water. This solution (0.5 ml) was mixed with 1.5 ml of 95% alcohol, 0.1 ml of 10% aluminum chloride hexahydrate ($AlCl_3$), 0.1 ml of 1 M potassium acetate (CH_3COOK), and 2.8 ml of deionized water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415 nm against deionized water blank.

Quercetin was chosen as a standard. Using a seven point standard curve (0 to 1000 ppm), the levels of total flavonoid contents in fruits and vegetables were determined in triplicate, respectively. The data were expressed as microgram quercetin equivalents (QE)/1 g of fresh matter from onion analysed.

Tannin analysis

Quantitative estimation of tannins was carried out using the modified vanillin–HCl in methanol method described by Price et al. (1978). The method is based on the ability of condensed tannins to react with vanillin in the presence of mineral acid to produce a red color. Ground pulse samples (1 g) were extracted with 20 ml of 1% HCl in methanol) for 20 min at 30°C in a water-bath. The samples were centrifuged at 2000 rpm form 4 min. The supernatant (1.0 ml) was reacted with 5 ml vanillin solution (0.5% vanillin + 2% HCl in methanol) for 20 min at 30°C. Blanks were run with 4% HCl in methanol in place of vanillin reagent. Absorbance was read at 500 nm on a UV/VIS spectrophotometer. A standard curve was prepared with catechin. Results were expressed in terms of catechin equivalents. Samples were analyzed in triplicate

Total phenolics acids content

Phenolics acids were determined according to the method of Netzel et al. (2006) and Sigh et al. (2012) with modification. Onion (1 g) were suspended in 0.3 M hydrochloric acid in 80% ethanol (2 ml) and placed in a sonicator for 20 min. The mixture was centrifuged at 2000 g for 10 min and the supernatant was transferred to a clean glass tube. The pellet was suspended in a second aliquot of 0.3 M hydrochloric acid in 80% ethanol (2 ml) and the process repeated. The combined supernatants were made up to 5 ml with 0.3 M hydrochloric acid in 80% ethanol and an aliquot (1 ml) was transferred to an Eppendorf tube and centrifuged at 15,700 g for 5 min, the absorbance was measured at 320 nm. The total phenolic acids, expressed as µg equivalents of chlorogenic acid per g of extract of fresh weight.

Total antioxidant capacity (DPPH)

The antioxidant capacity was measured by the DPPH radical method according to Kuskoski et al. (2006) and Faller and Fialho (2009). Briefly, a 100 µM DPPH solution was prepared with 80% methanol. In test tubes, 100 µl of each VE, fresh or after cooking, was placed, after which was added 3.9 ml of the DPPH solution (100 µM). The mixture was allowed to stand, in the absence of light, and the absorbance was measured at 60 min. The DPPH solution alone was measured before the addition of the samples (A0) and 80% methanol was used as blank. The antioxidant capacity was represented as the percentage radical scavenging capacity (I%) remaining after 60 min according to the equation, which represents the absorbance of the DPPH solution alone measured, and the absorbance for each sample at 60 min after the addition of the DPPH solution at 517 nm.

Antioxidant activity I% = (A0-A/A0) × 100

Anticholinesterase activity

A spectrophotometric method developed by Ellman et al. (1961) and Ertas et al. (2015) was established to indicate the acetylcholinesterase inhibitory effects. Aliquots of 150 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of sample solution and 20 µl of acetylcholesterase (AChE) or butyrylcholinesterase (BChE) or solution were stirred and incubated for 15 min at 25°C, then 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 10 µl) is added to mixture. In the next step, by the addition of acetylthiocholine iodide (10 µl) the reaction was started. At the end, final concentration of the tested solutions was 200 µg/ml. BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates. The experiments were carried out in triplicate. Galanthamine was used as a reference compound. The percentages of inhibition were calculated by using the following equation:

DTNB (Ellman's reagent), AchE or butyrylcholinesterase BChE

$$\text{Inhibition (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Statistical analysis

The results of the antioxidant, anticholinesterase activities and total phenolic-flavonoid, tannins and phenolic acids contents were expressed as means ± SEM.

RESULTS AND DISCUSSION

Effect of storage on the levels of total polyphenols and antioxidant activity

Effect of storage on the content of total polyphenols

The concentration values are read directly from the calibration curve established using the reference solution. The following equations were used to calculate total phenolic contents of the extracts: Absorbance = 0.048 gallic acid (µg) + 0.027 (R^2 = 0.991). Concentration of the sample is expressed in µg equivalent per gram of extract. The range is plotted for gallic acid concentrations between 0 and 1000 ppm. Onion contains a total polyphenol content of 90.88 ± 0.4µg EAG/g VE. This quantity decreases with increasing the shelf life of the onion. It appears that storage at 4°C to cause loss of total polyphenols. The levels of total polyphenols obtained after 12 days of storage was 70.22 ± 1.11 µg EAG/g (Figure 1).

The flavonoid content is higher in fresh onion; it corresponds to EQ 70 µg/g VE. The results showed decreased levels of flavonoids. Storage at 4°C causes a loss of flavonoids after 12 days, the rate of flavonoids was 49.25 ± 0.6 µg EQ/g VE of onion (Figure 2). Conservation has a negative effect on the content of tannins. The results indicate that the decrease in tannin contents depends on the length of storage (Figure 3). Conservation at 4°C of onion causes phenolic acids decrease over time of conservation (Figure 4).

The results obtained show that storage (at 4°C) causes a decrease of the levels of total polyphenols, flavonoids, tannins and phenolic acids. This loss may be due to the effects of enzymatic browning. Polyphenols are converted by the action of the enzyme. They become inaccessible phenolic groups which cause the decrease of the content of total polyphenols, flavonoids, phenolic acids and tannins (Spagn et al., 2005). According Spagna et al. (2005), PPO retains 55% of its activity at 4°C, which is the cause of the decrease in phenolic content during storage in refrigerator. Different authors also observed the negative effect of the storage period on
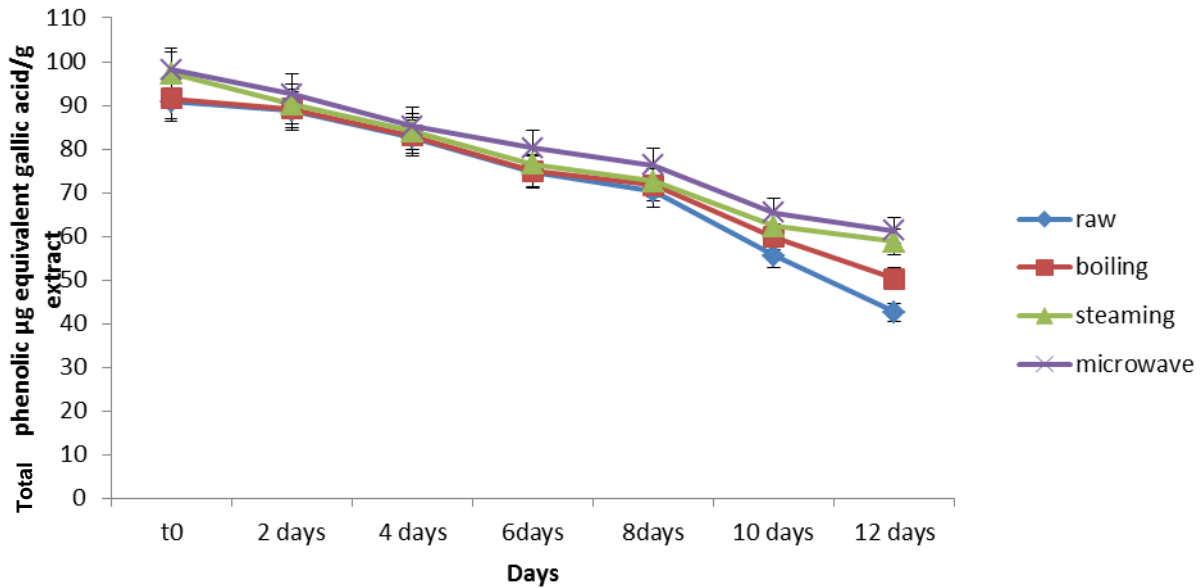
Figure 1. Effect of conservation and cooking on the levels of total polyphenols.
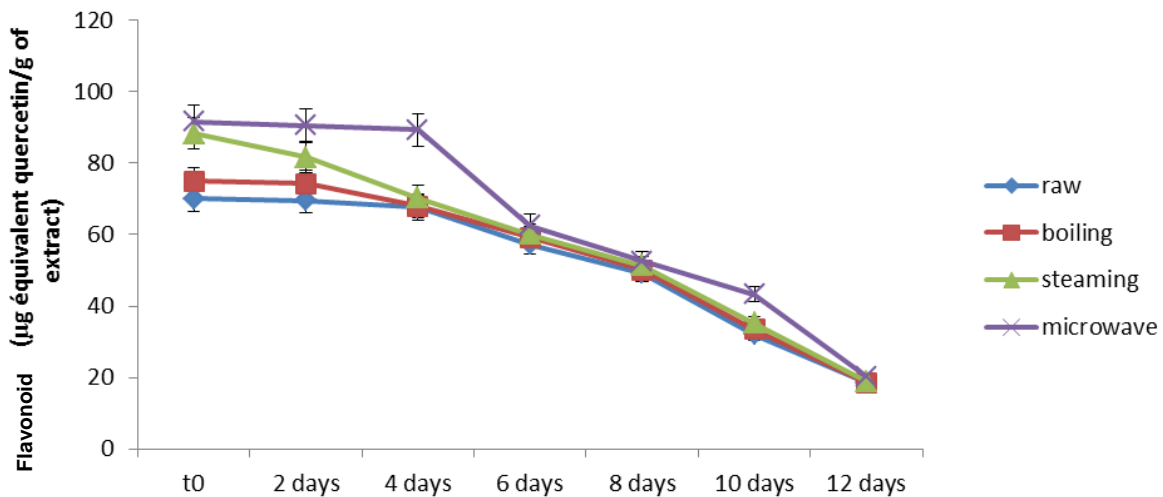

Figure 2. Effect of conservation and cooking on the levels of flavonoids.

the level of the analysed constituents in frozen vegetables.

Cisneros-Zevallos and Heredia (2009) showed that the conservation of onion reduces the levels of total polyphenols. Ferreres et al. (1996) studied the impact of storage content of flavonoids of onion. After 7 days, they found that the content of anthocyanins was significantly decreased. Ewald et al. (1999) showed that loss of the highest flavonoids of onion is obtained when the samples were subjected to a pretreatment before storage. According to Cheynier et al. (1998) and Spigno and De Faveri (2007), the conservation causes a decrease in tannins that could be due to hydrolysis of the polymers of condensed tannins, or a condensation of tannins with anthocyanes. However, some studies have shown that conservation causes increase in the total polyphenol content in some vegetables. The amount of polyphenols increases spontaneously after 3 days. This is due to the release of tissue senescence (Rodriguez-Arcos et al., 2002).

Antioxidant activity and conservation

It appears that storage contributed to decrease of antioxidant activity (Figure 5). This is due to polyphenols of onion. The main role of these compounds as reducing
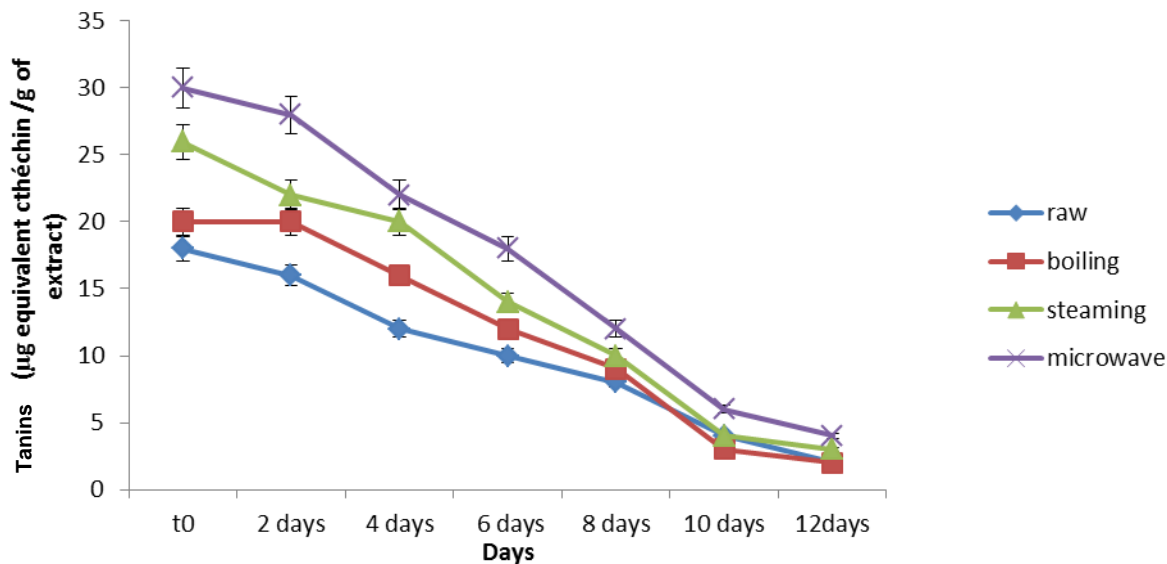
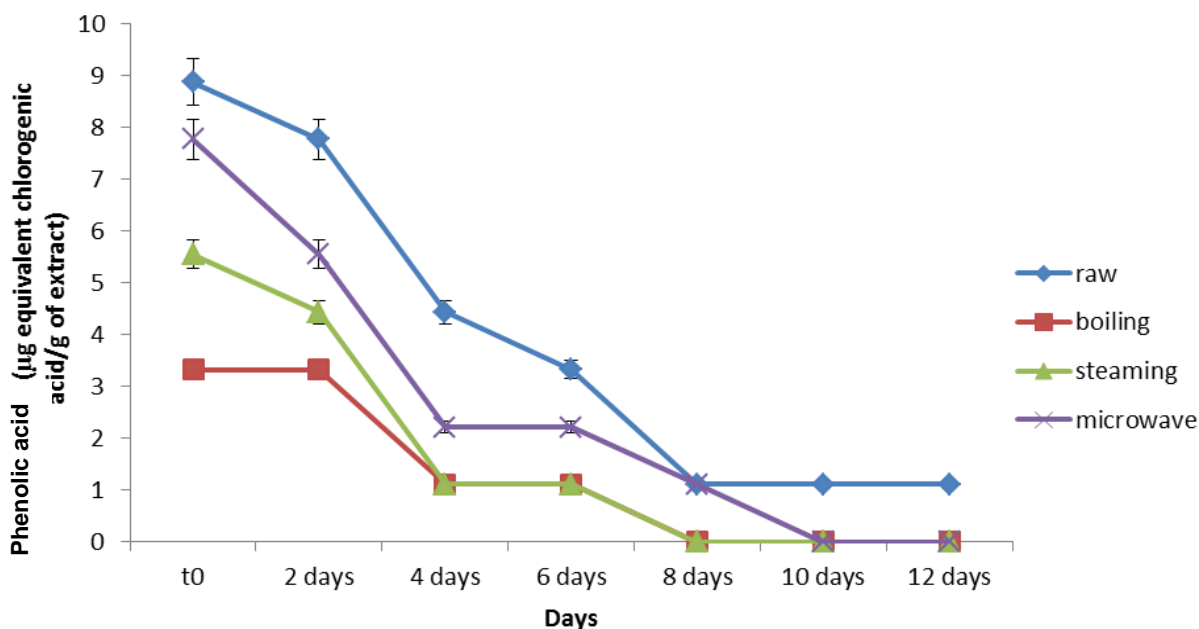**Figure 3.** Effect of conservation and cooking on tanins content.

Figure 4. Effect of cooking on the levels of phenolic acids of raw and stored samples.

free radicals is emphasized in several reports. These results are consistent with those obtained by Ewald et al. (1999), Villano et al. (2007), and Cisneros-Zevallos (2009) who found that the conservation of onion causes a decrease in antioxidant capacity.

Effect of cooking on the content of total polyphenols

Figure 1 summarizes the changes in the content of phenolic compounds during the cooking process.

Cooking increases the total polyphenol content for both lots tested (raw and stored onion). However, the use of microwave and steam were more effective as' to higher polyphenol contents.

Dewanto et al. (2002) attributed this increase in phenolics to the embrittlement of tissues with heat cooking which facilitates extraction of these compounds. This release would offset any loss by thermal degradation. The results indicate that the three cooking modes have positive impact on the flavonoid content of
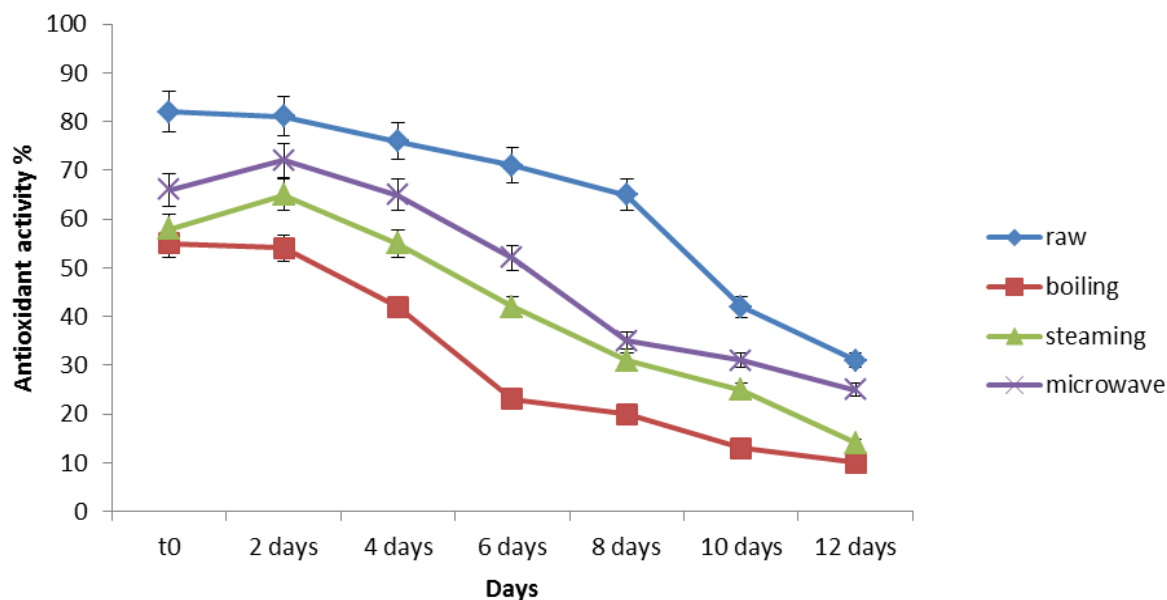
Figure 5. Effect cooking on the antioxidant activity of raw and stored samples.

samples stored at 4°C. However, the use of microwave and steam were more effectives. Flavonoids exist as glycoside in food; one or more hydroxyl groups are combined with sugars. The presence of this glycoside fraction makes flavonoids very soluble in water (Figure 2). Increase in the content of tannins is observed for the three cooking modes of raw or stored samples (Figure 3). However, the use of microwave in cooking onion has proved relatively effective in comparison with the other two methods: steaming and boiling water

The results show that the three cooking modes cause an increase in the levels of total polyphenols, flavonoids and tannins, but decrease the levels of phenolic acids (Figure 4). However, the use of microwave and steam were the most effective.Increased levels of total phenolic compounds could be explained by the ease with which they are extracted following certainly a strong weakening of the cell walls of onion by heat (Gahler et al., 2003). Cooking in boiling water is less effective as increase in phenolic compounds; this is due to the solubilization of polyphenols in the cooking water during this type of thermal treatment (Price et al., 1997; Makris and Rossiter, 2001). However, two other cooking methods (by steam and microwave) allow a better retention of soluble polyphenols in plant tissues.

Effect of cooking on the antioxidant activity

Three cooking modes cause a decrease in antioxidant activity of the two samples tested onion (raw and stored) (Figure 5). This result is consistent with that of Fialho and Faller (2009) which showed that cooking in water, steam or microwave decreased antioxidant activity. This activity

is dependent on the mobility of the hydrogen of the hydroxyl group of the phenolic compounds atom. However, there is no relationship between the polyphenol content and antioxidant activity. A small amount of polyphenols can generate strong antioxidant activity (Makris and Rossiter, 2001). Cooking leads to the modification of the amount of polyphenols, but it can also change the structure of polyphenols, which may affect the antioxidant capacity (Makris and Rossiter, 2001). This may explain the decrease in antioxidant activity despite the increase of total polyphenols, flavonoids and tannins.

In addition, each phenolic compound has a degree of affinity (high or low) for free radical DPPH which could affect the antioxidant variations observed (Heim et al., 2002).

Effect of storage and cooking on anticholinesterase activity

The level of anticholinesterase activity steadily decreased during refrigerated storage and after 12 days it was 1/4 of the value found in the raw material (Table 1). The influence of home cooking methods (boiling, microwaving and steaming) on the anticholinesterase activity of raw and stored onion has been evaluated. The raw onion showed a moderate activity which increased after most cooking treatments. The highest level of capacity was observed after microwaving (Table 1). This result indicates that extract of onion contain an acetylcholinesterase inhibitors, which are selective organophosphorus anticholinesterases. The mode of action of these compounds is to block the action of the acetylcholinesterase enzyme, leading to the excessive

Table 1. Anticholinesterase activity of the extracts and standards.

Parameter	Inhibition % against AChE						
	t0	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
Raw	55.21±0.25	44.5±0.85	41.22±1.1	31.5±0.75	22.5±0.15	22.32±0.65	14.26±0.12
Boiling	56.98±0.95	42.5±1.06	35.25±0.25	32.6±0.26	25.02±0.25	13±0.26	8.08±0.75
Steaming	59.48±1.01	49±0.45	32.02±0.46	22.25±0.13	19±0.89	18.55±0.98	18.12±0.65
Microwave	66.25±0.32	55±0.94	44.13±0.25	29.78±09	23.08±0.49	21.77±0.29	20.33±0.35
Galanthamine	88.02±0.22	-	-	-	-	-	-

Values are means ± standard deviation (SD), n = 3.

build-up of the neurotransmitter acetylcholine.

Phenolic compounds are involved in the protective effect of neurodegenerative diseases (Ramassamy, 2006). In addition, previous reports demonstrated the potential of phenolic compounds to inhibit acetylcholineterase activity (Williams et al., 2011). Because phenolic compounds have different intracellular targets, they can become an efficient approach to reduce the incidence of AD (Ramassamy, 2006).

Literature suggests that vegetables have neuroprotection effects. A prospective cohort study of 3718 human subjects aged 65 years and older based on food frequency questionnaire assessed cognitive functions at baseline and 3- and 6-year follow-ups. The rates of cognitive decline among persons in the fourth and fifth quintiles of vegetable intake were slower by 0.019 (P=0.01) and 0.018 (P=0.02) standardized units per year compared with that among persons in the lowest quintile; the overall mean change per year was a decline of 0.04 standardized units (Morris et al., 2006). Extracts of these fruits and vegetable fed to 19-month-old Fischer 344 rats for 8 weeks reversed age-related deficits in several neuronal and behavioral parameters. Blueberry particularly improves motor function that relies on balance and coordination (Joseph et al., 1999).

Conclusion

The effect of three modes of cooking and conservation at 4°C on total phenolic, flavonoid, tannins, phenolic acid content, antioxidant and anticholinesterase activities of extract of red onion were analyzed. Results indicated that red onion were rich sources of polyphenols and flavonoids and showed the promising antioxidant and free radical scavenging activities. Storage at 4°C reduced the levels of total polyphenols, flavonoids, phenolic acids and tannins. Decreased antioxidant activity was observed during storage temperature 4°C. The three modes of cooking used (water, steam and microwave) increased the levels of total polyphenols, flavonoids and tannins, but decreased the content of phenolic acids.

However, there was a decrease in antioxidant activity after using the three modes of cooking. The microwave

cooking mode has proven most effective as the increase of the polyphenols. The level of anticholinesterase activity decreased during refrigerated storage and after 12 days, it was 1/4 of the value found in the raw material. The raw inion showed a moderate activity which increased after most cooking treatments. The highest level of capacity was observed after microwaving. These results suggest that some common cooking treatments can be used to enhance the nutritional value of vegetables, increasing bioaccessibility of health-promoting constituents.

Conflict of interest

The authors have not declared any conflict of interest

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