Journal of Applied Pharmaceutical Science Vol. 5 (02), pp. 022-027, February, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.50205
ISSN 2231-3354 

In vitro antioxidant activity of the individual herbs of DIA-2, a herbal mixture containing standardized extracts of *Allium sativum* and *Lagerstroemia speciosa*

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ARTICLE INFO

Article history:
Received on: 15/11/2014
Revised on: 05/12/2014
Accepted on: 30/12/2014
Available online: 27/02/2015

Key words:
DIA-2, *Allium sativum*, *Lagerstroemia speciosa*, Diabetes mellitus, Antioxidant, Oxidative stress.

ABSTRACT

Hyperglycemia induced oxidative stress is one of the main mechanism involved in the pathogenesis of diabetic complications. Use of antioxidants as an adjuvant therapeutic approach in diabetes could prevent or delay the progression of oxidative stress induced diabetic complications. Many herbal drugs possess multiple pharmacological actions like anti-hyperglycemic, anti-obesity and anti-oxidant properties etc., which could act through multiple pathways and may offer beneficial effect in the management of multi-factorial diseases such as diabetes. Development of herbal formulation with ingredients having multiple actions could be a potential approach for improving therapeutic efficacy. We here report the free radical scavenging activity of a herbal mixture, DIA-2 and its individual herbs. DIA-2, a herbal formulation containing fixed combination of standardized aqueous extracts of *Allium sativum* (ASE) bulbs and *Lagerstroemia speciosa* (LSE) leaves. The present study was undertaken to investigate whether a combination of ASE and LSE shows a synergistic antioxidant effect than its individual herbs. *In vitro* antioxidant activity for individual herbs and DIA-2 was determined by DPPH radical scavenging, nitric oxide scavenging assay, total antioxidant capacity, reducing power assay and ABTS radical scavenging methods respectively. Our results suggest that DIA-2 could synergistically enhance the antioxidant activity and could be attributed due to the synergistic actions of the individual ingredients. The data obtained suggests the combined use of ASE and LSE as active ingredients in the development of antidiabetic herbal formulation, synergizing its therapeutic value in treating hyperglycemia and associated oxidative stress.

INTRODUCTION

Hyperglycemia is the main causative factor for oxidative stress during diabetes mellitus (DM). Chronic hyperglycemic conditions enhance the production of free radicals, resulting in end organ tissue damage leading to diabetic complications (Brownlee 2001; Chan *et al.,* 2008). Recent research studies indicate that current treatments available for diabetes have good control over hyperglycemia but not on the progression of diabetic complications, suggesting their inability to improve the altered antioxidative defense mechanisms (Waisundara *et al*., 2008).

Consequently, the current research and development on drugs has targeted on oxidative stress pathways for the prevention of diabetic complications (Omar *et al*., 2010). Use of antioxidants is considered as one of the therapeutic remedy for the prevention of diabetic complications till date (Xie *et al*., 2009). Use of synthetic antioxidants is not helpful and has been reported for their adverse effects (Radulović *et al*., 2007). In recent years, plants based medicines have been found to possess excellent antioxidant activities and used in combination to demonstrate significant therapeutic effects than when used alone (Yang *et al*., 2009).

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Plant drug combinations were widely used in the adjuvant therapy of type 2 diabetes mellitus for the prevention of complications (Szentmihalyi *et al*., 2010). DIA-2 is a polyherbal formulation formulated with an aim to combat hyperglycemia and hyperglycaemia induced oxidative stress. It contains standardised extracts prepared from dried bulbs of *Allium sativum* (ASE) and leaves of *Lagerstroemia speciosa* (LSE) in the ratio 1:1 w/w. The rationale for selection of these two herbs in the formulation was that, both the herbs were well known for multiple therapeutic activities so that they can synergistically act on various pathophysiological processes involved in the aetiology of this metabolic syndrome. Both the individual herbs namely ASE (Eidi *et al*., 2006; Chung 2006) and LSE (Priya *et al*., 2008; Saumya and Basha 2011) were known for their antioxidant effect and anti-diabetic effect. DIA-2 is distinct from other polyherbal formulation, that it contains standardised minimum number of ingredients. However, no studies have been reported so far on the combination of these two herbs. In the present study using *In vitro* antioxidant screening systems we attempted to test our hypothesis, whether individual herbs of DIA-2 could show a synergistic antioxidant effect.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used were of analytical grade obtained from M/s. Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.

Plant Extracts

Standardized extract of *Allium sativum* (ASE) and *Lagerstroemia speciosa* (LSE) were obtained from M/s. Amsar Pvt. Ltd, Indore, India and K. Patel Phyto Extractions Ltd, Mumbai, India, respectively. ASE is an aqueous extract of dried bulbs *Allium sativum* and LSE is 40% methanolic extract of dried leaves of *Lagerstroemia speciosa*. Both ASE and LSE were supplied in the powder form and claimed to contain 1.1% alliin w/w and 1.28% w/w corosolic acid, respectively.

Antioxidant activity of ASE and LSE

DPPH radical scavenging assay

The DPPH assay was performed as per the earlier described methods (Koleva *et al*., 2002). About 10 µL of each concentration (1.5–1,000 µg/10 µL) of test sample was added to 190 µL DPPH solution. After vortexing, the mixture was incubated for 20 min at 37°C. The control blank contained the solvent without test sample. The decrease in absorbance of the test mixture was measured at 517 nm. The percentage of DPPH radical scavenging activity of the test sample was calculated and the results were compared with standard Vitamin E (Merck, Mumbai, India).

The percentage inhibitions of free radicals were calculated by the formula: % Inhibition = $[(Ao - A_1)/Ao] \times 100$.
Where Ao was the absorbance of the control (containing all the reagents except the test extract), and A_1 was the absorbance of samples. The assays were carried out in triplicates, the IC_{50} value, the concentration of extracts that inhibits the formation of free radicals by 50% was calculated from the graph plotted as inhibition percentage against extract concentration.

Nitric oxide scavenging assay

The nitric oxide (NO) assay was performed as per the earlier described methods (Green *et al*., 1982). Briefly, 5 mM sodium nitroprusside in phosphate buffered saline was mixed with 3 ml of different concentrations (1.5 – 1,000 µg/mL) of the extracts (ASE, LSE) and incubated at 25ºC for 150 min. The samples from the above were allowed to react with Greiss' reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm. The percentage of NO radical scavenging activity of the test sample was calculated, and the results were compared with standard Vitamin E.

Phosphomolybdenum assay

The total antioxidant capacity assay was performed as per the earlier described methods (Prieto *et al*., 1999). Briefly, an aliquot of prepared extract/α-tocopherol was combined with α-Tocopherol reagent solution. In case of blank, solvent was used in place of the sample. The tubes were capped and incubated in a boiling water bath at 95°C for 60-90 min. Samples were cooled to room temperature, the absorbance of each was measured at 695 nm against the blank in a Perkin Elmer UV/visible spectrophotometer (Lambda 25,USA). The experiments were repeated in triplicates. The content of α tocopherol was calculated using a standard graph and the total antioxidant activity of the extracts was expressed as the number of gram equivalents of vitamin E (mg of vitamin E/g of extract).

Reducing power assay

The reducing power assay was performed as per the earlier described methods (Oyaizu *et al*., 1986). To 1 mL of prepared extract/ascorbic acid, 0.1 mL of DTC reagent (2, 4-Dinitrophenylhydrazine-thiourea-copper sulphate) was added and incubated at 37ºC for 3 hours. After incubation, 1.25 mL of 85 % H_2SO_4 was added under ice-cold condition. The mixture was kept at room temperature for 30 minutes. The absorbance was measured at 540 nm against a blank using UV/Visible spectrophotometer. The experiments were repeated in triplicates. The content of Vitamin C was calculated using a standard graph and the reducing power of the extracts was expressed as the number of gram equivalents of ascorbic acid (mg of ascorbic acid/ g of extract).

Antioxidant activity of DIA-2

ABTS radical scavenging assay

The ABTS assay was performed as per the procedures of the kit insert of Cayman Chemical Company, Michigan, USA (Miller *et al*., 1993; Miller and Rice-Evans 1997). About 10 µl of various concentrations (0-1000 µg/mL) of DIA-2 was added to 10 µL of metmyoglobin and 150 µL of ABTS (2 mM).

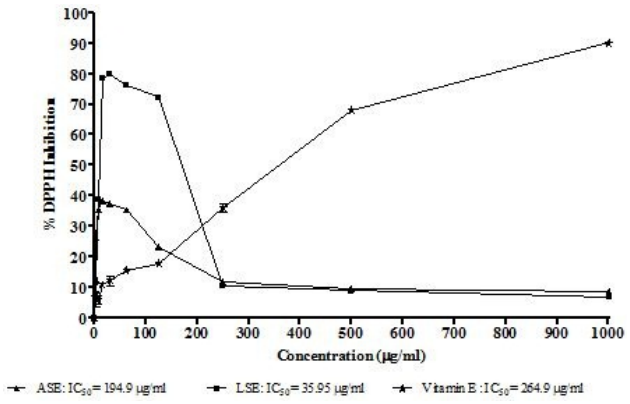

Fig. 1(a)

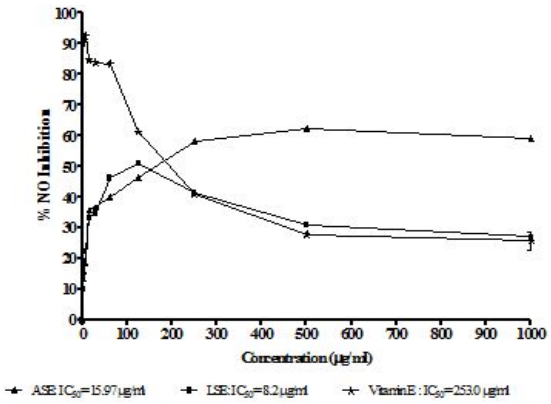

Fig. 1(b)

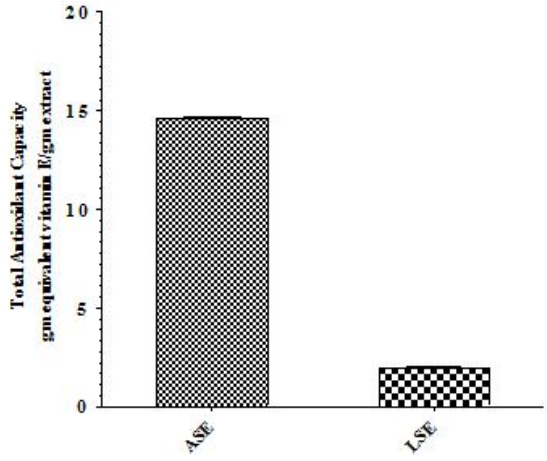

Fig. 1(c)

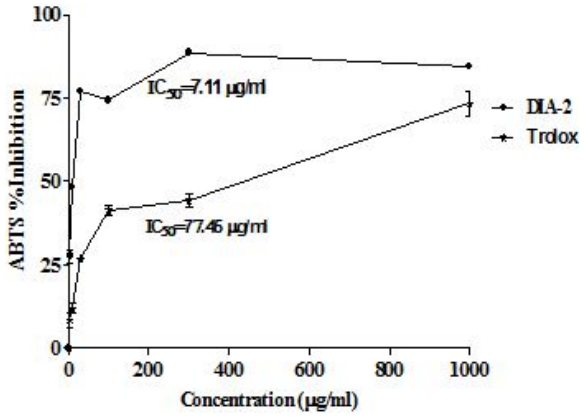

Fig. 1(d)

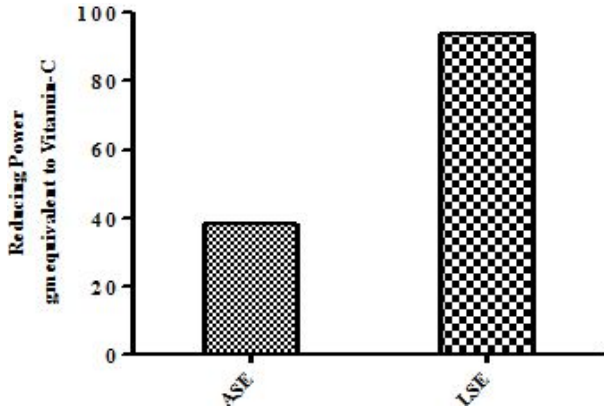

Fig. 1(e)

Fig. 1: Antioxidant effects of ASE and LSE employing (a): DPPH radical scavenging assay; (b): NO scavenging assay; (c) Phosphomolybdenum assay; (d) Reducing power assay; (e) ABTS radical scavenging activity. Results are mean ± SEM of three replicate analyses.

The reaction was initiated by adding 40 µL of H_2O_2 (441 µM), shake well and the amount of ABTS formed was measured at 690 nm. The percentage of ABTS radical–scavenging activity of the test sample was calculated and the results were compared with standard Trolox, a vitamin E analogue.

Statistical analysis

All values are expressed as the mean ± SEM. IC_{50} values were calculated using nonlinear regression analysis with the GraphPad Prism (Version 5.0) for Windows (GraphPad Software, Inc., San Diego, CA, USA, www.graphpad.com).

RESULTS AND DISCUSSION

Free radicals are implicated in the pathogenesis of various diseases including metabolic disorders like DM. Current research is directed towards finding naturally occurring drugs that could ameliorate hyperglycemia and hyperglycemia induced oxidative stress as well (Tiwari and Rao 2002). Many of the medicinal plants have been used in our diet are known to possess antihyperglycemic and antioxidant properties as well. Combination of herbal ingredients into a formulation to achieve synergistic effect is a common practice in the herbal industry till date. The success of an herbal product is also governed by its quality, safety and efficacy (Hardy *et al*., 2002). Modern medicine prefers to use single ingredients on the grounds that dosage can be more easily adjusted, drug interactions could be prevented and quality control measures could be achieved with ease. In case of herbal medicines, they synergistically work better than a single herb. It is very difficult to set the quality control measures in a polyherbal formulation as the number of ingredients may vary from 2 to 25 or more. Different forms of concentrated form of an herb are available as crude extracts (example: powders, tinctures etc), but still standardized extracts are widely accepted clinically since they produce reproducible therapeutic effects compared to crude extracts. Standardized extracts contain specific amount of one or group of phytoconstituents in an herb at a certain level. The clinical effectiveness requires delivery of an active dosage and use of standardized extracts forms a way to assure the delivery of an effective dosage (Firenzuoli and Gori 2007).

In the present study, we aimed to develop a polyherbal formulation with minimum number of standardized ingredients. One of the common methods to minimize the number of ingredients in a formulation is to choose an active ingredient that possesses more than one effect (Ofner III CM and Schwartz 1996). A synergistic herbal formula with minimum number of ingredients could also facilitate the manufacturer to develop a cost-effective formula. Another simple method to develop a polyherbal formulation is choosing ingredients which have shown efficacy in clinical models (Teixeira and Fuchs 2006). Both ASE and LSE are well known for their multiple therapeutic benefits and are well clinically accepted (Ashraf *et al*., 2011; Judy *et al*., 2003). Though several reports were available for ASE and LSE individually, data on their combination is not hitherto known. These spurt interest to develop a combination of ASE and LSE and to investigate their synergistic antioxidant effects, if any. Here we report antioxidant activity for ASE, LSE using various *In vitro* assay methods and for their 1:1 w/w fixed combinations using ABTS assay. An independent study on DIA-2 revealed that 1:1 mixture (ASE:LSE) is less cytotoxic to 3T3 L1 preadipocyte cells than 1:2 mixture (Kesavanarayanan *et al*., 2012), hence 1:1 mixture was selected for this study. Similarly the antioxidant effect of individual herbs and their equiproportional herbal mixture triphala were investigated using *In vitro* antioxidant assay methods (Naik *et al*., 2005).

The approach of scavenging the stable DPPH radicals is a widely used method to evaluate the hydrogen donating capacity of antioxidants, since it involves a relatively short time compared to other methods. DPPH radical scavenging ability expressed as IC_{50} value exhibited by LSE was 35.95 µg/mL; whereas ASE and Vitamin E showed an IC_{50} value of 194.9 µg/mL and 264.9 µg/mL respectively (**Fig-1a**). After addition of 150 µM of alcoholic solution of DPPH to various concentrations of test compounds (ASE, LSE and Vitamin E), we found that there was gradual decrease in absorbance in all the treatment groups. The decrease in absorbance may be due to the proton donating ability (Mathiesen *et al*., 1997) of phenolic constituents present in ASE, LSE (Kesavanarayanan *et al*., 2012) and Vitamin E (Mukai *et al*., 2005).

The effect of ASE and LSE on NO radical scavenging was determined by the decrease in intensity of pink coloured chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme measured at 540 nm. The antioxidant activity was compared with Vitamin E as standard. It was found that IC_{50} of both ASE (15.97 µg/mL) and LSE (8.20 µg/mL) showed an inhibitory activity on NO free radical compared to Vitamin E (253 µg/mL) [**Fig-1b**]. NO plays a dual beneficial/deleterious function, depending on their concentration (Bencini *et al*., 2010). Low concentrations of NO are essential for normal cellular homeostasis and higher concentrations have been identified as a key pathogenic factor leading to cellular damage in many chronic human diseases (Amiri *et al*., 2011). During chronic hyperglycemic conditions, there is over production of superoxide free radical, which is accompanied by an increased NO generation, resulting in the formation of the stronger oxidant peroxynitrite (Brownlee 2001).

The NO scavenging effect might be associated with organosulfur compounds (Ippoushi *et al*., 2002) and phenolic compounds (Conforti and Menichini 2011) found in ASE and LSE respectively. Our previous finding also reveals that ASE and LSE could inhibit the reactive oxygen species and super oxide radicals *In vitro* (Kesavanarayanan *et al*., 2012); suggesting its beneficial use in preventing superoxide radical induced tissue damage and superoxide radical induced NO production.

The phosphomolybdate is a quantitative method to access the total antioxidant capacity (Prieto *et al*., 1999) and the antioxidant capacity was expressed as grams equivalent of vitamin E per gram of extract (**Fig-1c**).

ASE (14.61 ± 0.08) was found to possess higher antioxidant activity when compared to LSE (1.93 ± 0.01). In the presence of ASE or LSE, Mo (VI) was reduced to Mo (V) and formed a green colour phosphomolybdenum V complex, which showed a maximum absorbance at 695 nm. Determination of the reducing capacity of a plant extract may reveal its potential antioxidant activity (Samarakoon *et al*., 2011). The highest reducing power was exhibited by LSE (94.07 ± 0.25) than ASE (38.48 ± 0.14) [**Fig-1d**], the highest activity of LSE compared ASE was attributed due to high phenolic content (Kesavanarayanan *et al*., 2012). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing ability is generally associated with the presence of reductones which breaks the free radical chain by donating a hydrogen atom (Harbaum *et al*., 2008).

Several formulations were formulated with fixed combination of standardized herbs and tested clinically for various disorders (Spasov *et al*., 2004; Gruenwald *et al*., 2005; Engelmann *et al*., 2006). DIA-2 is a polyherbal formulation containing standardized extracts of ASE and LSE. The antioxidant effect of DIA-2 was assessed using ABTS assay and compared with trolox. Earlier reports on the individual ingredients of DIA-2 using ABTS method (Gorinstein *et al*., 2006; Saumya and Basha 2011) are available hence the mixture alone was tested and compared with trolox. In ABTS assay, DIA-2 (IC_{50} = 7.2 µg/mL) showed stronger antioxidant capacity than trolox (IC_{50} = 77.5 µg/mL) [**Fig-1e**].
DIA-2 and its individual herbs showed prominent antioxidant effect at lower doses; decreased effect at higher doses might be attributed due the threshold or ceiling effect of antioxidants. Synergistic inhibition of reactive oxygen species production within the cell was also observed in an independent study by DIA-2 compared to it individual herbs (Kesavanarayanan *et al*., 2012). The organosulphur compounds (Imai *et al*., 1994) and phenolic compounds (Lu *et al.*, 2011) available with ASE are well known for its antioxidant activity.

Alliin (*A.sativum*) and corosolic acid (*L.speciosa*) are the phytochemicals available with the standardized individual herbal extracts of DIA-2. Both the phytochemicals are biologically available *in vivo* after oral administration (Lachmann *et al*., 1994; Yin *et al*., 2012) and the antioxidant mechanisms of these phytochemicals are not clearly understood so far. The antioxidant mechanism of all phytochemicals of garlic is not available. However, alliin is a principle sulfur-containing compound of garlic, which gets enzymatically converted to allicin (a biological active metabolite) in the presence of alliinase (Rybak *et al*., 2004). The antioxidant action allicin could be attributed due to its reaction with thiol containing proteins (Rabinkov *et al*., 1998). Both alliin and allicin could suppress the formation of free radicals probably *via* a thiol exchange mechanism (Chung *et al*., 2006). Corosolic acid has been reported to scavenge free radicals but the exact mechanism of action is not known (Ali *et al*., 2007). The synergistic antioxidant effect of DIA-2 might be attributed due to the presence alliin and corosolic acid available with ASE and LSE respectively. Our earlier report also showed that 14 days of oral treatment of DIA-2 could ameliorate the impaired glucose homeostasis and altered oxidant/antioxidant levels in high fat diet fed/streptozotocin treated diabetic rats (Kesavanarayanan *et al*., 2013).

CONCLUSION

The results obtained from our study suggested that a fixed combination of ASE and LSE could be an ideal candidate for the management of diabetes mellitus and to a greater extent, could be useful in preventing diabetic complications *via* its antioxidant effects. Further investigation on DIA-2 for its use in co-morbid conditions of diabetes mellitus such as hypertension could support its therapeutic use during such clinical conditions.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Dr Aishah Adam, Dean, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM) Puncak Alam Campus for her support.

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How to cite this article:

Kesavanarayanan K.S, Kavimani S, Prathiba D. *In vitro* Antioxidant Activity of the Individual Herbs of Dia-2, a Herbal Mixture Containing Standardized Extracts of *Allium sativum* And *Lagerstroemia speciosa*. J App Pharm Sci, 2015; 5 (02): 022-027.